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RESEARCH ARTICLE

Responses of *Manduca sexta* larvae to heat waves

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ABSTRACT

Climate change is increasing the frequency of heat waves and other extreme weather events experienced by organisms. How does the number and developmental timing of heat waves affect survival, growth and development of insects? Do heat waves early in development alter performance later in development? We addressed these questions using experimental heat waves with larvae of the tobacco hornworm, *Manduca sexta*. The experiments used diurnally fluctuating temperature treatments differing in the number (0–3) and developmental timing (early, middle and/or late in larval development) of heat waves, in which a single heat wave involved three consecutive days with a daily maximum temperature of 42°C. Survival to pupation declined with increasing number of heat waves. Multiple (but not single) heat waves significantly reduced development time and pupal mass; the best models for the data indicated that both the number and developmental timing of heat waves affected performance. In addition, heat waves earlier in development significantly reduced growth and development rates later in larval development. Our results illustrate how the frequency and developmental timing of sublethal heat waves can have important consequences for life history traits in insects.

KEY WORDS: Heat tolerance, Growth rate, Development time, Thermal stress

INTRODUCTION

Extreme temperature events are important determinants of individual survival, population persistence and geographic range limits of many organisms (Hoffmann and Srgo, 2011; Bailey and van de Pol 2016; Grant et al., 2017). With recent global warming, the frequency of extreme high temperatures has increased dramatically in many areas during the past three decades. For example, maximum daily temperatures for weather stations across the contiguous USA have been much above average (top 10%) in 19 of the past 30 years (1989–2018), compared with 2 of 30 years half a century ago (1959–1988); the two most extreme years recorded in the past century occurred during the past decade (data from https://www.ncdc.noaa.gov/extremes/). We can now attribute many specific extreme weather events to anthropogenic climate change, and climate projections indicate that the frequency of such events will continue to increase during the coming decades (Wang et al., 2013; Fischer and Knutti, 2015; Trenberth et al., 2015).

Assessing the biological responses of organisms to extreme temperature events is complex and multi-faceted (Bailey and van de Pol 2016; Chevin and Hoffmann, 2017; Grant et al., 2017; Stoks et al., 2017; Harris et al., 2018). For ectothermic animals, measures of upper thermal limits have been widely used to determine responses to acute temperature exposure (Terblanche et al., 2007, 2011). For example, lethal temperatures (LT_{50}) and thermal death time curves quantify the temperature and exposure time at which death occurs; the critical thermal maximum temperature (CT_{max}) specifies the temperature at which failure or 'ecological death' (loss of neuromuscular control, righting response, etc.) occurs (Overgaard et al., 2012; Rezende et al., 2014). The abundant literature on LT_{50} and CT_{max} for both terrestrial and aquatic ectotherms has allowed biogeographic and comparative analyses of upper thermal limits, and such metrics are increasingly used to predict the vulnerability of insects and other ectotherms to extreme high temperature events due to climate change (Sunday et al., 2011; Overgaard et al., 2014; Rezende et al., 2020).

In natural environments, daily temperature extremes do not occur at random, but are temporally structured: hot temperatures on one day are usually associated with hot temperatures on subsequent days, leading to heat waves that may last for days or weeks (Denny et al., 2009; Kingsolver and Buckley, 2017; Dowd and Denny, 2020). The biological consequences of extended or repeated heat waves, involving temperatures below critical upper thermal limits, are more poorly understood, but have been explored in several recent insect studies (Rezende et al., 2020). For example, repeated daily maximum temperatures well below CT_{max} can reduce survival, development rate and population growth in aphids (Ma et al., 2015, 2018). The developmental timing of high temperature events can also be important. For example, in *Manduca sexta*, high daily cycling temperatures during the egg stage delayed egg development, leading to longer development times to pupation (Potter et al., 2011). In contrast, in *Plutella xylostella*, the reproductive consequences of a single high temperature event were greater when the event occurred at later developmental stages (Zhang et al., 2015a,b). Despite their potential importance, the biological responses of ectotherms to repeated heat waves are largely unknown. What are the cumulative consequences of heat waves below upper thermal limits? How does exposure to prior heat waves affect subsequent performance?

