

Critical thermal limits affected differently by developmental and adult thermal fluctuations

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Summary statement

Thermal fluctuations during development in *Drosophila melanogaster* lead to detrimental cold and beneficial heat acclimation responses, while thermal fluctuations induce little acclimation response during adult exposure.

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. 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 effect of fluctuations at any life stage was gradually lost with prolonged adult maintenance suggesting a more prominent effect of fluctuations during developmental compared to adult acclimation in *Drosophila melanogaster*.

Introduction

The role of environmental temperature in regards 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 behavioral, 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, within years and/or to within days. Adaptation to fluctuating temperatures can affect insect life history and survival differently compared to 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 due to a limited scope for behavioral 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 changes 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 rely for most parts 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 the performance in a fluctuating environment (Kingsolver et al., 2009; Bowden et al., 2014; Estay et al., 2014; Folguera et al., 2011) as well as how the molecular mechanisms induced by fluctuations resemble or differ from

responses to constant temperature (Podrabsky and Somero, 2004; Sørensen et al., 2016b). The effect 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. 2016, but see Overgaard et al. 2011) and low temperature acclimation (Boardman et al., 2013). The outcome of thermal fluctuations likely depends on the exact experimental protocol (Colinet et al., 2015). We here focus on diurnal fluctuations with cold dark (night) and warm light (day) periods with varying amplitudes (± 4 or 8°C), however, factors such as the number of exposures, duration of each exposure, and the product of these also clearly contributes 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 the 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., 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 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 alternate 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 to 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 role of diurnal temperature fluctuations for thermal acclimation ability, we here investigated the relative contribution of means and variances of temperature to heat and cold tolerance acclimation 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 expect both mean and fluctuation of temperature to contribute to the thermal acclimation (Bozinovic et al., 2011; Niehaus et al., 2012; Sørensen et al., 2015; Slotsbo et al., 2016). Specifically, we expect that the heat and cold phase of a fluctuation will cancel each other out with respects to cold tolerance acclimation, and thus, that cold tolerance will be determined by the mean acclimation temperature. For heat tolerance, we expect the temperatures reached during the heat phase to be responsible for the accumulation of heat tolerance acclimation (Fig. 1). We expect 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 expect 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* collected in 2013 was used for the experiments (Schou et al., 2015). The population was maintained in the laboratory at 25°C (12L:12D) for several generations before experimentation. Density of the all flies used for experiments was controlled by transferring approximately 40 (\pm 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 that several vials of the same treatment group received 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 performed. In the first set the treatments were divided into four groups, 19C (19°C, constant), 19F (19 ± 4°C, fluctuating), 23C (23, constant) and 23F (23 ± 4°C, fluctuating), i.e. two main treatments (constant & fluctuating) and two main temperatures (19°C & 23°C) (Fig. 2A). In the second set we tested the same treatments, but with the 19F and 23F fluctuating treatments reaching amplitudes of ± 8°C. The fluctuations followed in both cases a sinus function across 12 hours. Thus, the 19F ± 4°C treatment would start at 19°C, increase to 23°C over six hours and then decrease to 19°C during the following six hours. For the following 12 hours, the temperature would follow a mirrored pattern reaching 15°C and generating a mean temperature of 19°C. A 12L:12D 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 anesthesia and returned to the same treatments for 2 days (recovery time) before using for heat and cold tolerance assays.

