SHORT COMMUNICATION

Plasticity of upper thermal limits to acute and chronic temperature variation in *Manduca sexta* larvae

Joel G. Kingsolver[‡], Heidi J. MacLean^{*}, Silvan B. Goddin and Kate E. Augustine

ABSTRACT

In many ectotherms, exposure to high temperatures can improve subsequent tolerance to higher temperatures. However, the differential effects of single, repeated or continuous exposure to high temperatures are less clear. We measured the effects of single heat shocks and of diurnally fluctuating or constant rearing temperatures on the critical thermal maximum (CT_{max}) for final instar larvae of *Manduca sexta*. Brief (2 h) heat shocks at temperatures of 35°C and above significantly increased CT_{max} relative to control temperatures (25°C). Increasing mean temperatures (from 25 to 30°C) or greater diurnal fluctuations (from constant to $\pm 10^\circ\text{C}$) during larval development also significantly increased CT_{max} . Combining these data showed that repeated or continuous temperature exposure during development improved heat tolerance beyond the effects of a single exposure to the same maximum temperature. These results suggest that both acute and chronic temperature exposure can result in adaptive plasticity of upper thermal limits.

KEY WORDS: Acclimation, Heat tolerance, Heat shock, Temperature exposure, Critical thermal maximum

INTRODUCTION

Temporal variation in natural environments is ubiquitous. Aspects of this variation are often predictable, making phenotypic plasticity an important mechanism by which organisms adapt to variable environments. Photoperiod, temperature and precipitation often serve as environmental cues that induce phenotypically plastic changes and improve organismal fitness in subsequent weather and climatic conditions (Tauber and Tauber, 1976). The time scales of environmental cues and of plastic responses are important factors that influence the reliability of cues for predicting future environmental conditions and the fitness consequences of plasticity (Levins, 1968; Moran, 1992). For example, some forms of developmental plasticity can reduce or even reverse the adaptive value of plasticity because of the time lag between detection of environmental cues and phenotypic expression (Kingsolver and Huey, 1998).

Physiological acclimation to temperature variation is a form of phenotypic plasticity found in many ectotherms. Acclimation may occur over a range of time scales. For example, acute, brief (1–2 h) exposure to high (or low) temperatures can increase subsequent tolerance to heat (or cold) in a wide variety of organisms (Angilletta, 2009). In many cases, such heat shocks induce the production of heat shock proteins (HSPs), which act as chaperones to protect key

proteins and improve tolerance and survival during subsequent heat exposure (Hofmann, 1999; Kregel, 2002; Hoffmann et al., 2003). In the absence of additional heat shocks, heat tolerance typically declines to baseline levels within a few days (Dahlgaard et al., 1998; Bahrmndorff et al., 2009; Karl et al., 2012).

Chronic exposure to constant, increased temperatures can also improve performance at higher temperatures (Hoffmann et al., 2003; Zerebecki and Sorte, 2011), but chronic exposure to stressfully high temperatures can reduce tolerance, growth rates and survival (Chown and Terblanche, 2007; Angilletta, 2009). More recently, several studies have examined the effects of diurnal temperature fluctuation on acclimation of physiological traits. For example, diurnal temperature fluctuations increase heat tolerance and HSP70 synthesis in woodlice, fruit flies and zebrafish (Schaefer and Ryan, 2006; Bozinovic et al., 2011; Folguera et al., 2011), and increase growth rate, developmental rate and heat tolerance in butterflies (Fischer et al., 2011). In these studies, it is usually not clear whether increased heat tolerance is the result of exposure to a maximum daily temperature, as opposed to effects of diurnal fluctuations per se. For example, does increased tolerance result from exposure to a single high-temperature event, to repeated events over several days, or to chronic exposure to increased temperatures throughout a life stage? The answer is relevant to understanding the differing responses of ectotherms to extreme events, heat waves and climate warming.