In this study, we addressed these questions using heat wave experiments with the tobacco hornworm, *Manduca sexta*. The effects of heat shocks and of constant and fluctuating rearing temperatures on growth, development, survival and thermal limits have been previously documented in this system. Our experiments explored how the number and timing of heat waves during larval development affect survival, growth and development, and whether prior heat waves affect performance later in larval development. Our results demonstrate the cumulative consequences of repeated heat waves, and the complex effects of heat waves on later larval performance.

MATERIALS AND METHODS

Study system

The tobacco hornworm, *Manduca sexta* (Linnaeus 1763) (Lepidoptera: Sphingidae), has been an important model system for the study of insect feeding, growth and development for more than six decades. It is distributed across tropical and temperate regions of the

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Nearctic (Rothschild and Jordan, 1903). Larval feeding is generally restricted to plants in the Solanaceae, and *M. sexta* is an important agricultural pest on tobacco (*Nicotiana tabacum*: Solanaceae) in southeast USA. Our current study used animals from a laboratory colony of *M. sexta* that has been maintained under standard larval rearing conditions (artificial diet, constant 25–26°C, 15 h light:9 h dark photocycle) by L. Gilbert and colleagues at the University of North Carolina for over 30 years. This colony was originally derived from field collections of eggs in eastern North Carolina during the 1960s (Kingsolver, 2007). Previous studies have shown that laboratory and field populations have diverged evolutionarily in terms of body size, immune response, acclimation capacity and other traits, but larvae from both laboratory and field populations are thermal generalists that can tolerate high temperatures (see next paragraph) (D'Amico et al., 2001; Kingsolver, 2007; Kingsolver and Nagle, 2007; Diamond et al., 2010; Diamond and Kingsolver, 2011; Kingsolver et al., 2016).

Many aspects of the thermal biology of *Manduca* have been previously explored (Casey, 1976, 1977; Reynolds and Nottingham, 1985; Kingsolver and Woods, 1997; Kingsolver and Nagle, 2007; Diamond and Kingsolver, 2010a), and this background informs and motivates the experiments described here. On host plants in the field, an individual *M. sexta* egg or larva may regularly experience fluctuations of 20–25°C or more over a single diurnal cycle, and larval temperatures above 40°C are frequently observed during summer conditions (Casey, 1976; Kingsolver et al., 2012b). *Manduca sexta* larvae from both laboratory and field populations have high heat tolerance, with upper lethal temperatures of 44–45°C (24 h exposure) and CT_{max} of 44–46°C (Casey, 1977); and prior heat shocks and developmental temperatures can alter heat tolerance (Kingsolver et al., 2016). In diurnally fluctuating rearing conditions of 25±10°C, laboratory and field *M. sexta* achieve high rates of growth, development and survival to pupation; but performance is strongly reduced in rearing conditions of 30±10°C (Kingsolver et al., 2015). At constant temperatures of 40°C, *M. sexta* are unable to sustain positive growth rates or to survive to pupation (Reynolds and Nottingham, 1985; Kingsolver and Woods, 1997). Given this background, our current experimental treatments focused on heat waves and heat pulses involving high temperatures between 40 and 44°C (with appropriate control groups). We use the term 'heat wave' (HW) to refer to 3 day heat events during larval development.

HW experiments

The goal of these experiments was to determine how the number and timing of 3 day HW events during larval development affect survival, development time and mass at pupation. In addition, these experiments evaluated whether HWs early in larval development affect survival, growth and development during later larval development. The experiments used two different sets of diurnally fluctuating thermal conditions (Fig. 1A): background conditions, with a 25±10°C diurnal cycle, with 2 h at the maximum (35°C)