Adult acclimation

To investigate the acclimation effects of exposure of adults to fluctuating temperatures and to investigate reversibility of the developmental acclimation effects (i.e. reversibility of tolerance gained from development upon change in adult acclimation regime) we further 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 adults (i.e. by changing between a constant and a fluctuating and *vice versa*) for a period of five 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 nine 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 five and nine days allowed enough time for a strong acclimation response to develop, whilst keeping within the timeframe 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 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 temperature of constant and fluctuating regimes were similar and individually transferred to small glass vials and immersed in temperature controlled aquaria set at 21°C (since 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 the rate of 0.1°C/ min. 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 analyzed CT_{min} and CT_{max} of each independent experiment by ANOVAs based on linear models. For developmental acclimation the parameters for analysis included dependent variables CT_{max}, 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 ANOVAs 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 (doi:10.5061/dryad.25b8f). 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} seems little affected by age in species of *Drosophila* flies within the age (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 temperatures could be seen at both the amplitudes and for both CT_{min} and CT_{max} (Table 1, Fig. 3). No effect of fluctuations was observed when the amplitude of fluctuations was mild ($\pm 4^{\circ}\text{C}$), whereas the $\pm 8^{\circ}\text{C}$ 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 $^{\circ}\text{C}$ in their CT_{min} scores compared to 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 $^{\circ}\text{C}$ 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 or *vice versa*) after emergence generally showed small and inconsistent effects for the 4 $^{\circ}\text{C}$ amplitude regime. Significant interactions between developmental and adult thermal regimes for CT_{min} at 19 $^{\circ}\text{C}$ (Table 2, Fig. 4C) and for CT_{max} at 23 $^{\circ}\text{C}$ were the only significant effects (Table 2, Fig. 5A). However, with an increase in the amplitude to $\pm 8^{\circ}\text{C}$, 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, Fig. 4D, 5D), and at the mean of 19 $^{\circ}\text{C}$ adult fluctuations also contributed with a further detrimental effect (Fig. 4D). The mean of 19 and 23 $^{\circ}\text{C}$ fluctuations lead to a CT_{min} loss of $\sim 0.3\text{-}0.4^{\circ}\text{C}$ and $\sim 0.5\text{-}0.6^{\circ}\text{C}$, respectively (Fig. 4D, 5D). A minor beneficial effect

of adult fluctuations corresponding to $\sim 0.15\text{-}0.2^\circ\text{C}$ improvement of CTmax 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 CTmin scores (Table 3, Fig. 6, 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 CTmin) and of the interaction between developmental and adult acclimation treatment (Table 3, Fig. 6D, 7D). For CTmax, shifts involving only the temperature only showed 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 19 and 23°C flies in our study are as expected. In contrast the 4°C amplitude of fluctuations 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 larger amplitude fluctuations.

While constant temperature reaction norms follow linear performance curves in regards to the critical thermal limits (Schou et al., 2016), reaction norms for fluctuating temperatures are

multidimensional, depending on amplitudes, rates of change, 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 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 temperatures (Sørensen et al., 2016a). Thus, even if significant effects of fluctuations were observed, their contribution was minor compared to the effects of the 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°C and 25°C, respectively, 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} suggested that the minimum and maximum temperatures reached during fluctuation affected these two traits differently. This supported our hypothesis that CT_{max} and CT_{min} were independently regulated based on mechanisms that were activated in response to the heat phase and cold phase of fluctuations (consult Fig. 1). Whilst the absolute changes to tolerance for both high and low temperature critical thermal limits in our study were very similar in absolute terms (0.3-0.4°C), due to 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 relative 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 23C (note that the 19F 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 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 to a much 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 temperatures were found to be highly reversible (Slotsbo et al., 2016), and the effect of the mean difference in the temperature far surpasses any effect of fluctuations. CT_{max} on the other hand was more strongly influenced by adult treatments, and acclimation developed during developmental stage was readily reversed with adult treatments. 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). In any case, since 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 temperatures similar to the responses observed for developmental acclimation, and only minor effects of fluctuations for 4°C and slightly more prominent for 8°C amplitudes (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 shifts from 23°C to 19°C. Larger effect at larger amplitudes are not surprising and corroborates other studies, e.g., for newt larvae where larger diel fluctuations during embryonic development resulted in faster swimming speed compared with those 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 (19F fluctuates to a minimum of 11°C) as opposed to the benign temperature conditions of 4°C amplitudes (here 19F fluctuates only to a minimum of 15°C). It could then be argued that since colder temperature acclimation results in beneficial acclimation, a temperature of 11°C reached during fluctuation should also elicit such a response. However, since the fluctuations also reaches a maximum temperature of 27°C (for 19F with 8°C amplitude fluctuations), the final acclimatory outcome is more likely to be a result of the interaction between the heat and cold phases of fluctuations.