Here, we addressed this question by examining upper thermal limits (critical thermal maximum, CT_{max}) for the larvae of *Manduca sexta*. A *Manduca* larva can experience a wide range of diurnal fluctuations and body temperatures during its larval life (Casey, 1976; Kingsolver et al., 2012; Woods, 2013), and repeated daily exposure to body temperatures above 35–38°C throughout larval development can reduce growth and survival rates (Kingsolver et al., 2015). *Manduca sexta* larvae also strongly increase HSP70 production in response to acute heat shocks of 38–44°C (Fittinghoff and Riddiford, 1990), and brief (30 min) exposure to temperatures above 44–45°C greatly increases larval mortality (Casey, 1976).

We conducted two sets of experiments. First, we used single, brief heat shocks to determine how heat shock temperature affects upper thermal limits. Second, we used constant and fluctuating rearing conditions to determine how mean temperature and diurnal temperature fluctuations throughout larval development affect upper thermal limits. We combined the data from these experiments to test whether single, repeated and continuous exposure to high temperatures have different effects on acclimation of upper thermal limits.

MATERIALS AND METHODS

Study system: *Manduca sexta* L.

Our studies used larvae from our laboratory colony of *M. sexta* maintained at the University of North Carolina Chapel Hill (Kingsolver, 2007; Kingsolver and Nagle, 2007; Kingsolver et al.,

Department of Biology, University of North Carolina, Chapel Hill, NC 27599, USA.
^{*}Present address: Institute for Bioscience, Aarhus University, Aarhus C 8000.

[‡]Author for correspondence (jgking@bio.unc.edu)

2009; Diamond and Kingsolver, 2010, 2012). This lab colony was originally derived from field collections near Raleigh, NC, USA, in the 1960s, and has been maintained on standard artificial diet at constant (25–26°C) temperature for over 300 generations (Kingsolver, 2007; Kingsolver and Nagle, 2007). Previous studies show that larvae from this colony have reduced survival rates at constant rearing temperatures of 35°C, relative to larvae from field populations in southeastern USA (Kingsolver and Nagle, 2007). However, lab and field populations of *M. sexta* have similar growth, development and survival responses to diurnal fluctuations during larval development (Kingsolver et al., 2009).

Measuring upper thermal limits: CT_{max}

Heat tolerance was determined using a ramping temperature assay to measure failure time and CT_{max} (Terblanche et al., 2007; Overgaard et al., 2012), defined as the temperature at which a larva begins to twitch or lose muscular control. We used a starting temperature of 38°C (with a 12 min acclimation period) and a ramping rate of 0.25°C min⁻¹ (Terblanche et al., 2007) using a programmable water bath (Thermo Scientific with AC-200 controller). Newly hatched larvae were reared in their treatment conditions (see below) until molt into the 5th (last) instar. During the morning of day 2 in the 5th instar, each larva was placed on filter paper at the bottom of a covered, weighted 1000 ml glass beaker, which was then placed into the water bath. Larvae from multiple treatment groups were measured during each ramping assay. Following the acclimation period, larvae were monitored once every minute until failure (began to twitch or lose muscular control) was observed. The time to failure and the temperature of the water bath at that time were then recorded for each larva. We note that CT_{max} is not a direct measure of mortality: nearly all larvae survived to pupation, when returned to control conditions following the CT_{max} assay (J.G.K., H.J.M., S.B.G. and K.E.A., unpublished results). However, loss of muscular control in the field can cause *M. sexta* larvae to fall off of their host plant, increasing the risk of mortality Casey (1976).

Experiments: acute and developmental treatments

Acute (heat shock) experiment

Larvae were reared from hatching in a constant temperature of 25°C and a 14 h light:10 h dark photocycle until molt into the 5th larval instar. On the first day of the 5th instar, larvae were weighed, placed in an environmental chamber without food, and subjected to a 2 h heat shock from 13:00 h to 15:00 h EDT, at one of seven different heat shock temperatures: 25 (control), 30, 32, 35, 38, 40 or 42°C. Following the heat shock, larvae were returned to their rearing conditions of 25°C. The morning of the next day (2nd day of 5th instar), heat tolerance of each larva was measured as described above. During each assay, larvae from both the control (25°C) group and one or two other heat shock treatment groups were measured. Sample sizes for the treatment groups were: $N=28$ (25°C), $N=3$ (30°C), $N=15$ (32°C), $N=10$ (35°C), $N=10$ (38°C), $N=17$ (40°C) and $N=10$ (42°C).