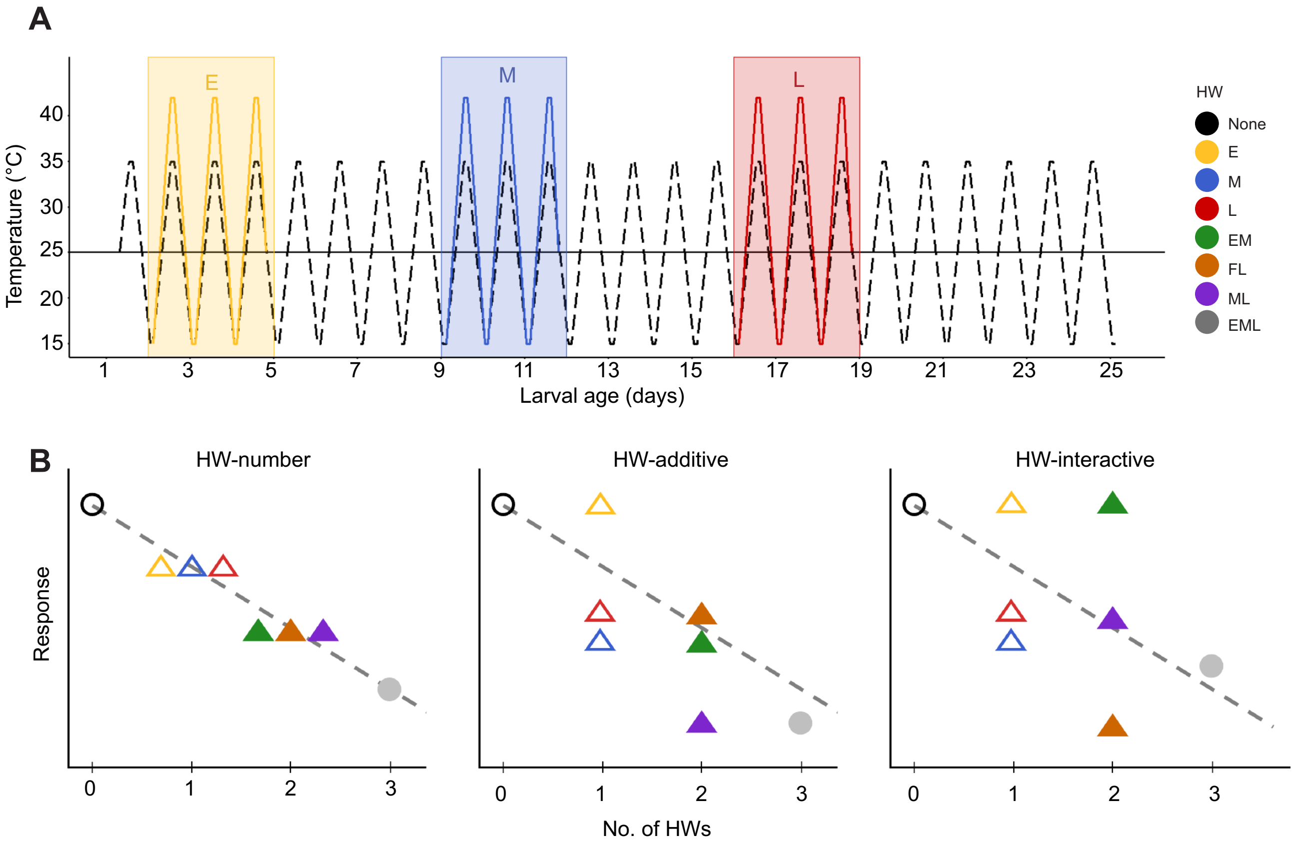

Fig. 1. Experimental design and alternative models for the heat wave experiment. (A) Diurnally fluctuating temperatures experienced during larval development (from hatching) for the different heat wave (HW) treatments. Background temperature conditions (black dashed line) are 25+10°C; a HW (colored solid lines) involved three consecutive days with maximal daily temperatures of 42°C. HWs may occur early (E, yellow line), middle (M, blue) and/or late (L, red) in larval development. All eight possible treatment combinations (from 0 to 3 HWs) are included. (B) Alternative models for the responses to HW treatments. HW-number model (left): response is determined only by the number of HWs (points jittered for clarity). HW-additive model (center): response depends on the timing of the HW, but the effect of multiple HWs is additive. HW-interactive model (right): response to HWs depends on exposure to other HWs. See Materials and Methods for further discussion.

temperature (from 13:00 h to 15:00 h) and 2 h at the minimum (15°C) temperature (from 01:00 h to 03:00 h); and HW conditions, with 2 h at the maximum (42°C) temperature (from 13:00 h to 15:00 h) and 2 h at the minimum (15°C) temperature (from 01:00 h to 03:00 h). For both sets of conditions, the temperature was ramped at a constant (linear) rate between the minimum and maximum values: as a result, mean temperature and ramping rate were also greater for HW than for background conditions. Larvae were maintained on the same photocycle (14 h light:10 h dark) in all temperature conditions. Background and HW conditions were maintained in two programmable environmental chambers (Percival model VL-36) throughout the experiments, and larvae were transferred between the two chambers as needed, depending on their treatment group (see below). An open container of water was placed in the bottom of each chamber to maintain high relative humidity (not measured).

