REVIEW

Ecologically relevant measures of tolerance to potentially lethal temperatures

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Summary

The acute thermal tolerance of ectotherms has been measured in a variety of ways; these include assays where organisms are shifted abruptly to stressful temperatures and assays where organisms experience temperatures that are ramped more slowly to stressful levels. Ramping assays are thought to be more relevant to natural conditions where sudden abrupt shifts are unlikely to occur often, but it has been argued that thermal limits established under ramping conditions are underestimates of true thermal limits because stresses due to starvation and/or desiccation can arise under ramping. These confounding effects might also impact the variance and heritability of thermal tolerance. We argue here that ramping assays are useful in capturing aspects of ecological relevance even though there is potential for confounding effects of other stresses that can also influence thermal limits in nature. Moreover, we show that the levels of desiccation and starvation experienced by ectotherms in ramping assays will often be minor unless the assays involve small animals and last for many hours. Empirical data illustrate that the combined effects of food and humidity on thermal limits under ramping and sudden shifts to stressful conditions are unpredictable; in *Drosophila melanogaster* the presence of food decreased rather than increased thermal limits, whereas in *Ceratitis capitata* they had little impact. The literature provides examples where thermal limits are increased under ramping presumably because of the potential for physiological changes leading to acclimation. It is unclear whether heritabilities and population differentiation will necessarily be lower under ramping because of confounding effects. Although it is important to clearly define experimental methods, particularly when undertaking comparative assessments, and to understand potential confounding effects, thermotolerance assays based on ramping remain an important tool for understanding and predicting species responses to environmental change. An important area for further development is to identify the impact of rates of temperature change under field and laboratory conditions.

Key words: ramping, thermotolerance, chill coma, heat, ecological relevance, ectotherm.

Introduction

The study of organismal thermal tolerance limits is important for a variety of reasons. At the most fundamental level it provides insights into the conditions that have been limiting for life over the course of its evolution (Pörtner, 2002; Boussau et al., 2008), and the functional basis for such limits (Prosser, 1986; Lutterschmidt and Hutchison, 1997a; Feder and Hofmann, 1999; Pörtner, 2001; Hochahcka and Somero, 2002; McNab, 2002). Comparative physiological investigations, now typically undertaken under the broader rubric of evolutionary physiology (Feder et al., 2000), have proven especially fruitful for understanding environment-related variation in thermal tolerance limits, the mechanistic underpinnings thereof, and their evolution (Scholander et al., 1950; Vernberg, 1962; Brattstrom, 1970; Bartholomew, 1987; Huey and Kingsolver, 1993; Hoffmann and Parsons, 1997; Somero, 2004; Kellermann et al., 2009). Investigations of thermal limits have also long been of interest to ecologists. Possibly owing to the close original association between ecology and physiology [reviewed partly in Huey (Huey, 1991) and Gaston et al. (Gaston et al., 2009)], it is

widely appreciated that mechanistic understanding of the distribution and abundance of organisms requires information about their physiological capabilities and the mechanisms underlying them (Gaston, 2009). This realization has been enunciated most clearly by Andrewartha and Birch, who wrote: 'If one wishes to explain the distribution and numbers of a certain species of animal, here is one way of going about the job which we have found especially profitable. It is best to describe the method as if it were done in three stages, although, in practice, it is usually best to have the three stages going forward at the same time: a) The physiology and behaviour of the animal must be investigated… b) The physiography, climate, soil and vegetation in the area must be investigated… c) The numbers of individuals in the population that has been selected for study must be measured as accurately as practicable' [p. 10 (Andrewartha and Birch, 1954)].

Thus, the significance of physiological tolerances for variation in species abundances and distributions, and their influence on the outcomes of interspecific interactions, has been a long-running theme in biological research (Park, 1962; Menge and Sutherland,

1987; Chase, 1996; Cerdá et al., 1997; Davis et al., 1998; Worthen and Haney, 1999; Somero, 2005; Gaston, 2009). However, the impetus for further understanding in the area has grown recently for several major reasons, of which four are most notable. First, early macroecological studies made a variety of assumptions about broad-scale variation in tolerances that had not been comprehensively investigated, and this stimulated renewed interest in large-scale comparative physiological work and the rise of macrophysiology (reviewed in Chown et al., 2004; Gaston et al., 2009). Second, growing concerns about the biodiversity impacts of global environmental change have stimulated research across several fronts seeking to understand the relative contributions of various factors that influence abundance structure and range limits, how these factors are likely to mediate the responses of organisms to such change, and what the outcomes of current and future environmental change are likely to be (Porter et al., 2000; Helmuth et al., 2005; Helmuth et al., 2010; Soberón, 2007; Pörtner and Farrell, 2008; Chown and Gaston, 2008; Kearney and Porter, 2009). Perhaps most notable among these studies are those that have indicated limited plasticity or evolutionary lability in some traits (Hoffmann et al., 2003; Stillmann, 2003; Hoffmann and Willi, 2008; Kellermann et al., 2009; Mitchell and Hoffmann, 2010); latitudinal variation in safety margins between current tolerances and present environmental conditions, which may have already been exceeded (Deutsch et al., 2008; Huey et al., 2009; Sinervo et al., 2010); and the importance of understanding the population-level consequences of spatial (intraspecific) variation in tolerances and of extreme events (Chown et al., 2010; Hoffmann, 2010; Helmuth et al., 2010). Third, several studies have found that spatial variation in diversity may in part be a consequence of the effects of niche conservatism, reflected in physiological traits such as thermal tolerance and desiccation resistance (Wiens and Graham, 2005; Wiens et al., 2006; Algar et al., 2009), thus further stimulating efforts to understand the nature and rates of evolution of physiological characteristics. Fourth, there has been considerable recent interest in the molecular basis of physiological tolerances to understand genetic changes underlying adaptive shifts (Hoffmann and Willi, 2008; Dalziel et al., 2009).

Understanding the fundamentals of animal physiology and their ecological implications therefore remain crucial elements of biology, and especially applied biology concerning mitigation of and adaptation to the impacts of global environmental change (Chown and Gaston, 2008; Williams et al., 2008; Wiens et al., 2009). The development of such understanding begins with measurement. Thus, comprehension of the extent to which different forms of measurement are likely to represent estimates of the same traits, and how measurement conditions may influence experimental outcomes, have not only long been of concern, but have also recently become the focus of renewed attention. That the measurement approach adopted may affect the results of the measurement is widely appreciated for a variety of physiological traits (reviewed in Chown and Nicolson, 2004). In the case of thermal tolerance, much previous work has been undertaken on how the rates of cooling might affect survival and freezing points (reviewed in Chown and Terblanche, 2007), and how particular experimental circumstances might affect estimates of upper thermal limits (e.g. Hoffmann et al., 1997) or might indeed represent measurements of different traits (Sgrò et al., 2010). More recently, concerns have developed especially about how the rate of cooling or heating and the initial temperature conditions in critical thermal limit experiments are likely to affect not only estimates of the mean values of these limits, but also estimates of the impact of acclimation, of variance in the traits, and of their heritability (Terblanche et al., 2007a; Chown et al., 2009; Mitchell and Hoffmann, 2010). Given that slow heating rates typically result in lower estimates of critical thermal maxima (CT_{max}) than faster ones (Terblanche et al., 2007a), and that the slower rates are more likely to represent conditions experienced by organisms in the field (Sinclair, 2001), the outcomes of this recent work have caused further qualms about the ability of organisms, especially tropical ones, to cope with increasing global environmental temperatures (Rezende et al., 2011). However, the study by Rezende and colleagues (Rezende et al., 2011) has suggested that these concerns may not be warranted because investigations of the impacts of heating rates on mean values, variances and heritability are confounded in several ways. Most notably, the confounding factors are thought to be the simultaneous effects of desiccation and starvation on organisms during slow ramping experiments [dynamic experiments in the terminology of Lutterschmidt and Hutchison (Lutterschmidt and Hutchison, 1997a)], and the statistical complexities that arise as a consequence of relationships among rate of temperature change, duration of the trial and CT_{max} (Rezende et al., 2011).

