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Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context

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Summary

1. Biologists have long been concerned with measuring thermal performance curves and limits because of their significance to fitness. Basic experimental design may have a marked effect on the outcome of such measurements, and this is true especially of the experimental rates of temperature change used during assessments of critical thermal limits to activity. To date, the focus of work has almost exclusively been on the effects of rate variation on mean values of the critical limits.

2. If the rate of temperature change used in an experimental trial affects not only the trait mean but also its variance, estimates of heritable variation would also be profoundly affected. Moreover, if the outcomes of acclimation are likewise affected by methodological approach, assessment of beneficial acclimation and other hypotheses might also be compromised.

3. In this article, we determined whether this is the case for critical thermal limits using a population of the model species *Drosophila melanogaster* and the invasive ant species *Linepithema humile*.

4. We found that effects of the different rates of temperature change are variable among traits and species. However, in general, different rates of temperature change resulted in different phenotypic variances and different estimates of heritability, presuming that genetic variance remains constant. We also found that different rates resulted in different conclusions regarding the responses of the species to acclimation, especially in the case of *L. humile*.

5. Although it seems premature to dismiss past generalities concerning interspecific and acclimation-related variation in critical thermal limits, we recommend that conditions during trials be appropriately selected, carefully reported and rigorously controlled.

Key-words: Argentine ant, broad-sense heritability, CT_{Max}, CT_{Min}, heating rate, macrophysiology, phenotypic plasticity

Introduction

The ability of organisms to remain active across an environmentally appropriate range of temperatures is a significant component of fitness (Kristensen, Loeschcke & Hoffmann 2007; Loeschcke & Hoffmann 2007). In consequence, biologists have long been concerned with thermal limits to activity, the shape of thermal performance curves and the mechanisms underlying variation therein (Andrewartha & Birch 1954; Cossins & Bowler 1987; Huey & Kingsolver 1993; Kingsolver & Huey 1998; Hochachka & Somero 2002; Ghalambor *et al.* 2006). Owing partly to concerns about how organisms will cope with modern climate change (Helmuth, Kingsolver & Carrington 2005; Parmesan 2006), renewed attention is being given to the nature, form and evolution of thermal performance (Angilletta, Niewiarowski & Navas 2002; Hoffmann, Sørensen & Loeschcke 2003a; Chown & Terblanche 2007; Ghalambor *et al.* 2007; Pörtner & Knust 2007). One theme emerging from the recent work is that, during assessments of thermal tolerance, basic experimental design may have a marked effect on the outcome of the work, either because different approaches assess different thermal tolerance traits (Hoffmann *et al.* 2003a; Chown & Nicolson 2004; Rako *et al.* 2007; Kristensen *et al.* 2008) or because variations on a single approach may affect the end result (e.g. Worland 2005; Rako & Hoffmann 2006).

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The rate of temperature change adopted in a trial appears to have an especially pronounced effect on estimates of critical thermal limits – a widely used measure of thermal activity range (Lutterschmidt & Hutchison 1997a; Beitinger, Bennett & McCauley 2000; Chown & Nicolson 2004; Somero 2005; Folk, Hoekstra & Gilchrist 2007). The typical expectation and finding has been that slow rates of temperature change improve either the critical thermal minimum (CT_{Min}) or maximum (CT_{Max}), owing to hardening, a short-term form of phenotypic plasticity (Kay & Whitford 1978; Kelty & Lee 2001; Powell & Bale 2006). However, a recent study has shown that the opposite may also be found. In the tsetse, *Glossina pallidipes*, slow rates of cooling elevated the CT_{Min}, while slow rates of warming reduced the CT_{Max} (Terblanche *et al.* 2007a; see also Overgaard *et al.* 2006, and Cocking 1959; Mora & Maya 2006 for data on fish). Although these results have highlighted the extent and form of the response of organisms to different rates of temperature change, and the importance of ecologically relevant experimental treatments for assessing thermal tolerance, they have been concerned largely with the mean values of critical limits, and short-term plasticity or hardening under a single set of conditions.