An individual HW was defined as three consecutive days in the HW conditions. We included HWs at three different time periods during larval development (Fig. 1A), defined in terms of the time since hatching (where the day of hatching is day 1): an early (E) HW, where larvae experience HW conditions from day 2 to 5; a middle (M) HW, with HW conditions from day 9 to 12; and a late (L) HW, with HW conditions from day 16 to 19. The transfer from background to HW conditions (or the reverse) occurred at ∼10:00 h in the morning, when temperatures in the two conditions were similar (Fig. 1). This experimental schedule allowed us to impose up to three HWs during the period of larval development. Given the larval development rates of *Manduca* in these conditions, the early HW occurred during the 1st or 2nd larval instars, the middle HW during the 3rd or 4th instars, and the late HW during the 5th (final) instar. Note that because of individual variation in larval development rates, all larvae in a treatment group experienced a HW at the same age, but with some variation in the larval instar.

To begin the experiment, eggs were obtained from the mating and oviposition cage (containing ∼30–40 mated females) of our laboratory colony and maintained at a constant 25°C until hatching. Upon hatching (day 1), each 1st instar larva was placed in an individual Petri dish with diet, and was randomly assigned to one of eight treatment groups, defined by the number and timing of HW events experienced (see Fig. 1): the control group, which experienced no HW (background fluctuating conditions throughout larval development); the E, M and L groups, which experienced a single 3 day HW at different times (as above); the EM, EL and ML groups, which experienced two 3 day HWs in different combinations; and the EML group, which experienced three 3 day HWs. Survival was monitored daily, and diet was checked and replaced every 2 days or as needed. Mass and age since hatching (development time) of each larva were measured at the start of the 3rd, 4th and 5th instar, and at wandering and pupation (Kingsolver et al., 2015). This experimental design allowed us to distinguish the effects of the number and timing of HWs on larval performance (see ‘Analyses’, below). For logistical reasons, the experiments were conducted in two blocks: control (N=40), E (N=40), M (N=40) and L (N=40) treatments; and control (N=44), EM (N=45), EL (N=46), ML (N=45) and EML (N=47) treatments. Preliminary analyses showed that there were no significant or detectable differences in survival, development time or pupal mass between the control groups in the two blocks, so these blocks were combined in the analyses.

Statistical analyses

The experiments were used to explore two sets of predictions about the effects of HWs on larval performance. First, we evaluated how the number and timing of HWs during larval development influenced survival to pupation, development time to pupation and pupal mass. Each of these response variables was modeled separately. We used a series of three overlapping but non-nested linear models with different predictor variables to evaluate three different hypotheses about the effects of HWs (Fig. 1B). The HW-number model tested the hypothesis that only the total number of HWs has an effect, not the timing or combination of HWs. In this model the number of HWs (0–3) is the predictor variable. The HW-additive model tests the hypothesis that HWs at different time periods may have different effects on performance, but that their combined effects are additive. In this model the presence/absence of a HW in each of the three time periods (early, middle, late) is a (binary) predictor variable. The HW-interactive model tests the hypothesis that combinations of HWs at different time periods may have different effects on performance, i.e. there are interactions among HWs at different time periods. This model used HW treatment group as the predictor variable (a factor with eight levels). Because our goal here was to identify the best model for the data, we took an information theoretical approach (Burnham and Anderson 2002) and used the Akaike information criterion (AIC) to identify the best (lowest AIC) model for each response variable; we also included an intercept-only model (without predictors) as a ‘null’ model for comparison. As a rule of thumb, if the difference in AIC (ΔAIC) between a pair of models is 2–3, this indicates ‘significantly different’ (P=0.05) models in a hypothesis-testing framework (Burnham and Anderson 2002). Development time and pupal mass were modeled using linear models (function lm in R), and survival to pupation was modeled using a generalized linear model with a logit link function (function glm in R).