Given these concerns, the proliferation of approaches to measuring thermal tolerance (e.g. Huey et al., 1992; Hazell et al., 2008), evidence that different measures may well be assessing different traits (Hoffmann et al., 1997; Loeschcke and Hoffmann, 2007; Sgrò et al., 2010), and repeated calls for consideration of realized field conditions in physiological ecology (e.g. Bale, 1987; Sinclair, 2001; Helmuth et al., 2010), we provide a critical review of thermal tolerance measures in an ecological context. That context is predominantly the way in which ecological realism has to be traded off against physiological confounding effects and the time scale of animal responses. We focus specifically on the available measures of tolerances to potentially lethal temperatures in terrestrial invertebrate ectotherms. Although sublethal effects are of considerable significance in a demographic context (see Baust and Rojas, 1985; Sinclair, 2001), the trials conducted are often similar to the ones discussed here, except that the extent of exposure to a given set of conditions (either time or maximum temperature) is varied (see Renault et al., 2004; Sinclair and Chown, 2005; Marshall and Sinclair, 2011). In particular we consider the environmental context of upper and lower thermal tolerance measures, where particular measures lie on the thermobiological scale (Vannier, 1994) (see also Hoffmann, 2010), the relationships among different measures and the evidence that they represent different traits, and the conditions under which confounding factors are likely to influence tolerance measures and estimates of mean, variance, heritability and acclimation effect. A further reason for this review is the fact that recent physiologically based prognoses of a bleak fate for tropical organisms under warming climates (Deutsch et al., 2008; Huey et al., 2009; Kearney et al., 2009; Dillon et al., 2010; Sinervo et al., 2010) are likely to substantially influence international policy through mechanisms such as the Intergovernmental Panel on Climate Change.

The environmental context

Classic mechanistic, experimental physiology frequently proceeds by holding as many conditions as possible constant and varying one parameter, such as oxygen tension, to understand its influence on an organism and the corresponding physiological responses that might be mounted to deal with the changed conditions. Much comparative physiology, which sets out to understand the patterns of and the mechanisms underlying adaptation, has proceeded in a

similar fashion (Bartholomew, 1987). That is, to understand the ability of organisms to deal with a certain set of circumstances, a standard set of conditions is used and either the population or species being assessed changes. Although such an approach has provided considerable insight into physiological variation and the mechanisms underlying it, as has the assessment of species or populations living in extreme *versus* less extreme environments (Bartholomew, 1987), it has also been criticized for a lack of ecological relevance. For insects, the selection of a 1°C min⁻¹ cooling rate to investigate the temperature at which freezing takes place and the survival of this event provides a salient example. Although initially set as a benchmark for comparative investigations of cold hardiness (see Salt, 1966; Block, 1990), this rate and neglect of duration of exposure and sub-lethal effects were criticized because of their lack of ecological relevance (Baust and Rojas, 1985; Bale, 1987; Renault et al., 2002). Additional concerns have since been raised about the effects of repeated exposures (Brown et al., 2004; Sinclair and Chown, 2005; Marshall and Sinclair, 2010), how the consequences of exposures to stress should be assessed (e.g. the time period over which survival should be assessed and the extent to which fecundity is affected) (Baust and Rojas, 1985; Bale, 1987; Renault, 2011), and the impacts of multiple stressors given that in a natural setting a variety of conditions change simultaneously (Ring and Danks, 1994).

Such tension is understandable. Many comparative physiological studies set out to examine the mechanisms underlying adaptive physiological variation, rather than to determine the explicit consequences of that variation for population dynamics. Therefore, the approach adopted was, and in many instances still remains, perfectly acceptable. However, the data generated from such studies have subsequently been pressed into service for other purposes, such as understanding the ecological implications of physiological variation. In this situation, attention must be paid to the ecological context of the measurements, but the lack of relevance of the comparative physiological data cannot simply be assumed. For example, in the cooling rates example discussed above, several studies have shown that rate variation has little impact on estimates of the freezing point (Salt, 1966; Slabber et al., 2007). Likewise, even if the conditions under which the organisms have been assessed do not entirely match those of the natural environment, the extent of the significance of this mismatch depends on the nature and scale of the question (Chown et al., 2003). Where broad spatial scale comparisons of thermal tolerance or performance are involved, the assumption is typically made that any error will not be directional, and therefore might add noise to the investigation but no directional bias (Huey et al., 2009). Although such an assumption seems to be reasonable, little broad-scale data exist to examine it, and the question of whether a standard set of conditions should be used to make comparisons among species and populations from different areas, or whether those comparisons should be made using relevant environmental conditions for each of the species and/or populations, remains one of macrophysiology's greatest challenges (Gaston et al., 2009). Nonetheless, just as the use of environmental niche models is imperfect, but necessary to peer into the future of environmental change (Wiens et al., 2009), so too must macrophysiological comparisons proceed with the data at hand (e.g. Deutsch et al., 2008; Huey et al., 2009). However, in so doing they must continue to give attention to the physiological complexities of animal responses to the environment, such as responses to repeated exposures (Brown et al., 2004; Marshall and Sinclair, 2010) and the fitness consequences of acclimation or acclimatization (Kristensen et al., 2008).

In the context of thermal tolerance limits of small ectotherms, microclimatic estimates of the conditions encountered by organisms, their rates of change, the durations of sub-lethal conditions, the return times of particular events, and the frequency with which biologically significant thresholds are crossed are all significant (Gaines and Denny, 1993; Vasseur and Yodzis, 2004; Chown and Terblanche, 2007; Pincebourde et al., 2007; Helmuth et al., 2010). In particular, they will play a major role in determining the extent to which a population will be able to grow in an area, mediated through physiological responses, bearing in mind that behavioural flexibility will influence exposure to a given set of conditions (Huey, 1991; Marais and Chown, 2008) and that migration is likely to substantially affect the extent of phenotypic flexibility and local adaptation (Chown and Terblanche, 2007; Chevin et al., 2010). Measuring such microclimate parameters and then applying them to estimates of key physiological traits, such as critical thermal limits, is therefore important for estimating the impacts of changing conditions on ectotherms. However, such an approach also introduces complexity, some of which has been at the forefront of recent discussions of measurement conditions (Terblanche et al., 2007a; Rezende et al., 2011).

First, although microclimatic measurements that are relevant to a particular organism or set of organisms are frequently made, and may include data from operative models (e.g. Chown and Klok, 2011), these data are often not long-term, nor are they readily available online as are data from standard meteorological stations for use in global comparisons. Moreover, they seldom include measures of variance. Downscaling approaches are capable of resolving these data deficits and are becoming increasingly available (e.g. Kearney and Porter, 2009). Further examination of their utility by comparison of model outputs with biophysical data under a range of conditions would do much to show how they could improve macrophysiological investigations, especially when macroclimatic conditions and those experienced by the organisms in question are likely to differ dramatically through space (Helmuth et al., 2010).