Developmental (rearing temperature) experiment

This experiment used a full-factorial experimental design with two factors: mean temperature (MT), with three treatment levels (25, 28 and 30°C); and diurnal temperature fluctuations (DTF), with two treatment levels [$\pm 0^\circ\text{C}$ (constant), and $\pm 10^\circ\text{C}$]. A 14 h light:10 h dark photocycle was maintained in all treatments. The $\pm 10^\circ\text{C}$ treatment group experienced a diurnal temperature cycle with 2 h at the maximum temperature (13:00–15:00 h EDT) and 2 h at the minimum temperature (01:00–03:00 EDT), with linear ramping

between these temperatures (see Fig. 1). Newly hatched larvae were assigned randomly to each of the treatment groups, and reared until molt into the 5th instar. On the morning of the 2nd day of the 5th instar, heat tolerance of each larva was measured as described above. During each assay, larvae from both the $\pm 0^\circ\text{C}$ and $\pm 10^\circ\text{C}$ treatment groups for a given mean temperature were measured. Sample sizes were $N=77$ for the $\pm 0^\circ\text{C}$ and $N=81$ for the $\pm 10^\circ\text{C}$ treatment groups.

Statistical analyses

All statistical analyses were performed using fixed-effects linear models in R (version 3.0.1). We used CT_{max} as the response variable in all analyses. Using failure time as the response variable gave qualitatively identical results in terms of significance testing. For analyses of the single heat shock experiment, heat shock temperature was considered as a continuous covariate; similar results were obtained when heat shock temperature was considered as a categorical factor. For analyses of the rearing temperature experiment, mean temperature and diurnal temperature range were considered as fixed (categorical) factors. Visual inspection of residuals was used to confirm that assumptions of normality and homogeneity of variance were met (Zuur et al., 2009).

In our two experiments, larvae in the different treatment groups differed both in the maximum temperature experienced during acclimation (i.e. prior to measurement of CT_{max}) and in the duration and temporal pattern of temperature exposure to maximum temperature (Fig. 1). For example, in the $\pm 0^\circ\text{C}$ rearing treatments, larvae were exposed continuously throughout larval development to a constant temperature; the maximum temperature experienced equaled the mean temperature (Fig. 1). Larvae in the $\pm 10^\circ\text{C}$ treatments repeatedly experienced a daily high temperature throughout their development, and the maximum temperature experienced was 10°C above the mean temperature (Fig. 1). Larvae in the heat shock treatment briefly experienced a maximum temperature (equal to the heat shock temperature) a single time, the day prior to measurement. By combining data from the two experiments, we can distinguish the effects of maximum temperature experienced and the type of exposure to maximum temperature: single=acute heat shock treatment; repeated= $\pm 10^\circ\text{C}$ diurnal temperature fluctuations throughout larval development; continuous=constant rearing temperature throughout larval

Fig. 1. Temperature as a function of time experienced by larvae for representative treatments in the heat shock and rearing temperature experiments. Five treatment groups are represented: 30 and 40°C heat shock (HS) treatments; 30 $\pm$ 10°C diurnally fluctuating rearing treatment; and 25 and 30°C constant ($\pm 0^\circ\text{C}$) rearing treatments. Note that prior to measurement of the critical thermal maximum (CT_{max} ; on day 2 of the 5th instar), the maximum temperature experienced was the same for *Manduca sexta* larvae in the 30°C heat shock and the 30°C constant rearing treatments ($=30^\circ\text{C}$), and for larvae in the 40°C heat shock and the 30 $\pm$ 10°C rearing treatments ($=40^\circ\text{C}$). See Materials and methods.