Second, we used this experiment to test whether HWs during earlier time periods influenced performance later in larval development. Note that the early and middle HW treatments occurred prior to the final (5th) instar (Fig. 1A). We used mass gain from the start of the 5th instar until wandering, and the development time from the start of the 5th instar until wandering, as metrics of late larval performance and as response variables, and considered the subset of individuals that did not experience a HW during the late time period (i.e. individuals in the control, E, M and EM treatment groups). For this subset, we used linear models and ANOVA to test whether mass gain or development time during the

Table 1. Comparison of three different models for the heat wave experiments assessing survival to pupation, development time to pupation and pupal mas

Model	d.f.	AIC
Survival to pupation		
Null (intercept)	1	259.0605
HW-number	2	255.8723*
HW-additive	4	256.2613
HW-interactive	8	258.7454
Development time to pupation		
Null (intercept)	2	1518.293
HW-number	3	1499.859
HW-additive	5	1475.256
HW-interactive	9	1470.019*
Pupal mass		
Null (intercept)	2	5295.795
HW-number	3	5259.617
HW-additive	5	5252.449*
HW-interactive	9	5253.677

The three models for the heat wave (HW) effects – HW-number, HW-additive and HW-interactive – are described in Materials and Methods. Asterisks indicate the best model based on the minimum AIC value.

final instar (to wandering) was influenced by HWs experienced during earlier time periods.

RESULTS

The best model for survival to pupation was the HW-number model, although the HW-additive model was quite similar (ΔAIC=0.39) (Table 1). Both of these models were substantially better than the null model (ΔAIC=2.8–3.2), suggesting that HWs at each time period contributed to reducing survival (Fig. 2A). HWs also significantly affected both development time to pupation and pupal mass (Table 1, Fig. 2B,C). The best model for development time to pupation was the HW-interactive model (Table 1), which was substantially better than the HW-additive (ΔAIC=5.2) or the HW-number (ΔAIC=29.8) model, and far superior to the null model (ΔAIC=48.2). These results indicate that both the timing and combination of HWs were important for development time. For example, treatments with two or three HWs had shorter mean developmental times compared with controls (no HWs); in contrast, a single HW did not increase mean development time (Fig. 2B). The best model for pupal mass was the HW-additive model, although the HW-interactive model was similar (ΔAIC=1.2); the HW-additive model was far superior to the HW-number (ΔAIC=7.2) or null (ΔAIC=43.3) models (Table 1). Inspection of the model coefficients (not shown) for the HW-additive model suggests that middle HWs had larger negative effects on mean pupal mass than early or late HWs (Fig. 2C). These results show that multiple HWs during larval development have substantial negative effects on survival, development time and body mass to the pupal stage, indicating the cumulative effects of HWs on performance throughout larval development.

For individuals that did not experience a late HW (during the 5th instar), development time and mass gain during the 5th instar (until wandering) were significantly affected by HW events experienced earlier in development (development time: F=6.9, P=0.009; mass gain: F=25.4, P<0.001). In particular, individuals that experienced both early and middle HWs had longer development times and smaller mass gain during the 5th instar (Fig. 3). This finding demonstrates that sub-lethal HWs earlier in development can reduce performance in later developmental stages that do not experience HWs: these larvae did not recover from prior HWs.

DISCUSSION

Like many other thermal generalists from temperate regions, *M. sexta* larvae can tolerate quite high temperatures relative to environmental