Are thermal fluctuations leading 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 of mean temperature, amplitude and frequency of fluctuations. For example, Kingsolver et al. (2016) have shown beneficial effects of repeated heat exposures on heat tolerance. Our results have shown that temperature fluctuations affect thermal acclimation responses independent of constant temperature reaction norms and with different impact in developmental and adult life stages. CT_{min} was more pronouncedly affected by the mean developmental temperature whereas CT_{max} was especially beneficially affected by the higher amplitudes, with the effects 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 from the reproductive status. However, 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 signs of reversibility during the adult stage when shifted to 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 the 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 as compared to 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 behavioral 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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Tables

Table 1. Results of the Analysis of Variances for ± 4 and $\pm 8^\circ\text{C}$ amplitudes for CTmin and CTmax after developmental acclimation. Assays were performed on adults two days post emergence. Treatment (Treat) represent constant and fluctuating regimes, respectively and Temperature (Temp) represent a mean of 19 and 23 $^\circ\text{C}$, respectively. ***: $P < 0.001$.

	CTmin ($\pm 4^\circ\text{C}$)	CTmin ($\pm 8^\circ\text{C}$)	CTmax ($\pm 4^\circ\text{C}$)	CTmax ($\pm 8^\circ\text{C}$)
Treatment ($F_{(d.f.)}$)	1.2 (1, 62)	28.1 (1, 73)***	0.2 (1, 62)	57.1 (1, 73)***
Temperature ($F_{(d.f.)}$)	660.7 (1, 62)***	263.4 (1, 73)***	16.2 (1, 62)***	31.4 (1, 73)***
Treat x Temp ($F_{(d.f.)}$)	0.3 (1, 62)	1.3 (1, 73)	0.2 (1, 62)	0.3 (1, 73)

Table 2. Results of the Analysis of Variances for ± 4 and $\pm 8^\circ\text{C}$ amplitudes for CTmin and CTmax after adult acclimation. Assays were performed five days post emergence. The flies used had a mean developmental temperature of 19°C (D19) or 23°C (D23) and were post emergence shifted between constant and fluctuating regimes (see Fig. 2). Developmental treatment (Dev. Treat) and Adult treatment (Ad. Treat) represent constant and fluctuating regimes administered during developmental and adult period, respectively. ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$.

	CTmin ($\pm 4^\circ\text{C}$)		CTmin ($\pm 8^\circ\text{C}$)		CTmax ($\pm 4^\circ\text{C}$)		CTmax ($\pm 8^\circ\text{C}$)	
	D19	D23	D19	D23	D19	D23	D19	D23
Dev. Treat ($F_{(d.f.)}$)	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)
Ad. Treat ($F_{(d.f.)}$)	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)*
Dev. Treat x Ad. Treat ($F_{(d.f.)}$)	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)

Table 3. Analysis of Variances for ± 4 and $\pm 8^\circ\text{C}$ amplitudes for CTmin and CTmax after adult acclimation. Assays were performed nine 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 19°C and 23°C (within same Constant and Fluctuating regimes). Developmental treatment (Dev. Treat) represents constant and fluctuating regimes during development and Adult temperature (Ad. Temp) represents a mean adult temperature of 19 and 23°C . ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$.

