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RESEARCH ARTICLE

Fluctuating temperatures and ectotherm growth: distinguishing non-linear and time-dependent effects

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ABSTRACT

Most terrestrial ectotherms experience diurnal and seasonal variation in temperature. Because thermal performance curves are non-linear, mean performance can differ in fluctuating and constant thermal environments. However, time-dependent effects – effects of the order and duration of exposure to temperature – can also influence mean performance. We quantified the non-linear and time-dependent effects of diurnally fluctuating temperatures for larval growth rates in the tobacco hornworm, *Manduca sexta* L., with four main results. First, the shape of the thermal performance curve for growth rate depended on the duration of exposure: for example, optimal temperature and thermal breadth were greater for growth rates measured over short (24 h during the last instar) compared with long (the entire period of larval growth) time periods. Second, larvae reared in diurnally fluctuating temperatures had significantly higher optimal temperatures and maximal growth rates than larvae reared in constant temperatures. Third, for larvae maintained at three mean temperatures (20, 25 and 30°C) and three diurnal temperature ranges (±0, ±5 and ±10°C), diurnal fluctuations had opposite effects on mean growth rates at low versus high mean temperature. Fourth, both short- and long-term thermal performance curves yielded poor predictions of the non-linear effects of fluctuating temperature on mean growth rates (compared with our experimental results) at higher mean temperatures. Our results suggest caution in using constant temperature studies to model the consequences of variable thermal environments.

KEY WORDS: Acclimation, Thermal performance curves, Fluctuating environments, Growth rates, *Manduca sexta*, Stress responses

INTRODUCTION

During their lives, most ecothermic organisms experience a wide range of environmental and body temperatures. This variation comes from diurnal and seasonal cycles as well as from stochastic variation in weather and climate at multiple temporal and spatial scales. As a result, terrestrial ectotherms have evolved to function over a range of temperatures. The thermal sensitivity of an organism is often characterized in terms of its thermal performance curve (Huey and Stevenson, 1979; Huey and Kingsolver, 1989): the rate of locomotion, growth or fitness as a function of body temperature (see Fig. 1). Comparative analyses indicate that key features of thermal performance curves are adapted to local climatic conditions. For example, thermal breadths are greater, and critical minimum temperatures are lower, for populations and species at higher than at lower latitudes (Sunday et al., 2011).

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Thermal performance curves for biological rates are non-linear, with an intermediate optimal temperature for performance, and reduced performance at both lower and higher temperatures (Fig. 1). As a result, mean performance in fluctuating temperatures may differ from performance at the mean temperature, an effect due to Jensen's inequality for non-linear functions (Ruel and Ayres, 1999; Martin and Huey, 2008). The consequences of such non-linear effects have been thoroughly explored for ectotherms in variable thermal environments, and are the basis for most predictions about responses to climatic variation and climate change (Deutsch et al., 2008; Kingsolver et al., 2013; Vasseur et al., 2014).

Thermal performance curves implicitly assume that performance in fluctuating conditions depends only on temperature, and does not depend on the pattern, duration or order of exposure to temperature. However, many studies have shown that the duration of exposure can influence performance even for non-lethal temperatures, and the thermal range for performance can decrease with increasing duration of exposure (Rezende et al., 2014). For example, for larval growth in insects, body temperatures that maximize growth rates at short time scales (4–24 h) can be suboptimal or even lethal over longer time scales (Reynolds and Nottingham, 1985; Kingsolver and Woods, 1997; Kingsolver, 2000; Petersen et al., 2000; Kingsolver et al., 2004; Kingsolver and Nagle, 2007). As a result, the shape and position of a thermal performance curve can depend on time scale. This has important consequences for understanding and predicting responses of ectotherms to variable environments or climate scenarios (Kingsolver et al., 2013). For example, thermal performance curves based on experiments using constant temperatures throughout development yielded poor predictions about mean development rates during diurnal fluctuating conditions in marsh frogs (Niehaus et al., 2012). Similarly, in *Manduca sexta* L., thermal performance curves for growth rates based on constant temperatures throughout larval growth do not accurately predict mean growth in alternating (day–night) temperature conditions (Kingsolver and Nagle, 2007; Kingsolver et al., 2009). These results call into question the common practice of using thermal performance curves measured at constant temperatures to predict responses of ectotherms to diurnal fluctuations and climate change (Deutsch et al., 2008; Sinervo et al., 2010; Vasseur et al., 2014).