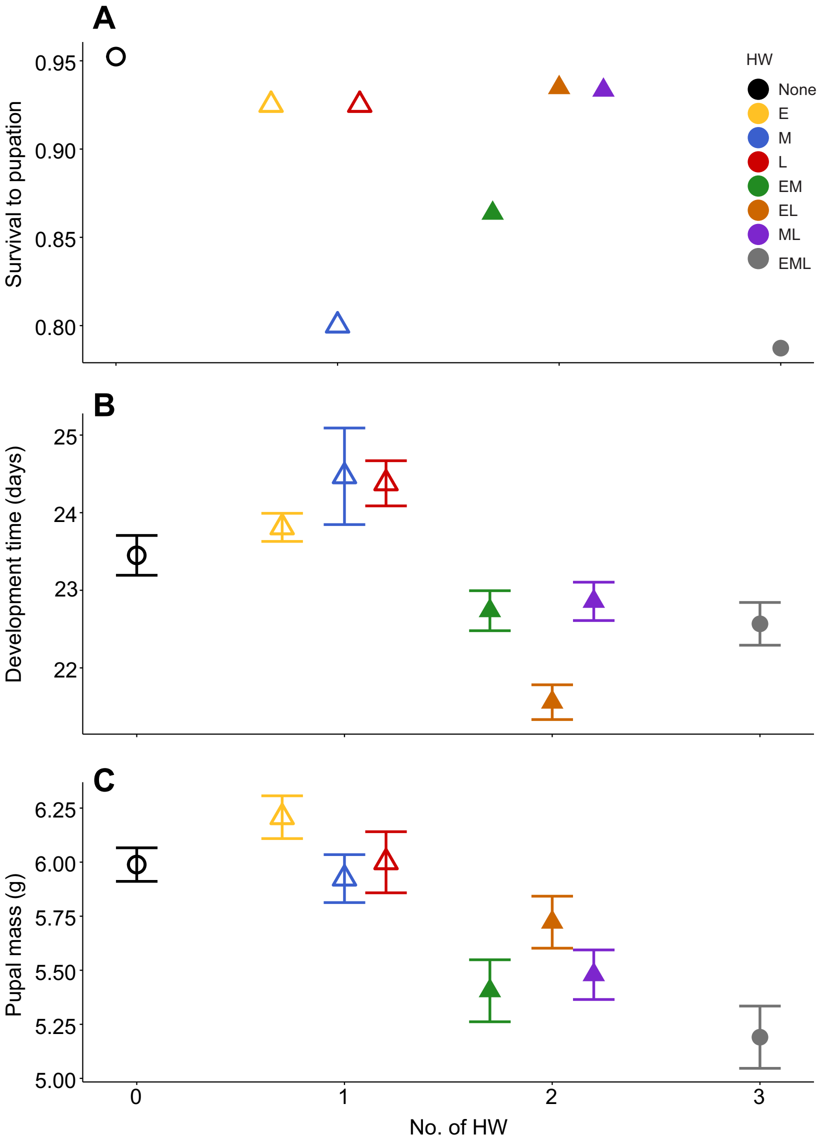

Fig. 2. Effects of the number of HWs (0–3) on survival, growth and development of *M. sexta* larvae. (A) Survival to pupation. (B) Pupal development time. (C) Pupal mass. Mean (±1 s.e.m.) values for each treatment group are given: each treatment group is labeled according to the HWs experienced. Points are jittered for clarity.