	CTmin ($\pm 4^\circ\text{C}$)		CTmin ($\pm 8^\circ\text{C}$)		CTmax ($\pm 4^\circ\text{C}$)		CTmax ($\pm 8^\circ\text{C}$)	
	D19	D23	D19	D23	D19	D23	D19	D23
Dev. Treat ($F_{(d.f.)}$)	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)
Ad. Temp ($F_{(d.f.)}$)	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)
Dev. Treat X Ad. Temp ($F_{(d.f.)}$)	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)

Figures

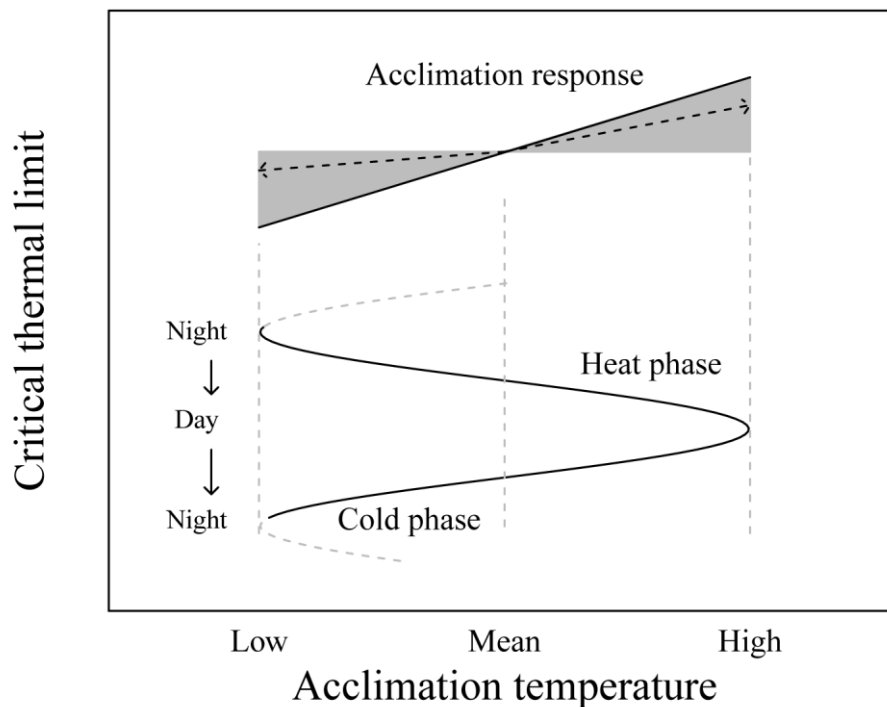


Figure 1. Conceptual diagram showing the hypothesized effect of fluctuating temperature acclimation relative to constant temperature acclimation. A thermal fluctuation cycle is depicted going from low temperature at night to high temperature during the day and back to low temperature the following night. The straight solid line above the fluctuation indicates a linear acclimation response for constant temperature (both critical minimum and 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 cold phase of thermal fluctuations, respectively. 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 CTmin and CTmax 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.