Fig. 2. CT_{max} as a function of temperature in the heat shock and rearing temperature experiments. (A) Acute heat shock experiment. The linear regression line (dotted) is also indicated. (B) Rearing temperature experiment. Filled circles, solid line: $\pm 0^\circ\text{C}$ diurnal temperature range (constant); open circles, dashed line: $\pm 10^\circ\text{C}$ diurnal temperature range. In each case, mean (± 1 s.e.m.) CT_{max} is shown for 5th instar *M. sexta* larvae.

development. The analysis considered maximum temperature as a continuous covariate and type of exposure (single, repeated, continuous) as a factor. We report the P -values based on the ANOVA for each analysis in the text.

RESULTS AND DISCUSSION

Single, brief heat shocks significantly increased CT_{max} ($P < 0.001$). Heat shock temperatures of 35°C produced increases in mean CT_{max} (Fig. 2A). We note that the 30°C heat shock treatment only had a sample size of $N=3$, much smaller than all other treatment groups ($N=10$ – 28).

The rearing temperature experiment showed that mean temperature ($P < 0.001$), diurnal temperature range ($P < 0.001$) and their interaction ($P=0.047$) all had significant effects on CT_{max} . Increasing mean temperature increased mean CT_{max} , and diurnal fluctuations increased mean CT_{max} at lower ($<30^\circ\text{C}$) mean temperatures (Fig. 2B).

Combining the data from these experiments revealed that maximum temperature significantly increased CT_{max} ($P < 0.001$) in

both experiments (Fig. 3). The type of experimental exposure and its interaction with maximum temperature also significantly affected CT_{max} ($P < 0.001$ for each): higher constant temperatures and larger diurnal fluctuations increased mean CT_{max} beyond the effect of maximum temperature alone (Fig. 3). These results suggest that both high temperatures and the pattern of exposure to those temperatures during development can alter heat tolerance.

Increased heat tolerance in response to a brief, recent heat shock (sometimes called heat-hardening) is a common form of physiological acclimation in insects and other ectotherms (Hofmann, 1999; Kregel, 2002). Our results for *M. sexta* suggest that heat-hardening may occur at relatively low temperatures: mean CT_{max} was increased by heat shocks of 35°C (Fig. 2). In the field, late-instar *M. sexta* larvae routinely exceed body temperatures of 35°C or more during midday conditions in summer, and temperatures above 40°C are not uncommon (Casey, 1976; Kingsolver et al., 2012; Woods, 2013). This suggests that environmental conditions that increase heat tolerance are commonplace in this species, resulting in acclimation to seasonal changes in environmental temperatures. This pattern may be widespread: organisms in highly variable thermal environments, including terrestrial insects and intertidal invertebrates, frequently mount HSP responses within the range of body temperatures normally experienced in the field (Hofmann, 1999; Dahlhoff and Rank, 2007; Tomanek, 2010).

Acclimation to different constant developmental temperatures has been widely observed in fish, insects and other ectotherms, although plastic changes in heat tolerance appear less substantial than in cold tolerance (Chown and Terblanche, 2007; Fischer et al., 2010; Overgaard et al., 2011; Seebacher et al., 2014). Fewer studies have explored physiological acclimation of heat tolerance to diurnal fluctuations in temperature (Hutchison and Michael, 1970; Hokanson et al., 1977; Podrabsky and Somero, 2004; Colinet et al., 2015). For example, in *Drosophila melanogaster*, diurnal fluctuations ($\pm 5^\circ\text{C}$) increased heat tolerance (measured as recovery time from heat coma) of adults, especially at higher mean temperatures (Bozinovic et al., 2011). Heat tolerance (heat knockdown recovery time) of adult *Lycaena* butterflies was greater when larvae were reared at high mean temperatures and with diurnal temperature fluctuations (Fischer et al., 2011). In adult Mediterranean fruit flies, increasing diurnal fluctuations for 10 days strongly increased CT_{max} , whereas increasing mean temperatures had little effect (Terblanche et al., 2010). Schaefer and Ryan (2006) showed that diurnal fluctuations in temperature during acclimation increased heat tolerance (CT_{max}) of zebrafish, at least at lower mean temperatures. Our results for *M. sexta* larvae parallel those for

Fig. 3. CT_{max} as a function of maximum temperature in the heat shock and rearing temperature experiments. Filled squares, dotted trend line: single heat shock; open circles, dashed line: diurnally fluctuating temperature; and filled circles, solid line: continuous constant temperature. Mean (± 1 s.e.m.) CT_{max} is shown for 5th instar *M. sexta* larvae. See Materials and methods and Fig. 1.