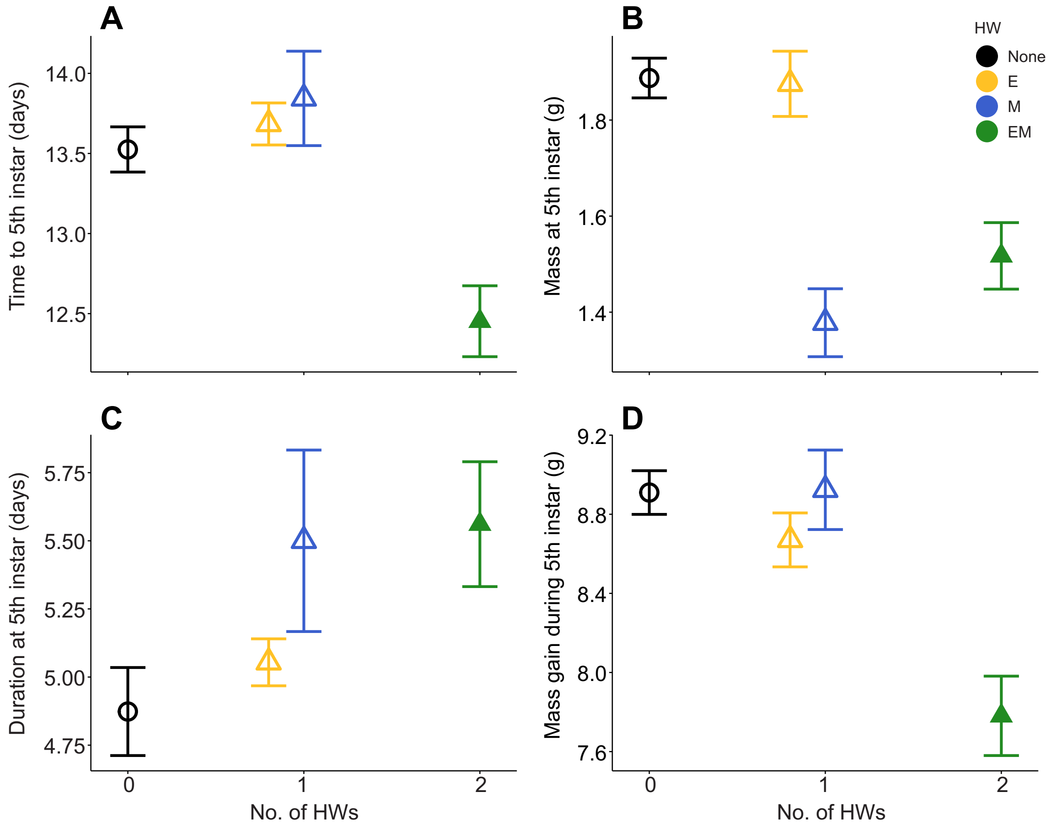

Fig. 3. Effects of the number of earlier HWs (E and M) on performance during the 5th (final) instar of *M. sexta*, for those individuals that did not experience a HW during the 5th instar. (A) Development time to the start of the 5th instar and (B) mass at the start of the 5th instar. (C) Duration of the 5th instar and (D) mass gain during the 5th instar. Mean (±1 s.e.m.) values for each treatment group are given: each treatment group is labeled according to the HWs experienced. Points are jittered for clarity.

and operative temperatures that they typically encounter in the field. Their CT_{max} based on ramping assays is 44–46°C, and prior exposure to short, sublethal heat pulses can increase CT_{max} by ∼1°C (Kingsolver et al., 2016; Agosta et al., 2018). They can survive 24 h heat pulses for temperatures up to 40–42°C (Casey, 1977), and can maintain positive growth over short (4 h) time periods at such temperatures (Kingsolver and Woods, 1997). Field studies in southwestern USA show that body (surface) temperatures of *M. sexta* larvae increase across instar (and size), because smaller larvae are more closely tied to leaf than to air temperatures (Woods, 2013). Modeling and operative model measurements suggest that late-instar *M. sexta* may occasionally experience body temperatures above 43°C during hot, midday summer conditions in some parts of its geographic range (Woods, 2013; Woods et al., 2018).

Extreme thermal conditions rarely occur in isolation in natural environments. In terrestrial environments, daily maximum temperatures are often temporally structured into HWs, where high maximum temperatures occur over a series of successive days. Terrestrial HWs frequently result from stationary high-pressure systems that produce anomalously high temperatures for days to weeks; the magnitude and duration of HWs are predicted to increase in many regions as a result of ongoing climate change (Wang et al., 2013; Fischer and Knutti, 2015; Trenberth et al., 2015). Within *M. sexta*'s geographic range in southwestern USA, repeated time periods with maximal daily air temperatures above 42°C already occur regularly (Woods, 2013; Woods et al., 2018).

An emerging literature is starting to document the potential consequences of HWs for insects (Ma et al., 2018). These studies indicate the different consequences of daytime versus night-time warming in diurnal fluctuating thermal environments (Speights et al., 2017). Low night-time temperatures can allow many insects to recover from the high daytime temperatures that approach upper thermal limits (CT_{max}) (Colinet et al., 2015). For example, increasing daily maximal temperatures (with a fixed night-time minimum temperature) strongly reduced survival and reproduction in aphids (Ma et al., 2015). Conversely, elevated night-time temperatures (with a fixed daily maximal temperature) reduced survival and reproduction (Zhao et al., 2014). Our results, using HWs in which daytime but not night-time temperatures were elevated, show that a single, 3 day HW had little detectable effect on development time, body size or survival to pupation (Fig. 2). This suggests that low night-time temperatures can ameliorate the negative impacts of exposure to temperatures well above the optimal temperature for short-term larval growth and development rates (34–38°C in *M. sexta*) (Kingsolver and Woods, 1997).