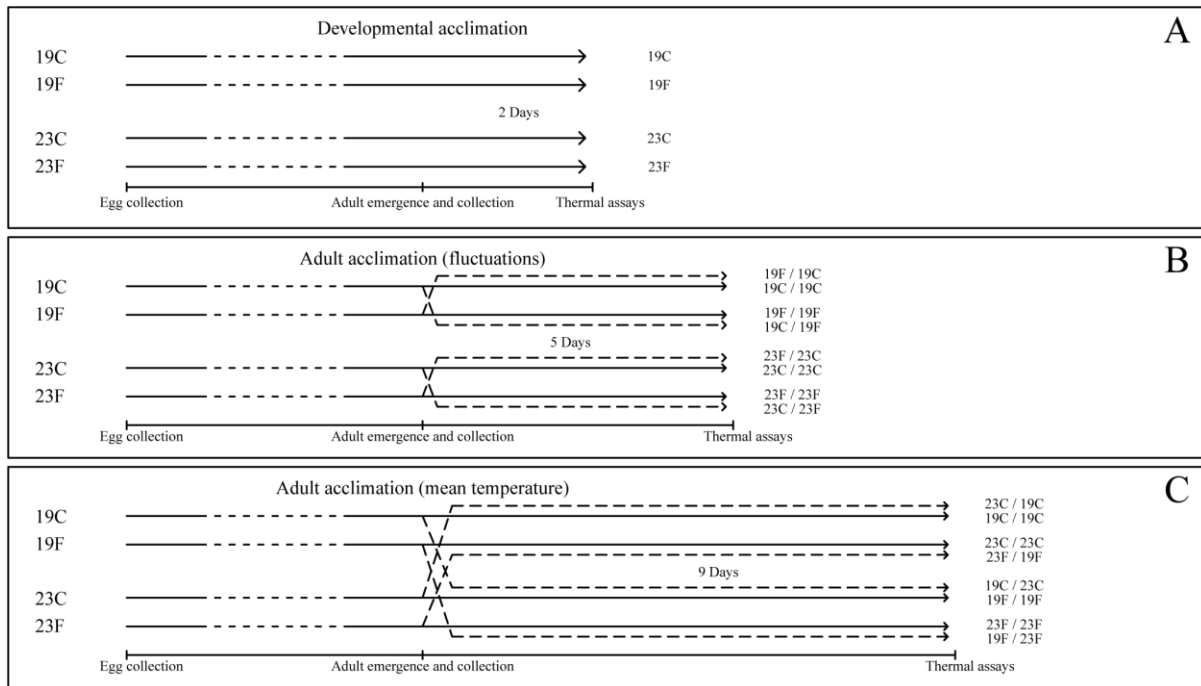


Figure 2. Experimental regimes used to investigate the effects of mean temperature and thermal variability in developmental and adult life stages of *D. melanogaster*. Thermal treatments were 19 or 23°C constant (19C & 23C, respectively), or fluctuating with a mean of 19 or 23°C (19F & 23F, respectively). Each set of experiments were performed with ± 4 or 8°C , respectively. The three experiments investigated effects of; Panel A: developmental acclimation, Panel B: thermal variability during adult acclimation, and Panel C: mean temperature during adult acclimation. Thermal assays were critical thermal minimum and maximum (CT_{min} and CT_{max}).

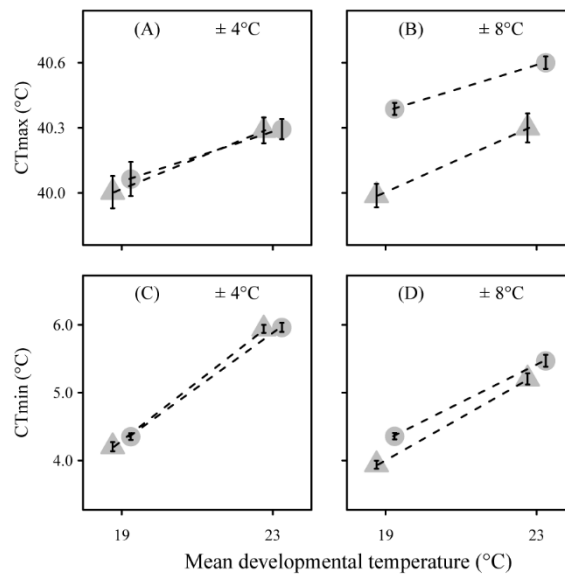


Figure 3. Critical thermal limits (mean \pm s.e.m) for constant and fluctuating temperatures assayed 2 days after emergence and plotted as reaction norms. Panel A and B: CTmax estimates at ± 4 ($N = 14, 14, 19, 20$) and $\pm 8^\circ\text{C}$ ($N = 20, 20, 20, 19$) amplitudes, respectively. Panel C and D: CTmin estimates at ± 4 ($N = 14, 14, 20, 20$) and $\pm 8^\circ\text{C}$ ($N = 20, 19, 20, 20$) amplitudes, respectively. Shaded circles indicate fluctuating treatment and shaded triangles indicate constant temperature treatment. N values correspond to each treatment, respectively.