However, when considering the evolution of traits, including those of thermal tolerance, it is not simply the mean value that is significant, but also the variance around that mean (Endler 1986; Garland & Kelly 2006). Indeed, heritable variation is an important prerequisite for natural selection (Endler 1986; Hoffmann *et al.* 2003b; Blows & Hoffmann 2005), a major means by which evolution can take place. Moreover, wherever genetic accommodation or assimilation might be involved in the evolution of new trait values (see West-Eberhard 2003; Pigliucci, Murren & Schlichting 2006; Ghalambor *et al.* 2007), how trait values respond to different environmental conditions via phenotypic plasticity is important.

If the rate of temperature change used in an experimental trial assessing CT_{Min} or CT_{Max} affected not only the mean of the trait, but also its variance, then estimates of heritable variation would also be profoundly affected. For example, if the genetic contribution to phenotypic variance remained relatively constant (Riska, Prout & Turelli 1989; Yassin *et al.* 2007), but total phenotypic variance declined with an increase in the rate of temperature change, then estimates of broad sense heritability would increase, recalling (see e.g. Hartl 1980) that broad sense heritability is calculated as:

$$h^2 = \sigma_g^2 / \sigma_p^2 \qquad \text{eqn 1}$$

Likewise, if the response to acclimation (a longer term form of plasticity than hardening, Hoffmann *et al.* 2003a) differed significantly among cooling or heating rates then at best interactions among hardening and plasticity would have been detected (e.g. Rako & Hoffmann 2006). That is, it might be concluded that the extent of acclimation differs significantly depending on the rate of temperature change used, with some rates resulting in larger acclimation effects than others. However, at worst, estimates of the extent and direction of plasticity would be completely confounded. That is, at different rates of change not only might the full extent of plasticity differ, but the acclimation response might be completely different at opposite ends of the rate of temperature change spectrum.

In consequence, and given the pressing significance of understanding the rate at which thermal tolerance traits might change in response to a changing world (Helmuth *et al.* 2005; Chown & Terblanche 2007), we here investigate the effects of variable cooling and heating rates on the phenotypic variance of CT_{Min} and CT_{Max}, respectively, and their interactions with acclimation. We include investigations of two species. First, we use a model organism, *Drosophila melanogaster*, in which hardening has been found when slow rates of cooling are used (Kelty & Lee 1999, 2001; but see also Overgaard *et al.* 2006). Second, because it is important to understand how broadly information from model organisms might generalize (Feder, Bennett & Huey 2000; Chown, Addo-Bediako & Gaston 2002), we also investigate workers of the Argentine ant, *Linepithema humile*. This species is not only phylogenetically and ecologically distant from *D. melanogaster*, but is also of considerable global significance as an invasive alien (Tsutsui, Suarez & Grosberg 2000; Holway *et al.* 2002), predicted to extend its range as global climates change (Roura-Pascual *et al.* 2004).

Materials and methods

STUDY ANIMALS AND ACCLIMATION CONDITIONS

The *D. melanogaster* flies used in this study originated from a mass laboratory population established in September 2002 (for details see Bubliy & Loeschcke 2005). Flies were taken from a line originally selected at constant 30 °C throughout development every second generation (see Sørensen, Nielsen & Loeschcke 2007). Briefly, egg–pupal development took place at constant 30 °C every second generation for 72 generations (36 selection events). Adults and un-selected generations were maintained at 25 °C. Thereafter, the line was kept unselected at 20 °C for 40 generations. At all times, the line was kept in high numbers (> 1000) to decrease drift and all maintenance took place on standard oatmeal-sugar-yeast-agar *Drosophila* medium at 12L : 12D photoperiodic cycle. Although it might be argued that these flies are somehow unrepresentative of field conditions, this is an especially vexing question. Flies held even under standard laboratory conditions show rapid laboratory adaptation (e.g. Harshman & Hoffmann 2000; Sgrò & Partridge 2000) and *D. melanogaster* is so broadly distributed (see Hoffmann *et al.* 2003a) that it is difficult to know what representative field conditions might mean. In consequence, we are of the view that providing explicit information on the conditions under which flies have been held is more important than attempting to determine whether or not these are fully representative of field conditions.