The effects of elevated temperatures during development naturally depend on the duration and frequency of exposure (Kingsolver et al., 2015; Ma et al., 2015, 2018). For example, in gypsy moths, exposure to higher (mean and daily maximal) temperatures had much stronger negative effects on survival, growth and development for 7 days compared with 2 days of exposure (Banahene et al., 2018). Using an elegant experimental design with aphids, that varied the duration and relative frequency of 'normal' (13–28°C diurnal fluctuation) and 'hot' (20–35°C diurnal fluctuation) time periods, Ma et al. (2018) demonstrated that the negative fitness consequences of a HW depend both on its duration (1–3 days) and on its temporal clustering. Responses to high temperature events may also vary with developmental timing and life stage (Zhang et al., 2015b; Banahene et al., 2018; Zhao et al., 2019).

Our results for *M. sexta* similarly show that the consequences of HWs depend on timing within the larval stage, especially when there are multiple HWs. A key finding of our study is that the effects of HWs on development time and pupal mass are not purely additive, and depend on the combination of HWs at different times during larval development (Fig. 2). For example, a single HW had little effect on mean pupal mass or development time, whereas two or three HWs strongly reduced both final size and development time. One possibility is that oxidative damage or metabolite accumulation following multiple HWs exceeds a physiological threshold and reduces subsequent growth rate (Feder and Hofmann, 1999; Harrison et al., 2012). More generally, growth and development rates may be non-linearly related to the frequency of HWs. Our experimental design with only three HWs does not allow us to distinguish between these possibilities.

One interesting finding is that HWs during the middle of larval development (3rd to 4th instar) may more strongly reduce survival to pupation than early or late HWs. For example, mean survival was lowest for those treatments that included a middle HW (Fig. 2A). The biological reasons for this apparent pattern are unclear. However, we note that the HW-number model – assuming that each HW has an equal effect on survival – was the best model for these data, so the statistical support for a greater effect of middle HWs is limited (Table 1).

Our results also demonstrate that exposure to HWs alters performance later in larval development. For example, exposure to HWs during the early and middle stages of larval development results in longer development times and smaller mass gains during the final (5th) larval instar (Fig. 3C,D). It would be informative to explore whether earlier HWs increase oxidative damage or heat shock protein concentrations during the final instar to reduce growth and development (Harrison et al., 2012).

The effects of HWs on pupal mass and development time demonstrated here may have important consequences for key components of fitness. In many organisms, larger size is associated with greater fecundity (Kingsolver and Pfennig, 2004): for example, pupal mass is positively correlated with egg production in *M. sexta* (Diamond and Kingsolver, 2010b; Kingsolver et al., 2012a). The effects of HWs on development time, and their consequences for fitness, are more complex. Field studies with *M. sexta* show that shorter larval development times (faster development rates) are correlated with higher survival to pupation, in part because rapid development reduces exposure to larval predators and parasitoids (Kingsolver et al., 2012a). For organisms (including *M. sexta*) that have multiple generations per year, shorter development times can allow more generations and increase the intrinsic rate of increase (Eck et al., 2015). Our results suggest two contrasting effects of HWs: multiple HWs decrease development time to pupation, but HWs during early or middle instars increase the duration of the final instar (Figs 2 and 3). How these factors combine to influence fitness in *M. sexta* is unclear, especially in field conditions where larval mortality rates are much higher (Kingsolver et al., 2012a). Collectively, these results illustrate the time-dependent and interacting impacts of sublethal high temperature events on key life history traits, and highlight that the temporal patterning and developmental timing of HWs are key to understanding their biological consequences for insects and other ectotherms.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.G.K.; Methodology: M.E.M., K.E.A., C.A.H.; Formal analysis: J.G.K.; Investigation: M.E.M., K.E.A., C.A.H.; Resources: J.G.K.; Writing - original draft: J.G.K.; Writing - review & editing: J.G.K., M.E.M., K.E.A., C.A.H.; Visualization: M.E.M., K.E.A.; Supervision: J.G.K.; Project administration: J.G.K.; Funding acquisition: J.G.K.

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Data availability
The data presented in this paper are available from the Dryad digital repository (Kingsolver et al., 2021): dryad.10.5061/dryad.2jm63xsp0

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