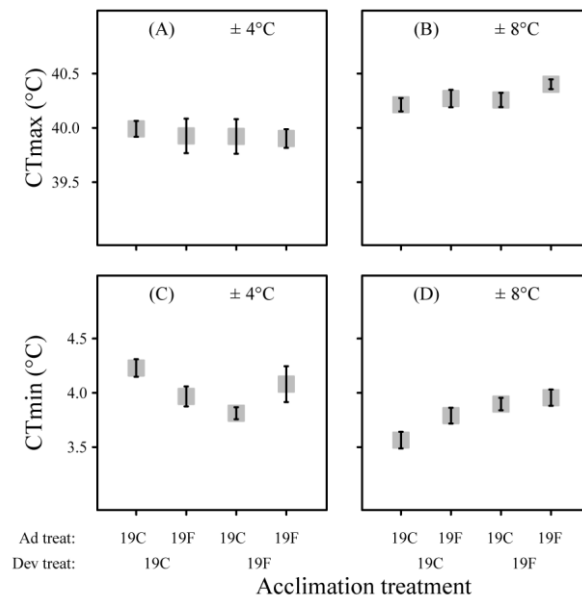


Figure 4. Critical thermal limits (mean \pm s.e.m) for treatment groups (mean temperature of 19°C) assayed 5 days after emergence. The flies were exposed to a full factorial combination of constant (19C) or fluctuating (19F) developmental acclimation (Dev treat), followed by constant (19C) or fluctuating (19F) adult acclimation (Ad treat) (see Fig. 2). Panel A and B: CTmax estimates at ± 4 ($N = 10, 10, 8, 10$) and $\pm 8^\circ\text{C}$ ($N = 20, 20, 19, 20$) amplitudes, respectively. Panel C and D: CTmin estimates at ± 4 ($N = 9, 10, 9, 9$) and $\pm 8^\circ\text{C}$ ($N = 20, 20, 16, 20$) amplitudes, respectively. N values correspond to each treatment respectively.

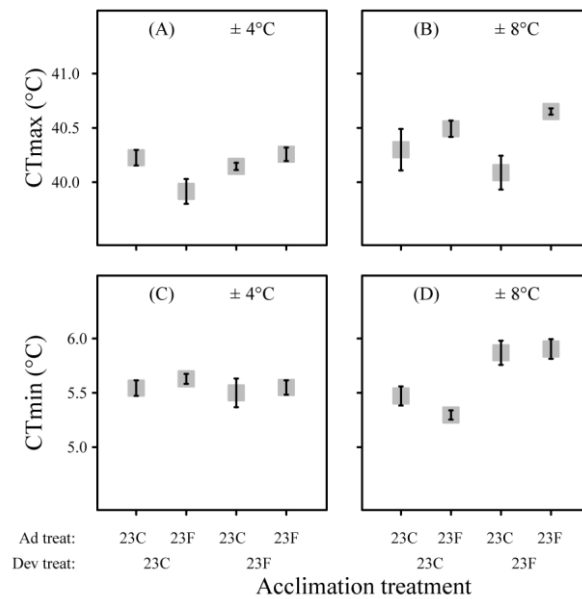


Figure 5. Critical thermal limits (mean \pm s.e.m) for treatment groups (mean temperature of 23°C) assayed 5 days after emergence. The flies were exposed to a full factorial combination of constant (23C) or fluctuating (23F) developmental acclimation (Dev treat), followed by constant (23C) or fluctuating (23F) adult acclimation (Ad treat) (see Fig. 2). Panel A and B: CTmax estimates at ± 4 ($N = 10$) and $\pm 8^\circ\text{C}$ ($N = 10$) amplitudes, respectively. Panel C and D: CTmin estimates at ± 4 ($N = 9, 10, 9, 10$) and $\pm 8^\circ\text{C}$ ($N = 9, 10, 10, 10$) amplitudes, respectively. N values correspond to each treatment respectively.