Second, a trade-off will always exist between what is or has been considered a reasonable approach for laboratory investigations and how this might relate to the situation in the field. For example, at a standard heating rate of 0.25°C min⁻¹, towards the slower end of the range of rates reported in the literature, the CT_{max} of the tsetse fly *Glossina pallidipes* is *ca.* 45°C (Terblanche et al., 2006; Terblanche et al., 2007a). Population investigations indicate that in this species, mortality reaches 100% by 40°C, well below the CT_{max}, although daily instantaneous mortality rates in the field do indeed increase with increasing temperature above *ca.* 27°C (Hargrove, 2004). However, at a rate of 0.06°C min⁻¹, much closer to that found in the field [mean of 0.050±0.010°C min⁻¹ recorded in Terblanche et al. (Terblanche et al., 2007a)], the CT_{max} declines to levels close to this value [*ca.* 40°C from a starting temperature of 35°C (Terblanche et al., 2007a)]. What appears to be a discrepancy between laboratory and field conditions, so questioning the relevance of the CT_{max} estimate, is clearly an artefact of the approach adopted in the laboratory. Therefore, when undertaking comparative work, or even when investigating thermal limits, rates of change are important to consider. Those recorded in the field are typically much lower than those used in the laboratory. Indeed, routinely used rates in the laboratory are among the fastest small ectotherms would likely ever encounter and a rate of 1°C min⁻¹ is far beyond what might be expected (Fig. 1), except perhaps in highly unusual situations, such as during the
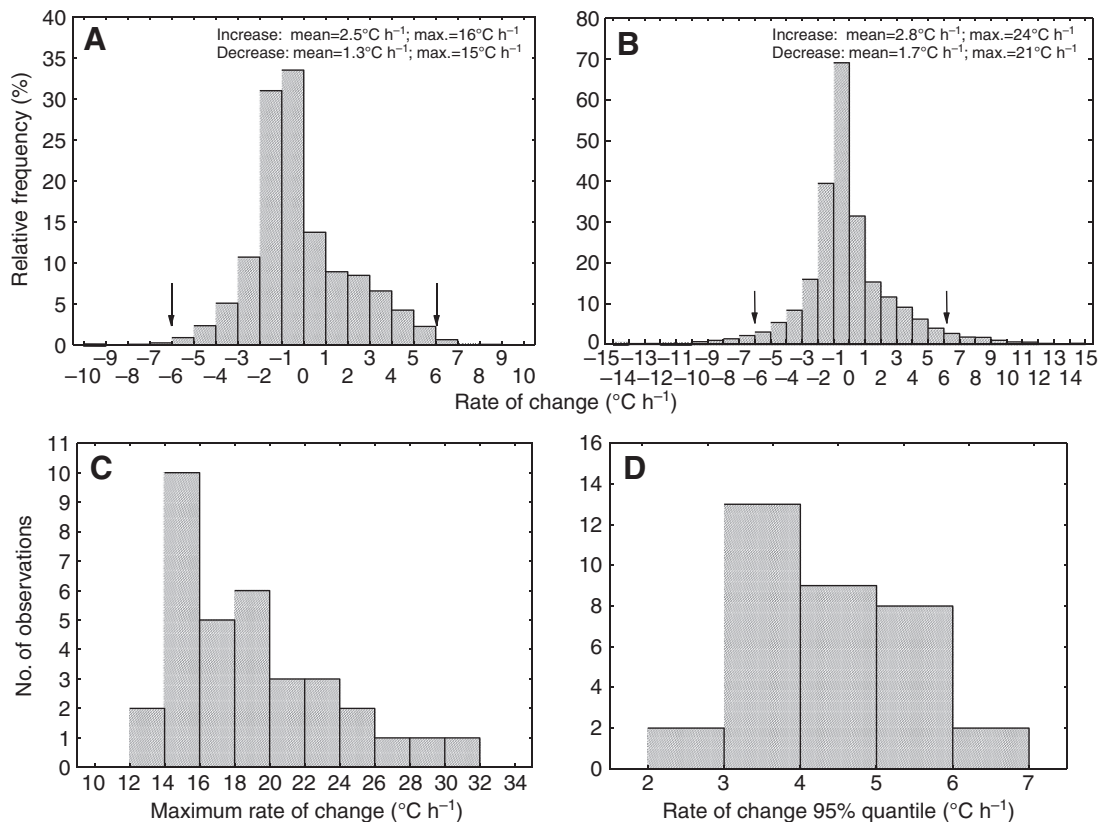
Fig. 1. Frequency distribution of the rate of change in temperature (°C h^{-1}) just below the soil surface in the Cederberg mountains of South Africa [see Botes et al. (Botes et al., 2006) for background information on the sites]. The data were recorded between 2002 and 2009 using Dallas Thermochron iButtons (DS1921 and similar models) and processed using custom-written software in the R environment. (A) Rates of change for the sea level site, (B) rates of change for the 1926 m site, (C) maximum rate of change (up and down, hence 34 values) recorded for each of the 17 sites, and (D) 95% quantile of the rates of change for each of the 17 sites. Arrows in A and B indicate 0.01°C min^{-1} change (6°C h^{-1}). Note that typical laboratory rates are 0.25°C min^{-1} (15°C h^{-1}), 0.5°C min^{-1} (30°C h^{-1}) or sometimes even 1°C min^{-1} (2°C h^{-1}); a mean rate typical of many of the sites investigated here corresponds to 0.0333°C min^{-1}.

passage of extreme weather fronts. In other words, lower rates are more likely to reflect the situation in the field except perhaps during rare, extreme conditions (see also Sinclair, 2001), and for thermal maxima the typical finding is that lower rates of warming result in lower values of the CT$_{max}$ or upper thermal limits (Terblanche et al., 2007a; Chown et al., 2009; Mitchell and Hoffmann, 2010) (but see Chidawanyika and Terblanche, 2011). At the limit, work on long-lived Antarctic marine species is showing that rates of change in the order of 1°C per month result in estimates of upper thermal limits of *ca*. 2°C, although at rates of 1°C day^{-1} (or *ca*. 0.0007°C min^{-1}), which is still slow compared with a laboratory experimental rate of 0.25°C min^{-1}, upper limits are *ca*. 12°C (Peck et al., 2009). Whether such effects apply to shorter-lived, terrestrial species is not known. Regardless, what these rate effects indicate is that investigations of thermal tolerances and forecasts of responses to temperature change need to: (1) consider rates of change explicitly in experimental designs (e.g. 1°C min^{-1} may be convenient for gathering much data in little time, but may be so far from what organisms encounter on a daily basis that the trials become irrelevant); (2) take them into account appropriately in statistical models (Terblanche et al., 2007a); or (3) at the very least acknowledge that they will add noise to any large interspecific analysis. Such time–temperature interactions have long been the subject of interest in physiological research [for insects see

the review in Chown and Nicolson (Chown and Nicolson, 2004)].

Third, organisms do not experience variation in one measure of the abiotic environment independently of others. Although investigations of cross tolerance are often undertaken (e.g. Cloudsley-Thompson, 1969; Hayward et al., 2001; Hoffmann et al., 2005; Chown et al., 2007a), altering more than a single variable at a given time is often not done because it does not always fit comfortably with the classic experimental approach (described in the first paragraph of this section), and can quickly become experimentally intractable (Gaston et al., 2009). Nonetheless, many factors often change simultaneously in the environment. For example, above 0°C, as temperature increases so does saturation deficit even if the water content of the air is kept constant. Below 0°C, a supercooled organism will experience greater drying than one that is frozen (Lundheim and Zachariassen, 1993). Moreover, ectotherms can also substantially alter these interactions in various ways. At the limit they may have an extended phenotype that substantially alters environmental conditions, such as is found in termites (Turner, 2000). More typically, behavioural adjustments can substantially alter the environment experienced by ectotherms, as has long been known (Huey, 1991; Angilletta, 2009). Behavioural flexibility may, in turn, dramatically affect the responses organisms are capable of mounting. For example, mobile adult kelp flies show little evidence of beneficial acclimation