The duration of thermal exposure can have both negative and positive effects on performance and fitness. Acute heat or cold shocks are stressful and can reduce performance and survival in many ectotherms (Feder and Hofmann, 1999). Repeated exposure to high, sublethal temperatures can also cause stress and reduce growth rates. Conversely, brief exposure to high but sublethal temperatures may induce heat shock protein (HSP) expression and increase survival when ectotherms are exposed to subsequent heat shocks, a form of adaptive acclimation called heat-hardening (Lindquist and Craig, 1988). The role of diurnal fluctuations in temperature (DFT) in causing time-dependent effects like stress and acclimation is poorly understood for most ectotherms. Several recent studies that

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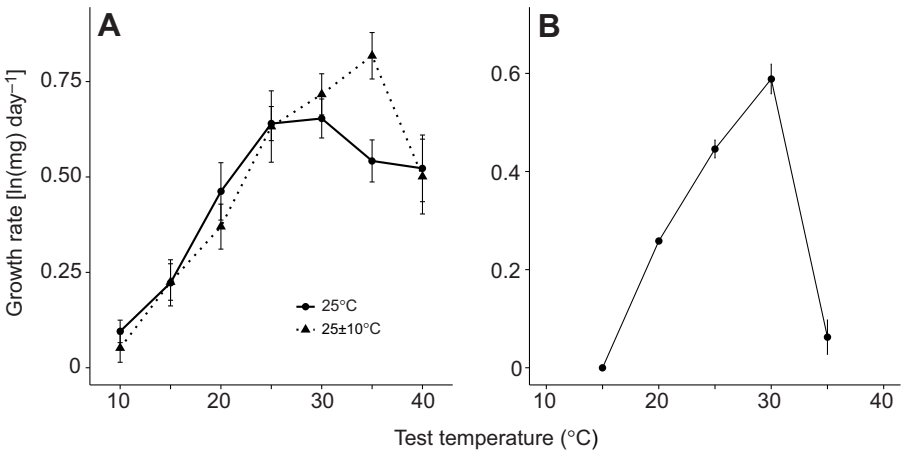

Fig. 1. Mean (±1 s.e.m.) larval growth rate as a function of temperature. (A) Short-term (24 h) growth rate (corrected for initial mass) at the start of the 5th instar, for larvae reared from hatching at constant (25°C) or fluctuating (25±10°C) rearing temperature. (B) Long-term (hatching to wandering) larval growth rate at constant temperature.

consider the consequences of diurnal fluctuations reveal a variety of positive, neutral and negative responses for metabolic rate, mortality and heat tolerance (Schaefer and Ryan, 2006; Cooper et al., 2010; Bozinovic et al., 2011; Folguera et al., 2011; Rojas et al., 2014; Xing et al., 2014). For example, DFT during development increased HPS70 protein synthesis, metabolic rate and mortality in a woodlouse (Folguera et al., 2011).

Here, we examined larval growth rates in the tobacco hornworm, *M. sexta*, to distinguish non-linear and time-dependent responses to diurnal fluctuations in temperature. We had four main goals. First, we measured short-term (24 h) growth rates of 5th (final) instar larvae to quantify the mean thermal performance curve (TPC), and compared this with previous TPCs for mean growth rate measured under long-term (throughout the period of larval growth) conditions with constant temperatures (Kingsolver and Nagle, 2007). Second, we examined whether constant versus fluctuating rearing conditions alter the mean TPC for short-term growth rate. Third, we quantified how mean temperature and diurnal fluctuations during larval development alter both short-term (24 h in the 5th instar) and long-term (throughout larval life) growth rates. Fourth, we used the estimated short-term and long-term TPCs for growth rate to predict expected mean growth rates under diurnally fluctuating conditions, and compared these predictions with the observed responses. Our analyses show that both non-linear and time-dependent responses determine mean growth rates under fluctuating conditions, and that time-dependent responses may be particularly important at higher mean temperatures and larger diurnal fluctuations.

RESULTS

Experiment 1: TPCs for short-term growth rate

Fifth-instar larvae maintained positive short-term (24 h) growth over a wide range of test temperatures from 15 to 40°C (Fig. 1A). Initial mass ($P<0.001$) and test temperature ($P<0.001$) had highly significant effects on growth rate. Rearing temperature did not have a significant effect ($P=0.162$), but there was a significant interaction between rearing temperature and test temperature ($P=0.007$). Mean growth rates for the constant (25°C) and diurnally fluctuating (25±10°C) rearing conditions were similar at test temperatures between 10 and 25°C, but differed at higher temperatures; in particular, both optimal temperature and maximum growth rate were higher under fluctuating rearing conditions (Fig. 1A). The fraction of larvae that maintained positive short-term growth at 40°C was only 0.6 for the constant rearing treatment compared with 0.9 for the fluctuating rearing treatment. These results suggest that daily exposure to higher temperatures during early larval instars improved performance at higher temperatures.

By contrast, for long-term growth measured over the period of larval growth for *M. sexta* from the same laboratory colony (data from Kingsolver and Nagle, 2007), the TPC was narrower and more symmetric, and growth rate was maximal at temperatures near 30°C (Fig. 1B). Note that thermal breadth, optimal temperature and maximal temperature for growth all depend on the time scale over which the TPC was measured.

Experiment 2: effects of mean temperature and diurnal temperature range

Survival to pupation exceeded 90% for all treatments with a mean temperature of 20 and 25°C, regardless of the diurnal temperature range. By contrast, for mean temperatures of 30°C, survival declined with increasing diurnal temperature range, from 100% for the ±0°C (constant) treatment to 62% for the ±10°C treatment. Most mortality occurred during the 4th or 5th instars. This suggests that daily exposure to temperatures above 30–35°C is stressful for *M. sexta* larvae.