The flies were bred under uncrowded conditions (*c.* 30 individuals per 7 mL medium) on agar-yeast-sugar-oatmeal medium. Upon emergence, virgin females were collected under CO_2 anaesthesia and transferred in groups of 20 to food vials. The vials were distributed equally among three acclimation temperatures of 15, 20 or 25 °C for 5–7 days. This acclimation period was selected because previous investigations of this species and others have shown that it is sufficient for the development of a full response (i.e. ongoing acclimation does not result in further change) to the altered conditions (Hoffmann & Watson 1993; Terblanche *et al.* 2006). Containers containing flies

were all positioned on the same shelf in the incubator eliminating shelf effects.

Whole *L. humile* colonies were collected in the vicinity of Stellenbosch (33°55′S18°51′E) and returned to the laboratory within 1–2 h. Colonies were then placed at one of four acclimation temperatures (15, 20, 25, 30 °C; 12L : 12D photoperiod) for 7 days. Each acclimated colony consisted of ~500 worker individuals housed in a plastic container ($20 \times 11 \times 8$ cm) lined with fluon (Northern Products, Woonsocket, Rhode Island) to prevent ants from escaping (Walters & Mackay 2003). A colony term was not included in the trials because this species is known to form supercolonies owing to low genetic diversity and selection against colony diversity (Tsutsui *et al.* 2000, 2003). Because the species is sensitive to dry conditions (Walters & Mackay 2003; Schilman, Lighton & Holway 2007), distilled water was made freely available in the containers in the form of moistened cotton wool. Moreover, each small plastic container was housed within a larger one ($25 \times 25 \times 14.5$ cm) lined with a sheet of moistened cotton wool. Ants were fed pin-head crickets once every second day, and a 20% sugar solution was also made freely available. During the acclimation period, containers were rotated daily among shelves within an incubator to avoid shelf effects. Because all workers are females, only females were assessed during the trials.

CRITICAL THERMAL LIMITS

The start temperature for critical thermal limit experiments in *D. melanogaster* (mean ± SD; 1.4 ± 0.05 mg, $n = 57$) was 20 °C, thereby eliminating any possible influence of variations in start temperature on experimental outcome (see Terblanche *et al.* 2007a). Female flies were placed individually in capped, empty, thin-walled 10-mL glass vials. All vials were placed in a horizontal rack and placed in a small tank with a glass front to allow flies to be monitored and scored as they reached their critical thermal limits. The temperature of the water in the tank was controlled by a programmable heating unit (Heto HMT 200 RS, Heto-Holten AS, Allerød, Denmark) which also ensured proper circulation. For tests of CT_{Min}, the water was cooled by 'cooling fingers' (Hetofrig, Heto, Birkerød, Denmark), with circulation provided by the unit described above. After 6 min equilibration time, temperature was increased (CT_{Max}) or decreased (CT_{Min}) at a constant rate of either 0.1, 0.25 or 0.5 °C min⁻¹ ($n = 50$ per rate and per trait) until the end point was observed, defined as the onset of muscle spasms in the case of CT_{Max} (Lutterschmidt & Hutchison 1997b) and loss of coordinated muscle function in the case of CT_{Min} (observed as an inability of flies to maintain an upright posture) (Klok & Chown 2003; Chown & Terblanche 2007). Based on their small body size, the body temperature of the insects was considered equivalent to the chamber temperature (see Stevenson 1985; Terblanche *et al.* 2007a). This in turn remained very close to water bath temperature, which was used to estimate the end point temperature, owing to the small vial size (ø ≈ 1 cm, with no more than a 5-s delay in equilibration).