Table 1. Measures of acute thermal tolerance in ectotherms

Endpoint measured	Description	Ramping or static stress usually applied?	Example
Survival	Immature or adult survival (or eclosion/pupation) following stress. For low temperature, =cold-death point (*sensu* Mellanby, 1939).	Either approach	Krebs and Loeschcke, 1999; Hammond and Hofmann, 2010
Loss of activity or movement	Organisms become inactive or lose a righting response as temperature changes. Threshold temperature for spontaneous movement (*sensu* Mellanby, 1939).	Ramping	Klok and Chown, 1997; Hazell et al., 2008; Yang et al., 2008
Recovery	Ability or time to recover locomotor activity or other trait such as fertility after stress. Chill coma tolerance (*sensu* Gibert and Huey, 2001).	Static	Gibert and Huey, 2001
Recovery temperature	Temperature at which animal recovers locomotor ability following a stress full temperature. Most often applied to low temperature dynamic trials (e.g. chill coma recovery).	Ramping	Klok and Chown, 2001
Knockdown temperature	Temperature at which animals are knocked down measured in different containers. For low temperature, =chill coma temperature (*sensu* Mellanby, 1939).	Ramping	Huey et al., 1992; Mitchell and Hoffmann, 2010
Knockdown time	Time to knockdown following stress exposure.	Either approach	McColl et al.,1996; Mitchell and Hoffmann, 2010
Change in metabolic rate	Sudden change in metabolic rate as thermal thresholds reached.	Ramping	Lighton and Turner, 2004; Sinclair et al., 2004; Stevens et al., 2010
Critical temperature or supercooling point	Exotherm associated with latent heat of crystallization.	Either approach	Lee and Denlinger, 1991

because they can move quickly to the cold sites they prefer, in contrast to their larvae (Marais and Chown, 2008). Of course, ideal habitat choice is not always possible and this may also have profound effects on fitness, at times necessitating quantification of the spatial structure of the thermal environment and the influence of interacting individuals (Angilletta, 2009). Other aspects of the environment may also determine how an individual experiences and makes use of the thermal environment. For example, in migratory locusts, low-quality diets lead individuals to seek out increasingly lower temperatures, whereas with an increase in diet quality warmer areas are sought (Coggan et al., 2011). Such interactions are rarely considered explicitly in assessments of thermal effects.

Measuring thermal limits

Thermal tolerance limits are most usefully considered in terms of the thermobiological scale (e.g. Cowles and Bogert, 1944; Vannier, 1994) or its equivalents. The scale can also be considered an integration of the performance curves that are typically used for various traits (for a review, see Angilletta et al., 2002). Typically, sublethal impacts on fertility and viability set in well before knockdown temperatures (or critical thermal limits), and these in turn are often less extreme than the lethal limits. However, these relationships depend critically on the duration of exposure, especially in lethal limit and critical thermal limit experiments, and on the extent to which the individuals in question have been exposed to sub-lethal stresses either over the short term (hardening) or longer term (either developmental acclimation or short-term acclimation), and their responses to these exposures. What exactly constitutes a hardening treatment and what constitutes acclimation has been discussed in a variety of contexts (e.g. Bowler, 2005; Sinclair and Roberts, 2005; Loeschcke and Sørensen, 2005) and is much like physiological time (Taylor, 1981) in that it depends to some extent on the duration of the stage in question. For example, an adult mayfly may live for only a few days, making insistence on a 7 day acclimation period meaningless. Much has also been made of classifications of the extent to which phenotypic plasticity

is fixed or reversible and whether it takes place among or within life stages [see discussions in Wilson and Franklin (Wilson and Franklin, 2002), Woods and Harrison (Woods and Harrison, 2002), Piersma and Drent (Piersma and Drent, 2003) and Chown and Terblanche (Chown and Terblanche, 2007)]. The most important point is that the conditions experienced by the organism or to which it is exposed in the laboratory should be well documented. In that case the terminology can be re-interpreted should new information come to light (Loeschcke and Sørensen, 2005). Sound documentation of experimental conditions also applies to the time the animals have spent in the laboratory. Even short periods may lead to acclimation to laboratory conditions (Terblanche et al., 2007b) and, after multiple generations in the laboratory, genetic adaptation may also take place (Sgrò and Partridge, 2001; Hoffmann et al., 2001). Nonetheless, in many cases responses to changed conditions, either through explicit acclimation trials or to laboratory conditions, may not be realized. Indeed, some traits may remain highly conserved irrespective of time of separation between populations (Klok and Chown, 2005) and in some instances there is little change under laboratory culture (Krebs et al., 2001).

Given the above, much variation exists in how responses across the thermobiological scale are measured (Table 1). Measurement approaches have a venerable history in the physiological literature and can often be traced back to key papers, or at least are often anchored to these key works. For example, for ectotherms, the criteria used for lethal thermal limits are often those laid out by Cowles and Bogert (Cowles and Bogert, 1944), although subsequent work has investigated these in more detail (Lutterschmidt and Hutchison, 1997a; Lutterschmidt and Hutchison, 1997b). Although much has been learned since many of the early studies, and the range of experimental approaches has diversified, much can still be gained from using terminology that was coined during the initial stages of development of the field. In particular, Mellanby (Mellanby, 1939) suggested that the term 'chill coma temperature' be used for temperature at which an insect is immobilized and the 'cold-death point' for the temperature below which exposure is lethal. He also pointed out that the lowest

temperature at which activity is possible (the chill coma temperature) is typically lower than that at which spontaneous movement takes place (called the threshold temperature for spontaneous movement), and argued that the two measures always be distinguished because they differ [for recent review of chill coma nomenclature see Hazell and Bale (Hazell and Bale, 2011), and for possible mechanisms underlying chill coma see MacMillan and Sinclair (MacMillan and Sinclair, 2011)]. Although a variety of solutions have been proposed to measure these different endpoints (see Huey et al., 1992; Hazell et al., 2008), typically they all have problems that need to be considered. For example, measurement of chill coma requires that movement really is no longer possible. A righting response test (e.g. Klok and Chown, 1997) may be the only way to assess this because many species do not move spontaneously in the absence of stimulation. Contrary to previous views (Hazell et al., 2008), these tests do not require removal of the animal from the testing conditions and nor is this typically done. Alternatively, the most effective way to assess the temperature threshold for spontaneous movement in continuously moving species is clearly through automated methods. For larger ectotherms, such as lizards, automated methods may not be viable, especially if the lethal temperature is close to the temperature at which spontaneous movement or movement of any kind ceases. Moreover, if insight into the underlying physiology is required, then methods such as thermolimit respirometry (Lighton and Turner, 2004; Klok et al., 2004) (Table 1) may be most appropriate because they provide proxies of the metabolic state of the animal concerned.

Bearing these complexities in mind, the distinction that is frequently drawn between static and dynamic measures remains useful, and in each case several important confounding factors or complexities may be relevant. Static measures of thermal limits (Table 1) typically involve exposing an individual to a given temperature for a given period after direct transfer to that temperature from the temperature at which the animal was kept. Such 'plunge' protocols then either measure the time survived at a given temperature or the temperature survived for a given period and usually a proportion survived for a sample of individuals is assessed using a typical dose response curve. The lethal temperatures or times are then identified at a given quartile (e.g. LT_{50}, LT_{25} or LT_{100}). The variable assessed is usually the survival after some fixed recovery period (24 h or more), though less frequently the fertility of individuals may also be examined. Often, only single response curves are generated and a mean and variance for a given quartile are estimated from the fit of the curve (e.g. using logistic regression). However, in other cases multiple assessments are made to obtain an idea of the true population variance (Slabber et al., 2007). Similarly, although time by temperature contour plots were previously commonly made (see Cossins and Bowler, 1987), this is often no longer done [but see e.g. Beitinger et al. (Beitinger et al., 2000), Jumbam et al. (Jumbam et al., 2008) and Stotter and Terblanche (Stotter and Terblanche, 2009) for obvious recent exceptions]. In comparing ramping to static experiments, one important consideration is the potential for cumulative heat (chill) damage that will depend on the rate of temperature increase (decrease), the actual temperatures and the duration of exposure at a particular temperature. If the damage repair mechanisms are impaired at extreme temperatures or have insufficient capacity to deal with the damage, the damage can accumulate, especially in longer exposures [see discussion in Renault et al. (Renault et al., 2004), Colinet et al. (Colinet et al., 2006), Marshall and

Sinclair (Marshall and Sinclair, 2011) and Renault (Renault, 2011)].