As expected, rates of change in mass and developmental stage were generally greater at higher mean temperatures (Fig. 2). Diurnal temperature range also strongly influenced mass and development time, but these effects depended on mean temperature (Fig. 2). At low (20°C) mean temperature, the age (development time) at which each stage was reached declined with increasing diurnal temperature range, especially at later stages (Fig. 2A). At intermediate (25°C) mean temperature, diurnal temperature range had relatively modest effects on age to most stages (but see below) (Fig. 2B). In contrast, at high (30°C) mean temperature, increasing diurnal temperature both increased development time and decreased mass at the later stages (Fig. 2C); these effects were particularly strong for the ±10°C diurnal range treatment. These results suggest that diurnal temperature fluctuations can have qualitatively different effects on growth and development rates, and that these effects can accumulate across life stages.

We used ANOVA to evaluate the effects of mean temperature and diurnal temperature range on development time and mass at the 5th larval instar and at pupation (see Materials and methods). Mean temperature, diurnal temperature range and their interaction all had highly significant effects on development time to 5th instar ($P<0.001$ in each case). Development time to 5th instar declined with increasing mean temperature, but the slope of this relationship varied with diurnal temperature range (Fig. 3). For example, at a mean temperature of 20°C, development time was fastest for

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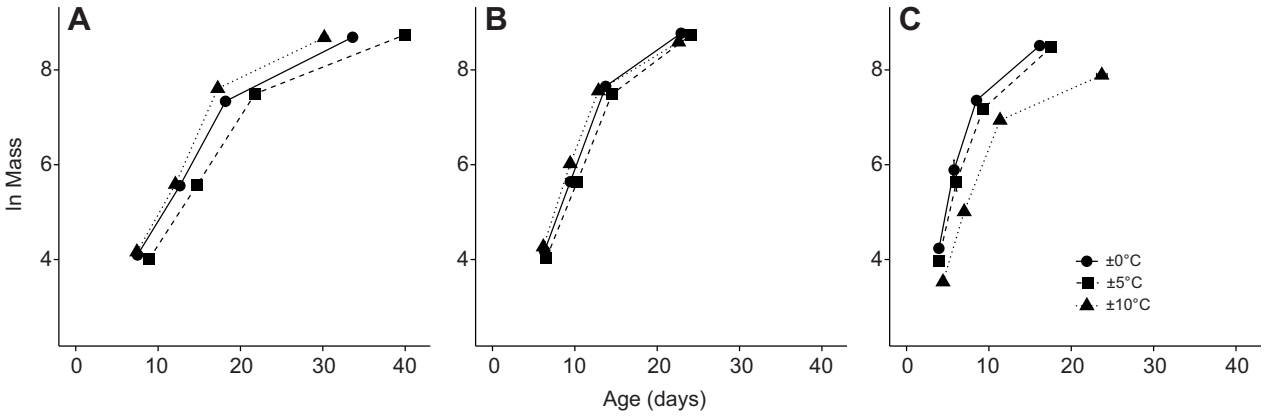

Fig. 2. Mass and development time at the start of the 3rd, 4th and 5th instar and at pupation, for different diurnal temperature ranges. (A) Mean temperature of 20°C; (B) mean temperature of 25°C; (C) mean temperature of 30°C. Mass was measured in mg. Means±1 s.e.m. are indicated.

the ±10°C treatment, whereas at a mean temperature of 30°C, development time was slowest for the ±10°C treatment. Similarly, mean temperature, diurnal temperature range and their interaction all had highly significant effects on mass at 5th instar (P<0.001 in each case). Diurnal temperature range had qualitatively different effects on mass for different mean temperatures (Fig. 3B). For example, increasing diurnal fluctuations resulted in larger size at lower mean temperatures, but smaller size at higher mean temperatures. These patterns of mean temperature and diurnal temperature range were qualitatively similar for time and mass at pupation (supplementary material Fig. S2). For both life stages, increased diurnal fluctuations at high mean temperatures prolonged development and reduced body size.

We considered two metrics of mean larval growth rate: short-term (24 h) growth rate during the early 5th instar (corrected for initial mass) and mean growth rate from hatching to molt into the 5th instar (see Materials and methods). ANOVA indicated highly significant effects of mean temperature, diurnal temperature range and their interaction on both metrics of growth rate (P<0.001 in each case). For short-term growth, increasing diurnal range had small effects on growth rate at lower mean temperatures but caused large reductions in growth at high mean temperatures (Fig. 4A). For mean growth rate to 5th instar, large diurnal fluctuations increased growth rate at low mean temperatures but had the opposite effect at high mean temperatures (Fig. 4B). For both metrics, growth rate was higher in constant than in fluctuating conditions when mean temperature was high. Mean growth rate to pupation (supplementary material Fig. S2) showed qualitatively similar patterns. These results confirm that repeated exposure to temperatures above 30°C can reduce average rates of growth and development.