For *L. humile*, an insulated, double-jacketed system which consisted of 11 isolation chambers for individual ants was connected to a programmable water bath (LTC 12 Grant Instruments Ltd., Cambridge, UK), which regulated water temperature around the chambers (see Klok & Chown 2003). Ten ants were placed singly into the chambers and a 40-gauge copper-constantan (Type T) thermocouple connected to an electronic thermometer (CHY 507 Thermometer, Taiwan) was inserted into a control chamber to monitor chamber temperatures. Based on their small body sizes (mean ± SD; 0.5 ± 0.1 mg, $n = 20$), the body temperatures of the

ants were considered equivalent to that of the chamber, with very little equilibration time required. The start temperature for all critical thermal limit experiments was 25 °C, which was maintained for 6 min, thereby eliminating any possible influence of variation in start temperature on the experimental outcome. Thereafter, temperature was increased (CT_{Max}) or decreased (CT_{Min}) at a constant rate of either 0.05, 0.1, 0.25 or 0.5 °C min⁻¹ until the end point was observed, defined as above (and observed as the loss of righting response and an inability of ants to retract their legs in a coordinated fashion when stimulated for CT_{Min}, and the onset of muscle spasms for CT_{Max}). For each trait, for each rate, and for each acclimation temperature, the trials were repeated until $n \sim 50$.

STATISTICAL ANALYSES

For each species, the effects of rate and acclimation temperature on each of the traits were assessed using a generalized linear model assuming a normal distribution of errors and using an identity link function. Initial investigations of normality using Shapiro-Wilks tests indicated that in a few instances, distributions deviated from normality. Hence, we did not use a general linear model or an ordered factor, orthogonal polynomial contrast ANOVA. The latter analysis is especially sensitive to departures from normality, while the others are less so (Quinn & Keough 2002; Littell, Stroup & Freund 2002). To assess the extent to which variances differed among the rate groups within each acclimation treatment, for each species, Levene's test was used, which is much less sensitive to departures from normality than others such as Bartlett's test (Quinn & Keough 2002).

Results

In *D. melanogaster*, both acclimation temperature and rate of warming significantly affected CT_{Max} (Table 1). The rate of warming had a large, positive effect on CT_{Max}, whereas the effect of acclimation temperature was smaller and more complex, but largely similar irrespective of the rate of warming (i.e. the interaction effect was not significant) (Fig. 1a). For CT_{Min}, the largest effect was that of acclimation, with low temperature acclimation reducing the CT_{Min} by comparison with higher acclimation temperatures (Fig. 1b). By contrast, rate had a much smaller, though significant positive effect on CT_{Min}, such that slower rates of change led to lower CT_{Min} values. The interaction term was not significant (Table 1).

Table 1. Outcome of the generalized linear model of the effects of acclimation temperature and rate of temperature change on CT_{Max} and CT_{Min} in *Drosophila melanogaster*