Static trials may also involve periods of temperature change. Thus, investigations may seek to understand the effects of multiple exposures to sub-lethal events, i.e. moving animals back and forth between more and less extreme conditions, and various components of performance and/or survival may be assessed (Brown et al., 2004; Sinclair and Chown, 2005; Marshall and Sinclair, 2010; Marshall and Sinclair, 2011). One subset of such work concerns the effects of rapid hardening. [Rapid cold hardening was first formally described by Lee et al. (Lee et al., 1987), although Mellanby (Mellanby, 1939) had already noted very rapid responses to changing acclimation temperatures; see Denlinger and Lee (Denlinger and Lee, 2010) for a review of rapid hardening effects.] Likewise, the period at more benign temperatures may be short, to examine the influence of thermal respite on survival and the mechanisms underlying it (Colinet et al., 2006; Kostál et al., 2007; Colinet et al., 2010). In these cases, and sometimes in routine static experiments, rather than a plunge protocol, the experiment may involve taking individuals to the experimental temperature and removing them from there at a fixed, controlled rate. Especially where investigations of cold hardiness are concerned, such controlled changes may have pronounced effects on the survival or viability of the individuals concerned and may trigger different mechanisms of resistance (Rako and Hoffmann, 2006).

Where temperatures tend to lie within the normal viable range, and static trials do not involve a plunge protocol so much as keeping individuals at a set temperature, fluctuating treatments have often been used to assess performance and fitness relative to the more constant conditions. Sometimes the fluctuating conditions result in fitness or performance improvements and sometimes they do not (e.g. Hoffmann et al., 2005; Terblanche et al., 2010; Renault, 2011). Importantly, the mean around which the temperatures fluctuate is significant because of the typical shape of performance curves and the effects of Jensen's inequality (see Ruel and Ayres, 1999; Savage, 2004).

Dynamic trials typically take a much wider form of approaches (Table 1). Usually this results from the fact that: (1) the rates of change involved vary substantially, such as from $0.000023°C\,min^{-1}$ (i.e. $1°C\,month^{-1}$) (see Peck et al., 2009) to the more typical $1°C\,min^{-1}$; (2) onset of and/or recovery from a given endpoint may be assessed (e.g. Klok and Chown, 2001); (3) animals may be exposed to what amount to static temperatures, but with the changes between them following some kind of ramping protocol (Worland et al., 1992); and (4) a wide range of endpoints may be involved, such as the crystallization temperature (or point where the animal freezes – also known as the supercooling point), chill coma temperature, temperature threshold for spontaneous movement, or chill coma recovery point. Dynamic trials typically use individuals, rather than samples of individuals, as the independent data and usually involve some measure of temperature. However, chill coma recovery time (David et al., 1998) is obviously a time-based measure.

Confounding factors

Irrespective of the approach to measuring thermal limits adopted, potential confounding factors should always be considered. Typically, experiments attempt to reduce these to ensure that the question posed is being answered. However, at times interacting effects are intentionally investigated (see The environmental context, above) although often this is not usually done. A typical example of the latter is understanding how acclimation conditions

or thermal history influences the rate of development of a particular thermal response, or how contact with ice crystals or exposure to ice, bacteria or a host plant might alter the freezing point of a cold-hardy set of individuals (see Chown and Nicolson, 2004; Denlinger and Lee, 2010). Knowledge of the role of interacting factors means that their effects can be circumvented where necessary. For both the upper and lower lethal limits, the effects of desiccation and starvation must be taken into account. For long-term experiments such as those undertaken by Peck et al. (Peck et al., 2009), the animals must be exposed to ideal conditions so that their survival is not being determined by factors other than the one of interest. Over the shorter term, the same situation holds, especially for estimates of upper lethal limits when experiments last for a significant period. As temperature rises, saturation deficit increases even given the same absolute quantity of water vapour in the air. Likewise, owing to the non-linear relationship between metabolic rate and temperature and water loss, higher temperatures mean higher rates of loss of water and storage compounds (Rezende et al., 2011). Clearly, confounding factors will be most significant when experiments have a long duration relative to the time it takes to reach a significant impact of starvation and/or desiccation on the individual in question. This in turn depends on the mass of the individual and the conditions of the experiment.

To address confounding factors, several approaches may be adopted, but they in turn may add further complexity. First, simply increasing relative humidity may actually have negative (rather than positive) influences on thermal limits (Mellanby, 1932). It cannot be assumed that very high humidity to avoid desiccation effects is a situation that all organisms will find suitable or will encounter under natural conditions. If high humidity is not encountered naturally by a species, the individual may increase its activity rate under high humidity, and thereby affect its thermal limits. Obviously if the animal relies on evaporative water loss for thermal regulation, the effect will be most pronounced, but it may also be important for species that are not thought to do so, such as mealworm larvae (Mellanby, 1932). The same situation may apply to fed *versus* fasted individuals (see Nyamukondiwa and Terblanche, 2009) (see below).

Second, the duration of the experiment will determine the magnitude of the confounding effect, and here it really is likely only to be problematic for small individuals in trials that last for a significant period [again first noted by Mellanby (Mellanby, 1932)]. This is best illustrated with an example. In insects, a general relationship exists between body mass (M_b, in g), temperature (T, in Kelvin, K) and metabolic rate ($\dot{M}_{O_2}$, in µW) [of the form $\ln\dot{M}_{O_2} = \ln M_b \times 0.72) + (-6384.98 \times 1/T) + 28.51$; $R^2 = 0.82$, s.e.m. estimate=0.932] (see Chown et al., 2007b; Irlich et al., 2009). If it is assumed that 5% of an insect's body mass [based on an average lipid content of *ca*. 12% of dry mass (Lease and Wolf, 2011); carbohydrate and protein storage of smaller, though significant, amounts (e.g. Folk and Bradley, 2004; Hahn and Denlinger, 2007); and an average water content of 65% of fresh mass (Edney, 1977; Studier and Sevick, 1992)] constitutes utilisable energy stores (and energy content is $20\,\mathrm{kJ\,g^{-1}}$), the likely time to death for an insect of a given mass can be estimated based on metabolic rate in the resting condition. Likewise, for desiccation rate (i.e. water loss, L_w), the same estimates can be made assuming $\ln L_w = (\ln M_b \times 0.54) + (-3782.73 \times 1/T) + 6.35$ ($R^2 = 0.47$, s.e.m. estimate= 1.203) [from data collected by Addo-Bediako et al. (Addo-Bediako et al., 2001)] and that tolerated water loss averages 30% of fresh mass (Edney, 1977). Acknowledging the variation around these relationships, it is clear that for insects of 20 mg and larger, and

assuming that survival might be much lower for desiccation (given the low coefficient of determination of the relationship), survival of 10 h can be expected at a constant temperature of 35°C (Fig. 2A). For insects of much smaller sizes (<10 mg) survival will likely be compromised under the very low humidity conditions typically used for tolerance trials, but only if trials continue for longer than several hours (which would be the case at rates of $0.01°\mathrm{C\,min^{-1}}$ or lower) (Fig. 2B).