Non-linear effects: predicting mean growth rates using TPCs

By integrating the performance curve for growth rate (Fig. 1) over the temperatures experienced over the diurnal cycle (supplementary material Fig. S1) we can compute the predicted mean growth rate for each temperature treatment in our experiment and compare this with our observed growth rates (see Eqn 1 and Materials and methods). Predictions based on the thermal performance curve for short-term (24 h) growth rate indicated that predicted mean growth rate increased with mean temperature regardless of diurnal temperature range, and that diurnal fluctuations had modest predicted effects on mean growth rate for the mean temperatures of 20 and 25°C, but not for 30°C (Fig. 5A). These predictions arise because short-term growth rate does not decline until temperatures exceed 35°C (Fig. 1A) – thermal conditions that only occur in the 30±10°C treatment. Predictions based on the long-term thermal performance curve for mean growth over the larval period (at constant temperature) showed that increasing diurnal fluctuations decreased predicted mean growth rate at higher mean temperatures (Fig. 5B). Predicted mean growth rate was largely insensitive to differences in mean temperature for the ±10°C treatments.

Comparing the observed and predicted mean relative growth rates for each treatment revealed two important results. First, for short-

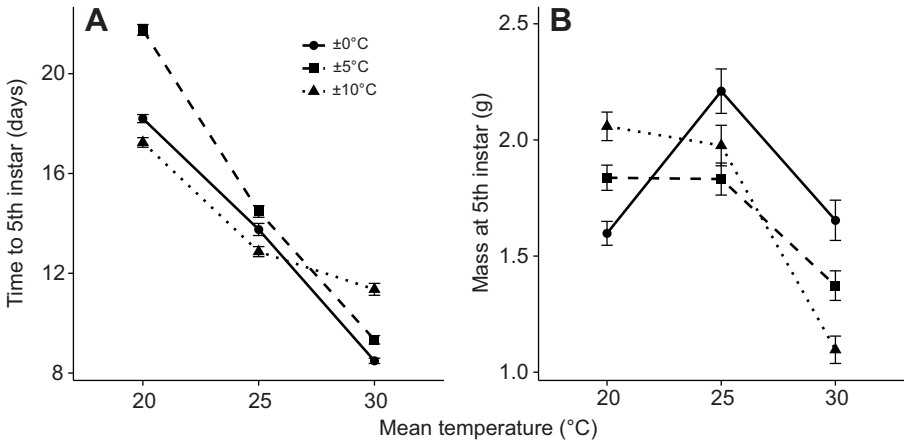

Fig. 3. Development time to 5th instar and mass at 5th instar as a function of mean rearing temperature, for different diurnal temperature ranges. (A) Time to 5th instar; (B) mass. Means±1 s.e.m. are indicated.

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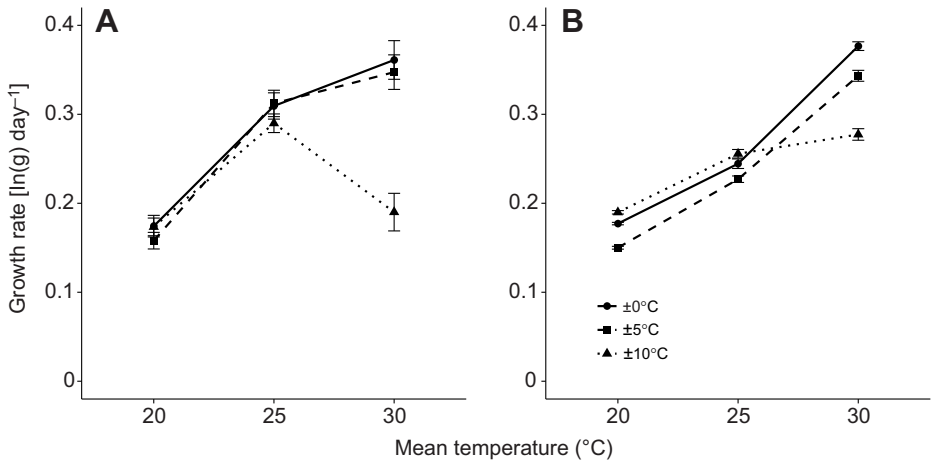

Fig. 4. Short-term growth rate during early 5th instar and mean growth rate from hatching to 5th instar as a function of mean rearing temperature, for different diurnal temperature ranges. (A) Short-term (24 h) growth rate; (B) long-term growth rate. Means±1 s.e.m. are indicated.

term TPCs (Fig. 6A), observed and predicted growth rates were similar (given the uncertainty of these estimates) for each treatment group with one exception: for the 30±10°C treatment, the observed mean growth rate was substantially below the predicted rate. Second, for the long-term TPCs, observed mean growth rates were substantially above the predicted rates for three of the six fluctuating treatments at higher temperatures (Fig. 6B). These results suggest that the non-linearity of thermal performance curves does not fully account for the effects of diurnal fluctuations on larval growth: time-dependent responses to temperature are also contributing to these effects. In addition, thermal performance curves based on long-term measurements at constant temperatures provided inaccurate and biased predictions of mean growth rate in diurnally fluctuating conditions.