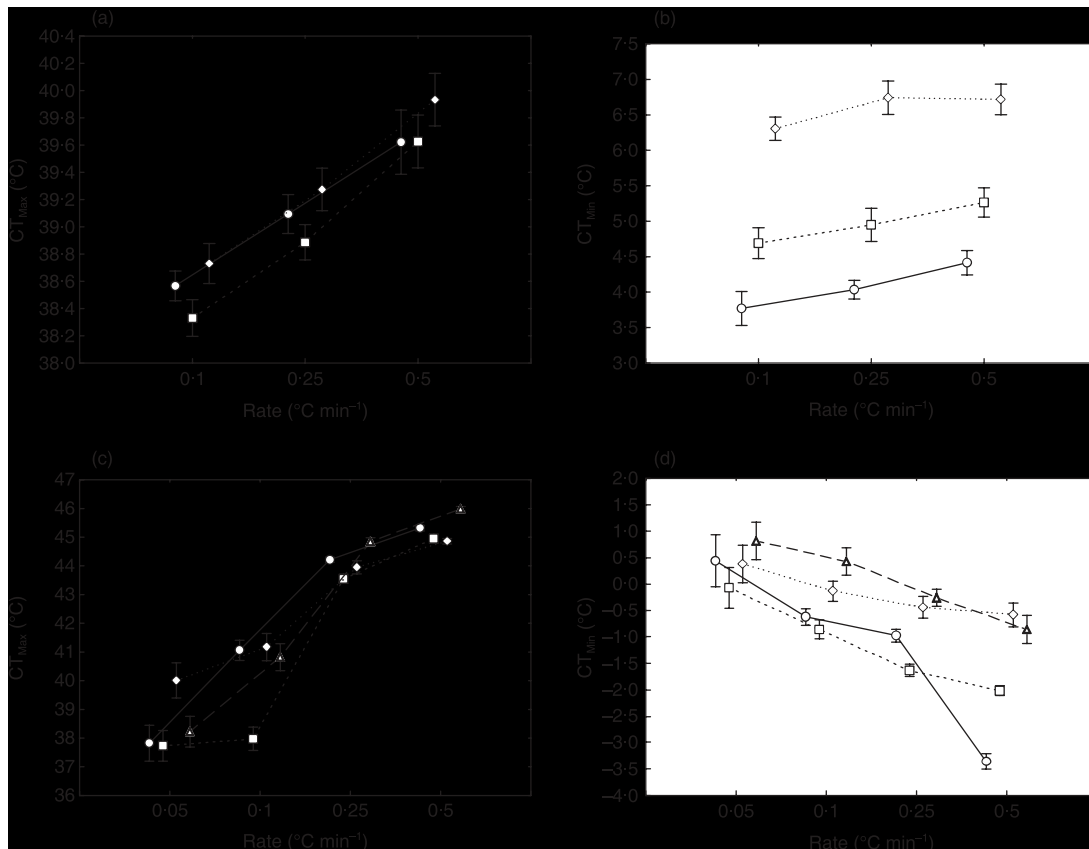| Trait	d.f.	χ^2	*P*
CT_{Max}			
Acclimation	2	29·8	< 0·0001
Rate	2	242·5	< 0·0001
Acclimation × rate	4	2·66	0·62
Deviance/d.f.	147·9/441 = 0·34		
CT_{Min}			
Acclimation	2	512·2	< 0·0001
Rate	2	42·0	< 0·0001
Acclimation × rate	4	4·7	0·319
Deviance/d.f.	229·58/441 = 0·52		

Fig. 1. The effect of acclimation temperature and rate of temperature change on (a) CT_{Max} and (b) CT_{Min} in *Drosophila melanogaster*, and (c) CT_{Max} and (d) CT_{Min} in *Linepithema humile*. Acclimation treatments are circles, 15 °C; squares, 20 °C; diamonds, 25 °C; triangles, 30 °C. Data shown are means ± SE, and symbols at each rate are staggered to ease interpretation.

Table 2. Outcome of a generalized linear model of the effects of acclimation temperature and rate of temperature change on CT_{Max} and CT_{Min} in *Linepithema humile*

Trait	d.f.	χ^2	P
CT_{Max}			
Acclimation	3	148·7	< 0·0001
Rate	3	1372·4	< 0·0001
Acclimation × rate	9	174·5	< 0·0001
Deviance/d.f.	1305·2/774 = 1·69		
CT_{Min}			
Acclimation	3	244·3	< 0·0001
Rate	3	441·3	< 0·0001
Acclimation × rate	9	157·8	< 0·0001
Deviance/d.f.	620·9/774 = 0·80		

Acclimation temperature and rate of temperature change affected both CT_{Max} and CT_{Min} in *L. humile* (Table 2). For CT_{Max}, the effect of rate of temperature change was positive and larger than that of acclimation temperature, which had a complex effect that varied depending on the rate of temperature change (Fig. 1c). In the case of CT_{Min}, the effect of rate of temperature change was negative. Moreover, the positive interaction meant the effect of acclimation temperature was small at the slowest cooling rates, whereas it was large at the

fastest ones (Fig. 1d), the opposite of what was found for CT_{Max}.