zebrafish: mean CT_{max} increased at higher mean developmental temperatures, and diurnal fluctuations increased mean CT_{max} at lower (25 and 28°C) but not higher (30°C) mean temperatures (Fig. 2B). As suggested by Schaefer and Ryan (2006), the interactive effects of mean temperature and temperature fluctuation on heat tolerance may emerge because at high mean temperatures, diurnal fluctuations can generate stressfully high temperatures. Recent studies of growth rate in *M. sexta* support this interpretation. For example, at a mean temperature of 25°C, diurnal fluctuations ($\pm 10^\circ\text{C}$) during larval development increase maximal growth rates and the optimal temperature for growth for 5th instar larvae, revealing beneficial acclimation to diurnal fluctuations (Kingsolver et al., 2015). By contrast, at a mean temperature of 30°C, increasing diurnal fluctuations during development strongly reduces rates of growth, development and survival (Kingsolver et al., 2015).

Of course, diurnal fluctuations also increase the maximum and minimum daily temperatures experienced by organisms (Marshall and Sinclair, 2012; Colinet et al., 2015). Do high temperatures experienced throughout development increase CT_{max} beyond the effects of a single, short-term exposure? By combining the results from our heat shock and rearing temperature experiments, our analyses suggest that changes in heat tolerance are strongly determined by maximum temperature (Fig. 3), whether the maximum temperature is experienced once (acute heat shock treatments), repeatedly every day (diurnally fluctuating treatments) or continuously throughout development (constant temperature treatments). However, rearing temperatures also have additional effects beyond maximal temperature: both repeated and chronic exposure to high temperatures during development significantly altered CT_{max} (Fig. 3).

Because our experimental treatments include exposure to increased temperature throughout larval development, our treatments differ in both the number and total duration of exposure to ‘stressful’ temperatures. Our study demonstrates that developmental acclimation to higher maximum temperature increases heat tolerance beyond that due to a single, short-term heat shock. Recent studies of cold tolerance of overwintering insect larvae suggest that the number, intensity and duration of cold exposure can have differing effects on tolerance and other traits (Sinclair and Chown, 2005; Marshall and Sinclair, 2012; Colinet et al., 2015). For example, in overwintering larvae of eastern spruce budworm, the number of cold exposures, rather than the intensity or duration of exposure, determines tolerance and survival to cold (Marshall and Sinclair, 2015). These experiments involve exposure within a single larval instar (2nd), and do not consider effects across development. Tolerance may also vary with age and development stage. For example, cold tolerance of adult female *D. melanogaster* declines rapidly with age, presumably as a result of senescence (Colinet et al., 2013). In our study, we standardized our measurements for individuals in the same developmental stage (2nd day of the 5th instar); because developmental temperatures alter rates of larval development, these individuals necessarily vary in chronological age.

The increases in CT_{max} due to acclimation that we observed are relatively modest: only 1–2°C above the mean control value (44.0°C). However, in many parts of its range in Central America and southern USA, body temperatures of *M. sexta* larvae in the field can often approach these temperatures (Casey, 1976; Kingsolver et al., 2012; Woods, 2013). For example, Woods (2013) estimated that in suitable habitat for this species in southwestern USA and northern Mexico, 5th instar larvae experience maximal body

temperatures above 44.3°C in 29% of the area; but maximal temperatures above 46°C are much more restricted (Woods, 2013). Casey (1976) found that 3rd instar *M. sexta* larvae from southwestern USA could survive a 30 min exposure to 44°C, but there was 100% mortality at 46°C. Thus, even small increases in CT_{max} could be important in reducing or avoiding heat stress and mortality.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.G.K. conceived and designed the experiments; H.J.M. set up the measurement apparatus and protocols; S.B.G., H.J.M. and K.E.A. performed the experiments; J.G.K. performed the statistical analyses; K.E.A. created the figures; J.G.K., H.J.M. and K.E.A. drafted and revised the manuscript.

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