DISCUSSION

Acclimation of thermal performance curves

Our results demonstrate that rearing temperature can alter short-term thermal performance curves. In particular, diurnally fluctuating (25±10°C) rearing conditions increased growth rates at higher temperatures, compared with constant (25°C) rearing conditions (Fig. 1). This suggests that daily exposure to higher temperatures during development can increase both optimal temperature and maximal growth rate at the optimum, an example of beneficial thermal acclimation (Huey et al., 1999). While many studies have evaluated beneficial acclimation to different constant temperatures (Huey and Berrigan, 1996; Angilletta, 2009), only a handful of studies have examined physiological acclimation to fluctuating versus constant temperatures. Diurnal fluctuations in temperature have been shown to increase HSP70 synthesis (Folguera et al., 2011) and heat tolerance (Schaefer and Ryan, 2006), reduce exploratory behavior (Rojas et al., 2014) and reduce maximum metabolic rates (Bozinovic et al., 2013), but to our knowledge beneficial acclimation of optimal temperature or maximal performance has not been previously reported (Cooper et al., 2010). Given the ubiquity of diurnal fluctuations in terrestrial environments, this issue deserves further study.

Time scale and thermal performance curves

Our studies confirmed that the time scale of temperature exposure can qualitatively alter the thermal sensitivity of performance. Both the optimal temperature and the thermal range for larval growth were greater for short-term (24 h) than for long-term (larval period) measurements of thermal performance curves (Fig. 1). It is not surprising that high rates of growth and performance cannot be sustained over longer time periods, as many factors may limit high rates of performance over time. What is important, however, is that performance typically declines more rapidly with increasing time at higher temperatures, such that the optimal temperature for performance declines over time. Similar effects of time scale on optimal and maximal temperatures have been reported for larval growth rates and thermal tolerance in other insects (Kingsolver, 2000;

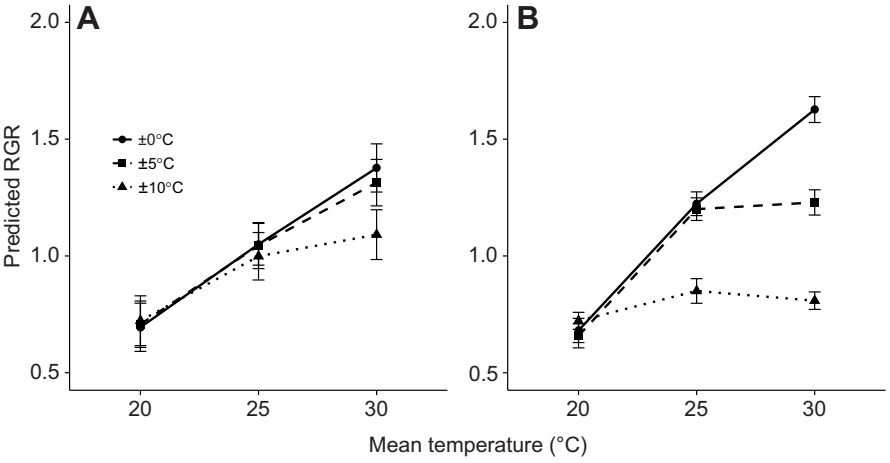

Fig. 5. Predicted short-term growth rate during early 5th instar and mean growth rate from hatching to 5th instar as a function of mean rearing temperature, for different diurnal temperature ranges. Predictions are based on the thermal performance curves in Fig. 1 (see Results). (A) Short-term (24 h) growth rate; (B) long-term growth rate. Means±1 s.e.m. are indicated. RGR, relative growth rate.

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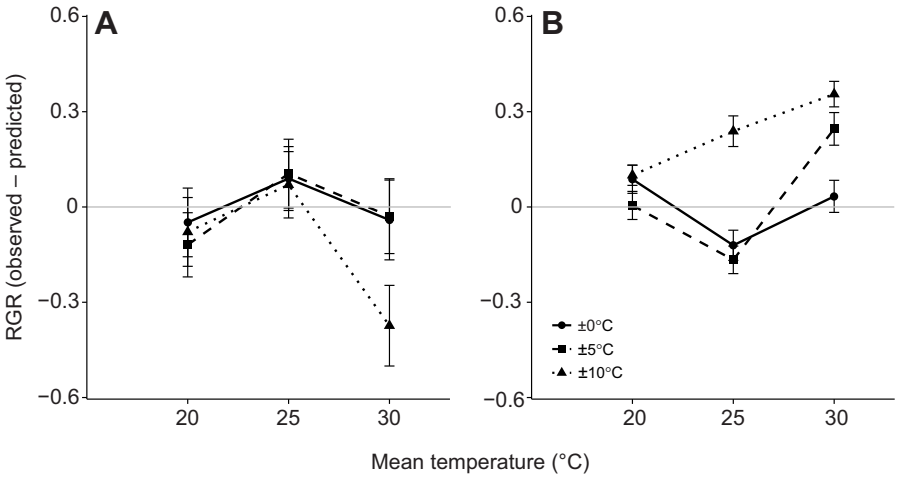

Fig. 6. Difference between observed and predicted values for short-term growth rate during early 5th instar and growth rate from hatching to 5th instar as a function of mean rearing temperature, for different diurnal temperature ranges. (A) Short-term (24 h) growth rate; (B) long-term growth rate. Means±1 s.e.m. are indicated.