The extent to which variances were heterogeneous among rates within acclimation treatments varied with the species and with the trait (Figs 2 & 3, see also Figs S1–4 of the Supporting Information). In *D. melanogaster*, Levene's test was not significant for CT_{Min} across rates within any of the acclimation treatments (Table 3). However, across the full range of acclimation treatments and rates, variances were heterogeneous (Levene's test $F_{(15,774)} = 13·86$, $P < 0·0001$). In contrast, variances were significantly heterogenous for CT_{Max} within each acclimation treatment (Table 3). Variance was largest at the fastest rate of warming, although it was marginally non-significant following acclimation at 25 °C (Fig. 2a and Fig. S1 of the Supporting Information). In the case of *L. humile*, although variances in CT_{Max} were heterogeneous among rates within acclimation treatments (Table 3), they showed the opposite trend to that found in *D. melanogaster*. That is, variances were largest at the slowest rates of warming, irrespective of acclimation temperature (Fig. 3a and Fig. S3 of the Supporting Information). Likewise, in the case of CT_{Min} the slowest rates of cooling resulted in the largest variances (Table 3, Fig. 3b and Fig. S4 of the Supporting Information). This effect was somewhat attenuated at the highest acclimation temperatures.
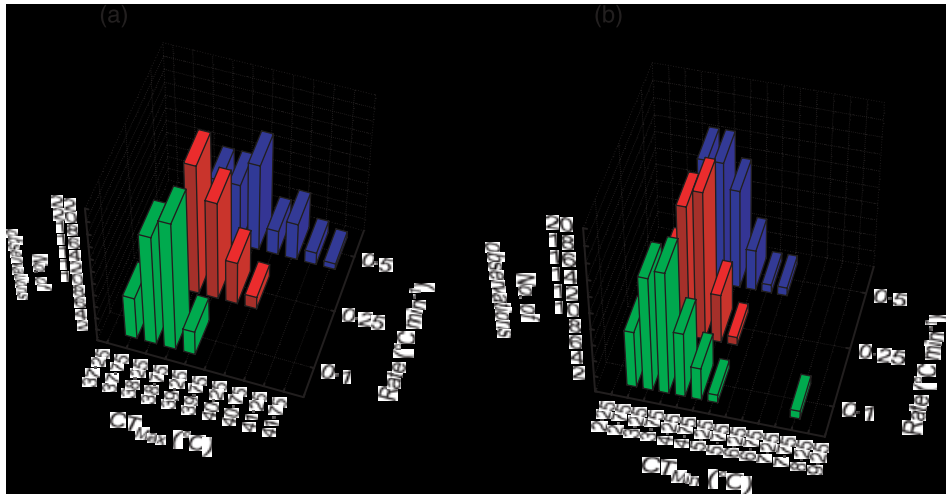
Fig. 2. Frequency distributions of (a) CT_{Max} and (b) CT_{Min} at different rates of temperature change following acclimation to 15 °C in *Drosophila melanogaster*. Frequency distributions for these traits following the other acclimation treatments can be found in Figs S1 and S2 of the Supporting Information.
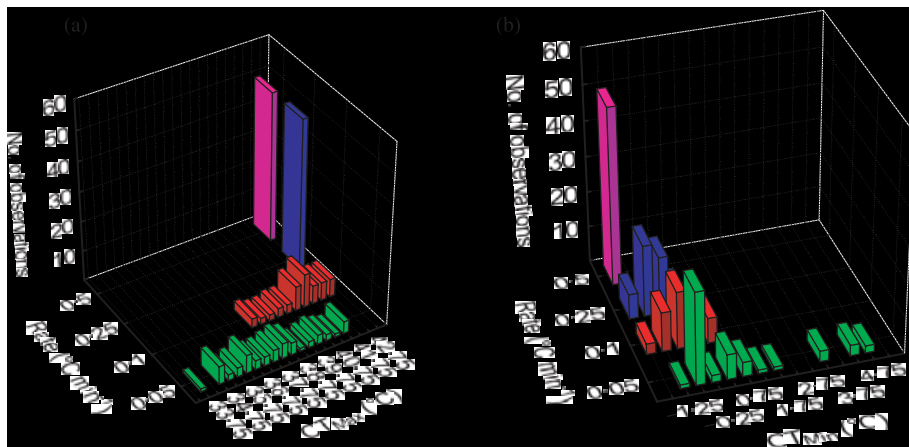

Fig. 3. Frequency distributions of (a) CT_{Max} and (b) CT_{Min} at different rates of temperature change following acclimation to 15 °C in *Linepithema humile*. Frequency distributions for these traits following the other acclimation treatments can be found in Figs S3 and S4 of the Supporting Information.