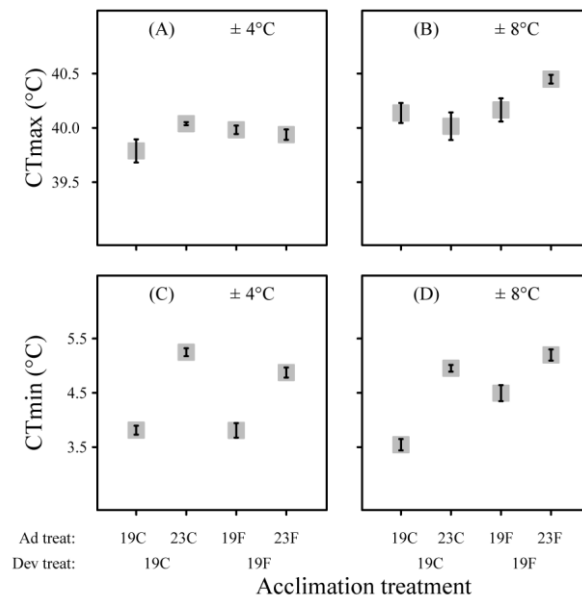


Figure 6. Critical thermal limits (mean \pm s.e.m) for treatment groups assayed after 9 days post emergence (mean developmental temperature 19°C). The flies were exposed to a combination of mean temperature exposures within thermal regime, i.e. constant (19C) developmental acclimation (Dev treat), followed by constant (19C or 23C) adult acclimation (Ad treat), or fluctuating (19F) (Dev treat), followed by fluctuating (19F or 23F) (Ad Treat) (see Fig. 2). Panel A and B: CT_{max} estimates at ± 4 ($N = 10$) and $\pm 8^\circ\text{C}$ ($N = 15$) amplitudes, respectively. Panel C and D: CT_{min} estimates at ± 4 ($N = 10$) and $\pm 8^\circ\text{C}$ ($N = 15$) amplitudes, respectively.

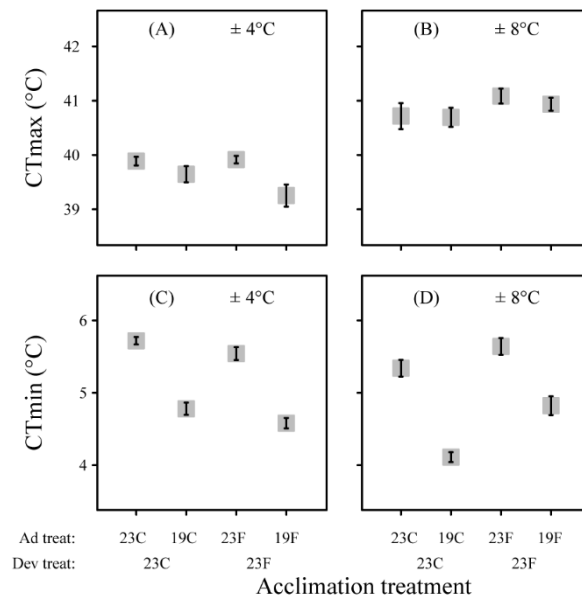


Figure 7. Critical thermal limits (mean \pm s.e.m) for treatment groups assayed after 9 days post emergence (mean developmental temperature 23°C). The flies were exposed to a combination of mean temperature exposures within thermal regime, i.e. constant (23C) developmental acclimation (Dev treat), followed by constant (19C or 23C) adult acclimation (Ad treat), or fluctuating (23F) (Dev treat), followed by fluctuating (19F or 23F) (Ad Treat) (see Fig. 2). Panel A and B: CTmax estimates at ± 4 ($N = 10$) and $\pm 8^\circ\text{C}$ ($N = 10$) amplitudes, respectively. Panel C and D: CTmin estimates at ± 4 ($N = 19, 20, 19, 20$) and $\pm 8^\circ\text{C}$ ($N = 10$) amplitudes, respectively. N values correspond to each treatment respectively.