A frequency distribution of body sizes of insects that have been investigated for various physiological investigations (see Chown and Gaston, 2010) indicates the form of the interspecific body size–frequency distribution (Fig. 2C), suggesting that for many species small size problems are unlikely to confound investigations. For example, using a similar process to that outlined in Rezende et al.'s gedanken experiment (Rezende et al., 2011), with a slightly different, tsetse-specific mass loss of 6% fat before death (Loder et al., 1998) and 45% of body mass before dehydration to death (J.S.T., unpublished) (Bursell, 1959), we also calculated time to death of *G. pallidipes* [the same species used in Terblanche et al. (Terblanche et al., 2007a)] under desiccating or starving conditions. This produces estimates of survival time well in excess of the duration of typical CT_{max} experiments, even under the slowest ramping rates used (Fig. 3). This qualitative result remains true irrespective of variation in these assumptions, which typically only affect the survival time by a few hours (data not shown).

Despite these general findings, for small species problems might still exist owing to the impacts of desiccation (Mellanby, 1932), and this has been suggested to be the case especially for *D. melanogaster* and the Argentine ant *Linepithema humile* (Rezende et al., 2011). However, it is not at all clear that this will necessarily be the case. In their specific criticisms of Mitchell and Hoffmann (Mitchell and Hoffmann, 2010) and other work, Rezende et al. (Rezende et al., 2011) simulated the effect different ramping rates would have on estimates of energy and water loss over time for flies, using desiccation and starvation estimates made at different temperatures. They used data from a study by Da Lage et al. (Da Lage et al., 1989) investigating the impact of temperature on desiccation and starvation resistance in *D. melanogaster* to estimate energy and water lost during thermal ramping experiments and survival data from the desiccation experimental protocol to make assertions about likely levels of desiccation stress. However, unlike in Mitchell and Hoffmann (Mitchell and Hoffmann, 2010), Da Lage et al. (Da Lage et al., 1989) placed flies into sealed vials with silica gel desiccant to measure desiccation resistance and used the same design without desiccant for the starvation design. The sealed environment in which individual flies were exposed to thermal stress in Mitchell and Hoffmann (Mitchell and Hoffmann, 2010) meant that flies were not necessarily losing water to the environment throughout stress. If desiccation during the stress were an issue, a ramping protocol might have been expected to affect *Drosophila birchii* and *Drosophila bunnanda* to a greater extent than the other species due to the known low level of desiccation tolerance in these species (Kellermann et al., 2009). However there is no evidence to suggest these species are impacted greatly by the ramping stress, and the least starvation-resistant species (*D. birchii*) tested in Mitchell and Hoffmann (Mitchell and Hoffmann, 2010) survives for more than 40 h without food (Griffiths et al., 2005). This makes a negative impact of desiccation and starvation on thermal stress using the ramping protocol unlikely.
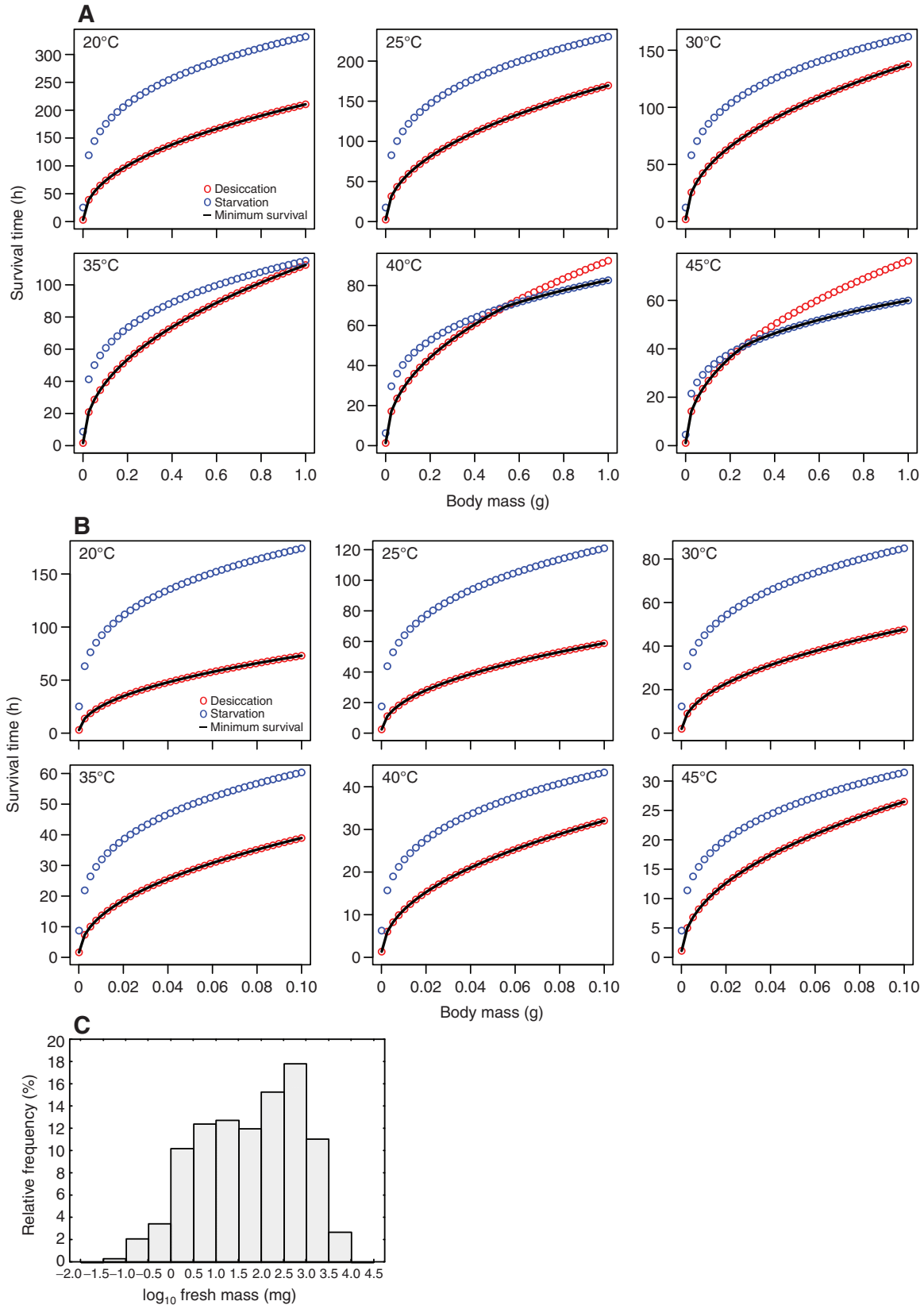
Fig. 2. Survival of insects of a given mass, of either starvation (blue circles) or desiccation (red circles) under a variety of temperature conditions, based on consensus equations relating metabolic and water loss rates to mass and temperature (see 'Confounding factors' for details). The black survival line indicates minimum survival time. In A, body mass ranges between 0 and 1 g, whereas in B the mass range is from 0 to 0.1 g, providing an indication of what happens in the case of smaller insects. In C, a frequency distribution is provided of $\log_{10}$ insect fresh body masses (mg) of 668 species studied in the insect physiological literature and used by Chown and Gaston (Chown and Gaston, 2010) to examine the taxonomic signal in mass. Here, species are from 21 orders and 134 families.
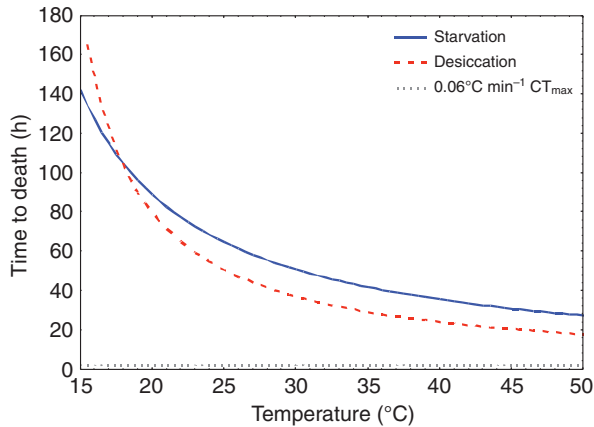
Fig. 3. Calculation of time to death from starvation or desiccation for *Glossina pallidipes* (Diptera: Glossinidae) (mass=45 mg) relative to the maximum duration experienced in an ecologically relevant heating rate (0.06°C min^{-1}) CT$_{max}$ experiment (Terblanche et al., 2007a). These calculations make the assumption that metabolic rate and desiccation rate increases exponentially (i.e. Q_{10}=2) with temperature, and that flies can withstand losing 6% of their body mass before dying of starvation, and 45% of their body mass as water before dying of dehydration (Bursell, 1959; Loder et al., 1998). Note the horizontal stippled line marks the longest time to death in CT$_{max}$ experiments from Terblanche et al. (Terblanche et al., 2007a).