Discussion

Typically it has been argued that slow rates of change enable some form of hardening, which results in an increase in CT_{Max} or a decline in CT_{Min} (Kay & Whitford 1978; Kelty & Lee 1999, 2001; Powell & Bale 2006). Our results bore out these arguments only for CT_{Min} in *D. melanogaster*, in keeping with previous findings for this species (Kelty & Lee 1999). However, the effect size here (*c*. 0·5 °C) was much smaller than that found previously (2·6 °C, over rates varying from 0·1 to 1·0 °C min^{-1}) possibly reflecting differences between the flies used here and the Oregon-R strain used by Kelty & Lee (1999). Which outcome should be considered 'representative' for the species is more difficult to discern because even the absence of specific selection regimes may result in laboratory adaptation (see e.g. Harshman & Hoffmann 2000), and because the species is so widespread globally.

By contrast, faster rates of heating resulted in higher CT_{Max} values in *D. melanogaster* and in *L. humile*, and faster rates of cooling lowered CT_{Min} in the latter. These findings are in keeping with previous results for tsetse (Terblanche *et al.* 2007a). What the ultimate cause is of the among-species difference in the response of CT_{Min} to rate variation remains unclear. It may well have to do with the absence of rapid cold hardening (Lee, Chen & Denlinger 1987) in more tropical species (Terblanche *et al.* 2007a), or perhaps its absence in species that show substantial behavioural avoidance of low temperature extremes (e.g. Hawes *et al.* 2006; Terblanche, Marais & Chown 2007b), as does the Argentine ant (Witt & Giliomee 1999; Krushelnycky *et al.* 2005). Whatever the explanation for the variation among rates, it is clear that future studies can ill afford to neglect the effects of the experimental protocol on the resultant outcome.

Table 3. Outcome of Levene's test for homogeneity of variances among rates of temperature change within each acclimation treatment for CT_{Max} and CT_{Min} in *Drosophila melanogaster* and *Linepithema humile*

Species/acclimation treatment	*F*	*P*	d.f.
D. melanogaster CT_{Max}			
15 °C	9·17	0·0002	2, 147
20 °C	5·96	0·0032	2, 147
25 °C	2·57	0·0797	2, 147
D. melanogaster CT_{Min}			
15 °C	2·22	0·113	2, 147
20 °C	0·01	0·988	2, 147
25 °C	3·04	0·051	2, 147
L. humile CT_{Max}			
15 °C	54·41	0·0001	3, 196
20 °C	27·41	0·0001	3, 192
25 °C	51·31	0·0001	3, 192
30 °C	56·53	0·0001	3, 194
L. humile CT_{Min}			
15 °C	28·93	0·0001	3, 195
20 °C	22·71	0·0001	3, 195
25 °C	5·33	0·0015	3, 190
30 °C	5·87	0·0007	3, 194

Indeed, these effects extend far beyond a change in means. Here, variances in both traits were affected substantially by the rate of temperature change adopted and this differed markedly among the species, traits, and to a smaller extent among the acclimation treatments. In *D. melanogaster*, the effects of rate on the variance of CT_{Min} were small and apparent only across the full trial, whereas increasing rates of temperature change resulted in increasing variances for CT_{Max}. By contrast, in *L. humile*, higher rates of change resulted in smaller variances of the critical thermal limit estimates. Why these differences among species and traits exist is not yet clear, although the differences among traits and species suggest that the changing variances are not simply an artefact of changing experimental durations. Nonetheless, the implications of these changing variances are important. If the genetic contribution to phenotypic variance remains constant (Riska *et al.* 1989; Yassin *et al.* 2007), which presumably it would amongst a random sample of individuals exposed to different rates of temperature change, then a decline in phenotypic variance (either with an increase or a decline in rate of temperature change as documented here) would lead to a substantial change in the estimate of heritability (see eqn 1). For example, following acclimation to 15 °C, the variance in CT_{Max} in *L. humile* ranged from 4·9 (at 0·05 °C min^{-1}) to 0·11 °C (at 0·5 °C min^{-1}). If the genetic variance is assumed to have a value of 0·1, then the estimate of broad sense heritability would vary from 0·02 at the slowest rate of change to 0·91 at the fastest. In other words, one set of experiments might lead researchers to conclude that CT_{Max} is not heritable, while another might provide evidence that it is a highly heritable trait. The same problem would apply in the case of narrow sense heritability, and particularly when the observed phenotypic variance is used in preference to the summed values of additive and residual variance (see Wilson