Kingsolver et al., 2004; Rezende et al., 2011, 2014). More generally, optimal and maximal temperatures are frequently greater for short-term (e.g. rates of locomotion) than for long-term (e.g. development rates or fitness) aspects of performance (Angilletta, 2009).

Two general mechanisms may account for these time-dependent effects of temperature. First, exposure to high temperatures can lead to production of HSPs and other stress responses (Feder and Hofmann, 1999). HSPs can increase survival in response to subsequent heat shocks, but chronic high temperatures and high levels of HSPs can reduce development rate and survival (Krebs and Feder, 1998). In *M. sexta*, 1 h heat shocks at 42°C produced high rates of HSP synthesis; and 1 h heat shocks at 38°C caused increased rates of HSP synthesis in some individuals (Fittinghoff and Riddiford, 1990). Although high, constant rearing temperatures can increase HSP expression (Karl et al., 2012), the effects of repeated (e.g. daily) exposure to high temperatures for gene expression and protein production for HSP genes is generally unknown (Dahlhoff and Rank, 2000; McMillan et al., 2005). We note that the physiological costs and benefits are likely due to synthesis and levels of HSP proteins rather than of HSP mRNA levels per se (Feder and Hofmann, 1999; Bahrndorff et al., 2009).

Second, time dependence of temperature may arise when different component processes have different thermal sensitivities. For herbivorous insects, growth rate is the outcome of a series of processes, including rates of ingestion, protein breakdown, amino acid uptake, excretion and metabolism (Woods and Kingsolver, 1999). In *M. sexta*, these rates differ substantially in their thermal sensitivity, especially at higher temperatures (Kingsolver and Woods, 1997). For example, short-term ingestion rates are greatest at 34–38°C and decline at temperatures above 38°C; protein digestion rates increase with increasing temperature to 42°C; and amino acid uptake rates are greatest at 38–42°C. As a consequence, different processes can limit growth rate at different temperatures (Kingsolver and Woods, 1997). Because gut passage time in *Manduca* is of the order of 5–6 h at intermediate temperatures (Woods and Kingsolver, 1999), a temporal switch to a new temperature generates time-dependent changes in growth rate at the new temperature. These two mechanisms are not mutually exclusive: both may contribute to time-dependent responses to temperature, but their relative importance is unknown.

Responses to diurnal temperature fluctuations

Our results illustrate that diurnal temperature fluctuations can have important impacts on size, development rate and other life history traits. A key finding is that diurnal fluctuations had qualitatively different effects at different mean temperatures. Diurnal fluctuations had little effect on growth and development rates at intermediate (25°C) mean temperatures; increasing fluctuation increased these rates at low mean temperatures, but had the opposite effect at high mean temperatures. The negative effects of diurnal fluctuations at high mean temperatures suggests that repeated exposure to temperatures above 30–35°C is stressful for *M. sexta* larvae, a result also found for *M. sexta* eggs (Potter et al., 2009, 2011).

Empirical studies with insects and other ectothermic animals reveal a variety of responses to diurnal fluctuations in rearing temperatures (Worner, 1992). Two factors are important in interpreting this literature. First, for both technical and conceptual reasons, most 20th century studies used alternating (square-wave) fluctuations in temperature (Stamp, 1993; Worner, 1992). Alternating temperatures poorly represent diurnal patterns of environmental and body temperature variation for most terrestrial and aquatic ectotherms, and can diminish or exaggerate the effects of fluctuations on mean performance. Recent experimental studies increasingly use more realistic patterns of diurnal temperature. Second, most studies considered the effects of diurnal fluctuations at only one or two mean temperatures. Given the shape of most thermal performance curves, we would expect that effects of diurnal fluctuations would be smallest at intermediate, non-stressful temperatures (Xing et al., 2014). Our results highlight that changes in mean temperature can qualitatively alter the consequences of diurnal fluctuations.