To test the potential association between thermal stress and desiccation/energy expenditure more generally in *D. melanogaster*, we used flies from a culture originating in Gordonvale, North Queensland, previously described in Mitchell et al. (Mitchell et al., 2011). The culture was maintained as a massbred population at a density of 400 individuals per 250 ml bottle. We used a cornmeal fly medium [Bloomington *Drosophila* medium adjusted by doubling the quantity of soy flour and replacing light corn syrup with dextrose (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm)]. The medium was treated with antifungal agent (0.14% w/v Nipagin). We used this medium because flies did not become stuck in it when it was heated. Flies for rearing were kept on 70 ml of fly medium in each bottle in a room set at 25°C, 24 h light and 70% relative humidity (RH). For the experiments we used mated 4–7-day-old flies. Sexes were separated without use of CO$_2$ to avoid additional stress (Champion de Crespigny and Wedell, 2008). The experimental procedures closely followed those described in Mitchell et al. (Mitchell et al., 2011) for both ramping and static temperature stress experiments. Briefly, flies were put into small vials with or without 2 ml of fly medium added. All vials were plugged with a plastic cap and additionally sealed with a small strip of Parafilm. We placed vials with flies in glass containers and knockdown times were recorded to the nearest second. Humidity and temperature were recorded using an iButton temperature and humidity data logging system (http://www.maxim-ic.com/products/ibutton/). For the static assay, water temperature was set at 39°C into which flies in glass vials were suddenly immersed. For the ramping assay, temperature was initially set at 28°C and then ramped at a rate of approximately 0.06°C min^{-1} until it reached 39°C and all flies were knocked-down.

Changes in temperature and humidity in the vials at times when flies were exposed varied markedly depending on the availability of food. In the ramping assay run with food, humidity was always >94% RH when food was present and dropped from 60% RH to 35% RH when it was absent. In the static assay, the equivalent figures were >96% RH in the presence of food, and between 61% RH and 33% RH in its absence. The time to knockdown varied significantly depending on whether food was present in the vials (Fig. 4). For both sexes, the presence of food in the static assay halved the knockdown times. In the ramping assay, there was also a decrease in knockdown times in the presence of food although the effect was less marked. Therefore in these assays there is clearly no detrimental effect of energy expenditure and a moderate level of humidity rather than high humidity on thermal resistance.

Using a much larger fly species, *Ceratitis capitata* (Diptera, Tephritidae), we undertook similar experiments to investigate the effect of relative humidity on thermal tolerance estimates under ramping and static protocols. In these experiments we compared knockdown time (KDT; min) for adult males only (N=20 per treatment group per trial) held in vials with and without additional humidity, provided by moist or dry cotton wool, respectively, for static and ramping protocols. In the ramping protocol we used a starting temperature of 25°C with a 10 min holding period, followed by a ramping rate of 0.12°C m^{-1}, whereas in the static experiment flies were directly transferred from 25 to 43°C. Effects of moisture provisioning were non-significant in the ramping protocol ($F_{1,38}$=1.17, P>0.28), but significant in the static protocol ($F_{1,38}$=17.38, P<0.001; Fig. 5A). The significant effect of moisture level on KDT might be considered a consequence of water availability limiting desiccation-associated mortality. However, it might also be a consequence of impacts on thermal equilibration, with moisture treatments remaining cooler than dry treatment vials. Thus, we repeated this static heat knockdown experiment, but including temperature recordings (type T 36-SWG thermocouples connected to Picotech USB loggers recording temperature at 1 s intervals in N=8 vials) for moist and dry heat tolerance assays at 43°C. Moist treatments were, on average, 4.5°C

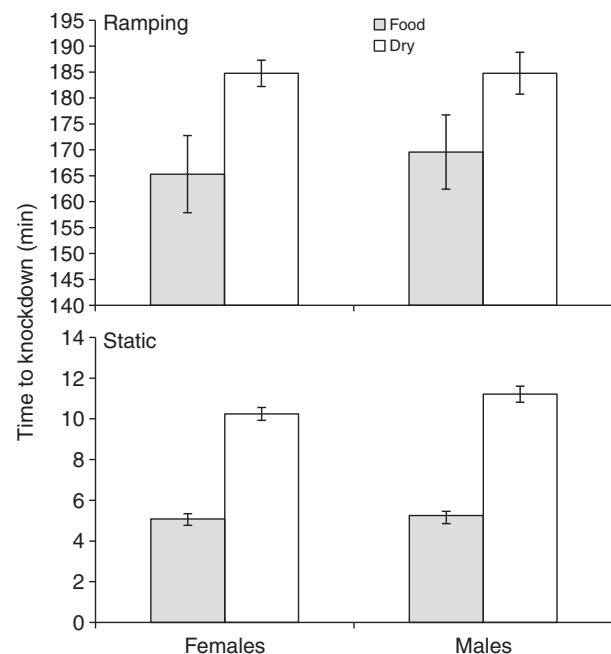Fig. 4. Comparison of knockdown time (means ± s.d.) under heat stress of *Drosophila melanogaster* adults held in vials with and without food (ramping N=60; static N=40).

cooler than dry treatments under the exact same conditions and at the same time point, resulting in apparently 'improved' knockdown times in the moist treatment group. For example, at 600 s the moist treatment was at 38.4±1.9°C (mean ± s.d.) whereas the dry treatment was at 43.0±0.9°C. At 1100 s into the trial, the moist treatment was at 39.9±1.6°C whereas the dry treatment was at 43.6±1.1°C (Fig. 5B). Thus, KDT must by necessity be longer
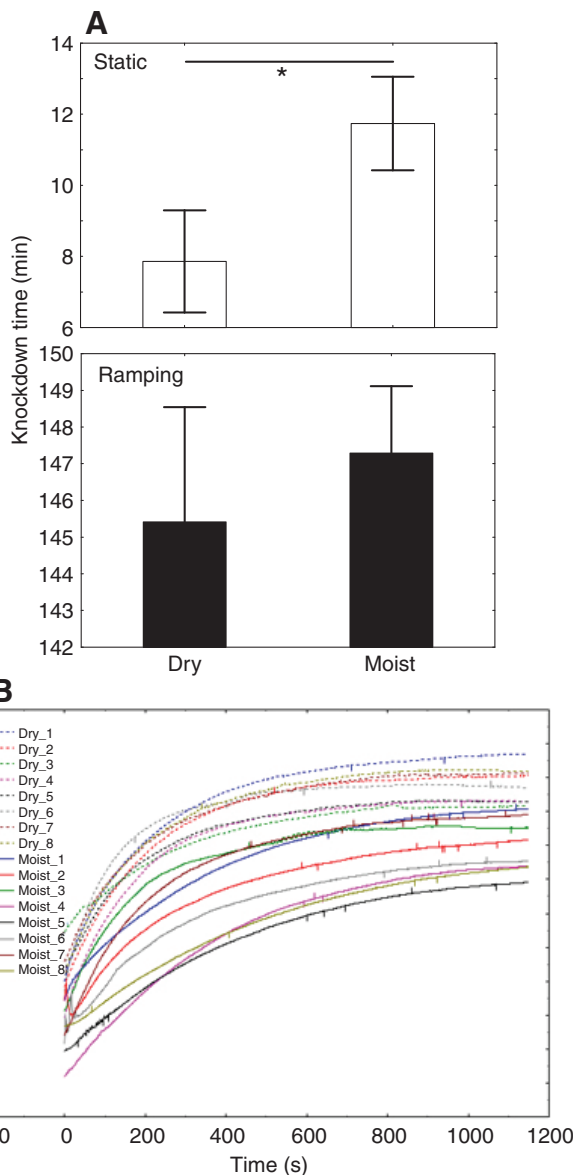