2008). Both conclusions would have obvious downstream effects on predictions about the extent to which the trait might evolve (Endler 1986; Falconer & Mackay 1996; Blows & Hoffmann 2005). Clearly, a different set of experiments involving laboratory selection (Gibbs 1999), full sib investigations (Falconer & Mackay 1996) or isofemale lines (Hoffmann & Parsons 1988) could be designed to assess realized heritability. However, unless the phenotypic variance was partitioned identically in each case, the experimental rates of change would still have an effect on estimates of heritability. We know of no work that has sought to investigate the effects of rate of temperature change on variance partitioning using laboratory selection, full sib assessments or isofemale lines.

In addition to their effects on variances, changing rates also had significant and pronounced effects on estimates of the response to acclimation of CT_{Min} and CT_{Max} in *L. humile*. Previous work investigating the effects of acclimation on critical thermal limits, typically undertaken using rates $\geq 0·25$ °C min^{-1}, has demonstrated that CT_{Max} is much less responsive to acclimation treatments than is CT_{Min} (e.g. Klok & Chown 2003; Terblanche *et al.* 2006). These findings are largely in keeping with what we documented here for *L. humile*, and with other investigations of geographic trait variation and responses to selection (e.g. Gilchrist, Huey & Partridge 1997; Kingsolver & Huey 1998; Addo-Bediako, Chown & Gaston 2000; Chown 2001; Kimura 2004). Nonetheless, they also illustrate that different species may have dissimilar responses to both experimental conditions and natural environmental variation (compare the above with the outcomes of work by Hoffmann, Anderson & Hallas 2002 and Calosi, Bilton & Spicer 2008).

Perhaps more importantly, this study has demonstrated that the experimental approaches adopted might substantially affect the conclusions drawn from a particular investigation of acclimation effects on thermal tolerance means and variances, over and above the differences expected from assessments of traits that have different genetic underpinnings (Rako *et al.* 2007). Although it seems premature to dismiss past generalities concerning interspecific and acclimation-related variation in critical thermal limits based on this finding, the latter does beg the question of how different present understanding would be if previous studies had used rates relevant to the environment within which the species occur. Clearly, merit exists in using standardized methods for comparing species and populations, but strong arguments can also be presented in favour of investigating thermal limits using environmentally relevant conditions (Sinclair 2001). Resolving these questions remains a key issue for macrophysiology (Chown, Gaston & Robinson 2004; Chown & Gaston 2008).

Of course, it may be argued that other methods of assessing dynamic thermal tolerance traits (see Lutterschmidt & Hutchison 1997a), such as chill coma recovery (David *et al.* 1998), should be used to avoid problems associated with rates of change used in critical thermal limit experiments. However, because cooling and heating rates are involved in these

assessments too, the problem is unlikely to be resolved. Another alternative might be to suggest that dynamic assessments of thermal tolerance limits should not be used at all. However, because static and dynamic methods are likely to be assessing completely different basal and induced traits, that have different genetic bases (Rako *et al.* 2007), such an approach would not resolve the matter either. To our minds, the most appropriate approach would either be to provide a comparison of outcomes using both a standard rate and an environmentally relevant one, or to be explicit about what the purpose of the work is. Later, comparative investigations would then simply have to include heating or cooling rates in the statistical analyses, much as census area is included in comparisons of avifaunal density because of the profound effect that area has on estimates of density (Gaston, Blackburn & Gregory 1999).

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Critical thermal maxima in *Drosphila melanogaster*.

Fig. S2. Critical thermal minima in *Drosphila melanogaster*.

Fig. S3. Critical thermal maxima in *Linepithema humile*.

Fig. S4. Critical thermal minima in *Linepithema humile*.

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