Diurnal temperature fluctuations are generally expected to influence mean performance, including mean growth rates, because thermal performance curves are non-linear (Ruel and Ayres, 1999; Martin and Huey, 2008). We used thermal performance curves for larval growth rate measured at two different time scales (Fig. 1) to predict how these non-linear effects would alter mean growth rates for diurnally fluctuating conditions (Eqn 1). Comparison with our experimental results (Fig. 4) suggests that both short-term and long-term thermal performance curves do not adequately predict the qualitative effects of fluctuations on mean growth rates, especially at higher mean temperatures (Fig. 5). The failure of these non-linear models suggests that time-dependent effects of temperature fluctuations are strongly influencing growth and development at higher temperatures. These results emphasize that the time scale over which thermal performance curves are measured should be specifically considered when predicting responses to fluctuating temperatures (Niehaus et al., 2012). Similarly, the causes and consequences of time-dependent responses of ectotherms to diurnal and stochastic variation in temperature require further attention.

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Time-dependent responses have important implications for predicting the biological consequences of climate change. Numerous recent studies have used thermal performance curves to predict the fitness and ecological responses of ectotherms under future climate scenarios (Deutsch et al., 2008; Sinervo et al., 2010; Huey et al., 2012; Kingsolver et al., 2013; Bonebrake et al., 2014; Vasseur et al., 2014). For example, thermal performance curves for fitness (measured at constant temperatures over a generation) have been used to predict geographic patterns in fitness responses of insects to climate change, including diurnal fluctuations in temperature (Deutsch et al., 2008; Vasseur et al., 2014). The use of generation-scale TPCs to predict responses to diurnal and other short-term thermal fluctuations may overstate the negative consequences of daily maximum temperatures (Fig. 1). One alternative is to use environmental data averaged over time scales that match the time scales of TPCs. For example, because many (non-diapausing) insects have generation times on the order of one to several months, Kingsolver et al. (2013) used mean monthly temperature data to predict fitness responses of insects to climate change. However, this approach cannot address the potential impacts of diurnal thermal fluctuations and heat waves on organisms. Integrating the short-term responses of ectotherms to diurnal and stochastic thermal variation across the life cycle to predict lifetime fitness remains an important challenge in thermal biology and climate change research.

MATERIALS AND METHODS

Study system: *M. sexta*

The tobacco hornworm, *M. sexta*, occurs in Central and South America and the southern USA, with eastern populations extending north into New York and Massachusetts. Larvae feed primarily on host plants in the family Solanaceae; in the southeastern USA, cultivated tobacco and tomato are dominant host plants for *M. sexta*. After hatching, *M. sexta* larvae grow rapidly through five (occasionally more) larval instars, increasing from ~1 mg to ~8–12 g in body mass in a few weeks under optimal conditions. Towards the end of the final instar, larvae stop feeding and wander off the host plant to pupate nearby in the soil. A facultative pupal diapause is determined by larval photoperiod, such that *M. sexta* populations have multiple generations per year in most areas. Because pupae do not feed, maximum larval mass at wandering largely determines pupal and adult size and the number of eggs (oocytes) produced by females (Davidowitz and Nijhout, 2004; Diamond and Kingsolver, 2010a,b).

Rates of larval growth and development for *M. sexta* are strongly influenced by environmental temperature (Reynolds and Nottingham, 1985; Stamp, 1993). Field studies in both the southeastern and southwestern USA show that larval body temperatures frequently vary by 20–25°C during a single diurnal cycle, and the body temperature of an individual may vary by 30°C during its larval life (Casey, 1976; Diamond and Kingsolver, 2010b; Kingsolver et al., 2012). Diurnal fluctuations in temperature during rearing can alter mean growth and development rates (Stamp, 1994; Kingsolver et al., 2009), but the contributions of non-linear and time-dependent effects to growth and development have not been explored.

Our experiments used *M. sexta* larvae reared on standard artificial diet (Bell and Joachim, 1976) from a laboratory colony maintained at the University of North Carolina since the 1980s; the colony was originally established from field populations near Raleigh, NC, USA, in the 1960s. Previous studies show differences in size, mean growth rate and heat tolerance between colony and field populations (Kingsolver and Nagle, 2007; Diamond and Kingsolver, 2010a), but colony and field populations have similar growth responses to alternating rearing temperatures (Kingsolver et al., 2009) (see Discussion).

Experiment 1: TPCs for short-term growth rate

Larvae were reared from hatching in a programmable environmental chamber (Percival 36VL) on a long-day (14 h light:10 h dark) photocycle at one of two rearing temperatures: a constant 25°C temperature, or diurnally fluctuating temperatures (25±10°C: see Experiment 2, below). Larvae were maintained in plastic bins with abundant diet that was changed every 2 days; the number of larvae per bin was adjusted to minimize potential aggressive interactions or competition for food among larvae. On the morning following molt into the 5th (final) larval instar, each larva was placed in an individual Petri dish and randomly assigned to one of seven test temperatures in environmental chambers: 10, 15, 20, 25, 30, 35 or 40°C. After a 2 h acclimation period at the test temperature without food, the larva was weighed (Mettler Toledo AT261) and returned to its dish with fresh diet at its test temperature. The larva was re-weighed after 4 h and again after 24 h (the time of each weighing was also recorded, to determine the duration of the test period). A total of 10–20 larvae were measured at each test temperature. Larvae whose initial mass was greater than 2.5 g were excluded from the analyses, as they were likely to be older than the first day of the 5th instar (Kingsolver and Nagle, 2007). We also excluded larvae that did not feed or had negative growth during the first 4 h of the test period.