Fig. 5. (A) Comparison of knockdown time (means ± 95% CL) under heat stress of *Ceratitis capitata* (Diptera: Tephritidae) male adults (*N*=20 per treatment group per trial) held in vials with and without additional humidity (provided by moist cotton wool) for static and ramping protocols. Ramping rate was 0.12°C min⁻¹ in the latter case (from a start temperature of 25°C with a 10 min holding period) whereas in the static experiment flies were directly transferred from 25 to 43°C. Effects of moisture provisioning were non-significant in ramping ($F_{1, 38}$=1.17, P>0.28), but significant in the static protocol ($F_{1,38}$=17.38, P<0.001). (B) Temperature recording during moist and dry static heat tolerance assays inside plastic 40 ml vials (*N*=8 per treatment group) on a thermal stage at 43°C. Note that moist treatments are, on average, 4.5°C cooler than dry treatments under the exact same conditions and at the same time point, resulting in apparently 'improved' knockdown times in the moist treatment group.

in flies experiencing the moist treatment. In consequence, any recommendation to include moisture or food in vials to prevent desiccation or starvation may also have inadvertent effects different to those typically envisaged (such as desiccation stress) (see Rezende et al., 2011), perhaps even confounding the major results and interpretation. These results are not definitive tests of Rezende et al.'s (Rezende et al., 2011) model because, for example, flies at high temperatures and high humidities may not wish to feed. However, they do suggest limited effects of dehydration over the period of the heat tolerance assays.

One additional problem in the logic of Rezende et al.'s (Rezende et al., 2011) gedanken experiment is that it leaves little room for a negative relationship between heating rate and upper thermal tolerance estimates. Terblanche et al. (Terblanche et al., 2007a) predicted such a relationship in ectotherms with marked heat hardening or high temperature acclimation responses, and it does appear to be characteristic of some species. For example, Chidawanyika and Terblanche (Chidawanyika and Terblanche, 2011) showed a marked increase in CT_{max} with slower heating rates in codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) (Fig. 6), likely a consequence of a pronounced heat hardening response at 36°C (Chidawanyika and Terblanche, 2011), and probably involving upregulation of heat shock protein 70 (Yin et al., 2006). Although they claim such effects are incorporated into their model [see e.g. eqn 4 in Rezende et al. (Rezende et al., 2011)] and, ultimately, that 'desiccation can potentially overshadow thermal acclimation effects' (p. 118) this presumes that the data upon which the model was built is an accurate reflection of such processes in other species. Given the magnitude of variation in high temperature tolerance and its plasticity to, for example, standardized high temperature pre-treatments among *Drosophila* species (e.g. Nyamukondiwa et al., 2011), such generalizations are clearly problematic.

Heritabilities and assays

Rezende et al. (Rezende et al., 2011) undertook a further gedanken experiment to explore the effects of differences in heating rate on the heritability and evolvability of heat resistance and compared their results with estimates provided in Mitchell and Hoffmann (Mitchell and Hoffmann, 2010). Their first point is that heritability under slow ramping may reflect genetic variation in desiccation and energy expenditure rather than heat resistance. However, the

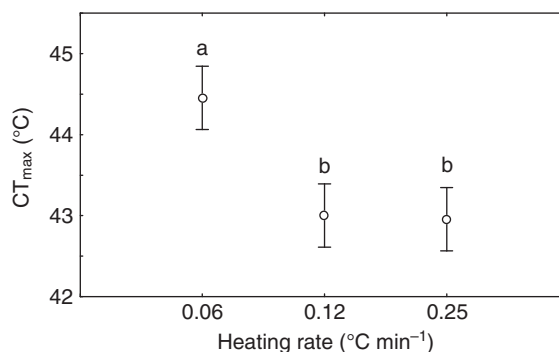Fig. 6. The effects of variation in heating rates on mean (±95% CL) critical thermal maxima of adult codling moth *Cydia pomonella* (Lepidoptera: Tortricidae). Data points represent means of *N*=20 in each rate treatment of mixed gender moths [redrawn from Chidawanyika and Terblanche (Chidawanyika and Terblanche, 2011)]. Different letters indicate statistically significant differences at *P*<0.01 following ANOVA.

absence of any decrease in resistance when food was provided (above) suggests that this was not the case in the ramping experiments. Moreover, although Mitchell and Hoffmann (Mitchell and Hoffmann, 2010) observed no heritable variation under ramping, variation in desiccation resistance might instead be expected to lead to heritable variation (rather than decreasing it) because the heritability and additive genetic variance for desiccation resistance in *D. melanogaster* is quite high when compared with heat resistance in assays where flies are rapidly heated (Hoffmann, 2000).

Rezende et al. (Rezende et al., 2011) also consider a situation where there is only additive genetic variation for heat resistance, but desiccation and energy expenditure reduce the maximum temperature reached by the flies. Based on a non-linear association between temperature and energy expenditure, they argue in their gedanken experiment that the environmental variance might increase under ramping, but the genetic component for heat resistance might decrease, consistent with the direct estimates of environmental and additive genetic variances as in Mitchell and Hoffmann (Mitchell and Hoffmann, 2010). This argument certainly seems valid for the model they consider. However, genetic variance components might just as well be expected to go up under ramping if, say, there is genetic variation for traits that minimize damage during ramping as CT_{max} is approached. Thus, although it may be possible to come up with one explanation for changes in phenotypic, environmental and genetic variance, empirical data typically show a range of patterns for changes under different environmental conditions (Hoffmann and Merila, 1999) and it is important to measure traits that are important ecologically under a realistic set of conditions.

Conclusions

Determining the thermal tolerance of organisms is clearly of considerable current concern, not only because it forms the foundation for much of mechanistic thermal physiology, but also because thermal relationships have such a wide variety of impacts on organismal responses, and because much concern exists about a failure of many organisms to cope with current environmental change. Much scope exists for different factors, such as ramping rate and humidity to influence the outcome of any given trial. However, what that influence will be varies from species to species. Mostly, no single way around potential confounding factors exists, other than to try to avoid them through sound experimental design or consider them explicitly in multiple assessments, particularly where they are likely to be ecologically important. Whatever is done, the most useful approach is to make sure that the methods report explicitly what was done experimentally. Where large comparative or meta-analyses are undertaken, explicit methodological differences can then be accounted for using appropriate statistical techniques (such as the consideration of closed *versus* open systems in comparative analyses of insect metabolic rates) (e.g. Chown et al., 2007b). However, for many studies this might not be possible owing to inadequacy of data or degrees of freedom. In consequence, much scope exists for determining in an experimentally explicit way the effects of different approaches (such as heating rates or static *versus* dynamic trials) on assessments of ecologically relevant thermal tolerances. If forecasts of physiologically mediated environmental change impacts are to be improved, such work will have to be done urgently.

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