We modeled the effects of rearing and test temperatures on growth in two ways. First, we quantified growth rate over the 24 h test period as $(m_f-m_i)/d$, where m_i and m_f are initial and final body masses and d is the duration of the test period (approximately 24 h). We modeled growth rate using linear models (lm function in R) with initial mass as a covariate, and including effects of rearing temperature, test temperature (as a 4th order polynomial), and the interaction of rearing and test temperature. The interaction effect tests whether rearing temperature alters the effects of test temperature. Preliminary analyses indicated that a 4th-order polynomial provided a better fit (based on AIC) to these data than a 3rd-order polynomial or a cubic spline. Modeling growth rate on a log-scale [i.e. $\ln(m_f/m_i)/d$] gave qualitatively similar results, and yielded very similar adjusted R^2 values (R^2=69.5%). Second, we considered $\ln(m_f)$ as a response variable, and used linear models with $\ln(m_i)$ as a covariate, and including effects of rearing temperature, test temperature (as a 4th-order polynomial), and the interaction of rearing and test temperature (Raubenheimer and Simpson, 1992). Because these two analyses yielded qualitatively similar results, we only present the results from the first analysis.

To determine thermal performance curves for growth rate, we computed a size-adjusted growth rate for each larva. We first regressed $\ln(m_i)$ on proportional growth rate [$g=\ln(m_f/m_i)/d$], and used the residuals (r) from this model. The size-adjusted growth rate G for each larva is then defined as $G=r+\mu$, where μ is the mean growth rate for all larvae in the study or rearing environment (see Results).

Experiment 2: effects of mean temperature and diurnal temperature range

This experiment had two factors, each with three levels: mean temperature (20, 25, 30°C) and diurnal temperature range (±0, ±5, ±10°C). A long-day (14 h light:10 h dark, with the lights on between 06:00 h and 20:00 h) photocycle was used for each treatment. The diurnal cycle for each temperature treatment involved 2 h at the low temperature (between 01:00 h and 03:00 h); a linear (ramping) temperature increase from the low to the high temperature (03:00 h–13:00 h); 2 h at the high temperature (13:00 h–15:00 h); and a linear (ramping) temperature decrease from the high to the low temperature (15:00 h–01:00 h) (see supplementary material Fig. S1 for examples). After hatching, each larva was randomly assigned to a treatment group, and reared individually in a Petri dish with abundant diet; diet was changed every 1–2 days depending on treatment. Mass and development time (since hatching) were measured at the start of the 3rd, 4th and 5th instars and at pupation. In addition, short-term (24 h) growth rate in the treatment conditions was measured, starting in the morning following molt into the 5th instar. Our analyses focused on two metrics of growth rate: mean growth rate from hatching to 5th instar (=mass at 5th instar/time to 5th instar), and 24 h growth rate in early 5th instar. Because rearing treatment affected mass at the start of the 24 h growth rate measurement (see Results), residual analysis was used to correct for effects of initial mass on growth rate. Response variables were modeled using ANOVA in R, with both mean temperature and diurnal temperature range considered as factors.

Modeling non-linear effects

We can use thermal performance curves to predict mean growth rates expected under fluctuating thermal conditions. Systematic differences between the predicted and observed mean growth rates would indicate the contributions of time-dependent effects on growth. We used estimates of the thermal performance curve for growth rate in *M. sexta* from our laboratory colony at two scales: short-term (24 h) growth rate in early 5th instar (from Experiment 1) and long-term (over larval life) growth rate from hatching to wandering (the end of larval growth) (Kingsolver and Nagle, 2007) (see Fig. 1). Each TPC was modeled using a 4th-order polynomial (see above); this allowed us to compute a predicted value, and an estimated standard error on that value, for any value of temperature. If $G(T)$ is the thermal performance curve for growth rate and $T(t)$ is temperature T as a function of time t over the diurnal cycle for a particular temperature treatment, then the predicted mean growth rate g is:

$$g = \int G(T)T(t)\mathrm{d}t, \quad (1)$$

where the integration is over the diurnal cycle. We compared the predicted mean growth rates with the observed values from our experiments for our two metrics of growth: mean growth rate to 5th instar and 24 h growth rate in early 5th instar (see above). Because our main goal was to evaluate the use of TPCs for predicting mean growth rates under fluctuating temperatures, we computed relative growth rate for each treatment standardized by the overall mean growth rate for the three constant temperature treatments. The difference between observed and predicted relative growth rate is an indicator of the contribution of time-dependent effects on growth rates at different temperatures.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.G.K. conceived and designed the experiments; J.K.H. and K.E.A. performed the experiments; J.G.K. performed the statistical analyses and modeling; J.K.H. and K.E.A. created the figures; J.G.K., J.K.H. and K.E.A. drafted and revised the manuscript.

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Supplementary material

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