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Growth, developmental and stress responses of larvae of the clouded sulphur butterfly *Colias eriphyle* to repeated exposure to high, sub-lethal temperatures

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Abstract. The optimal temperature at which an organism grows and develops is commonly correlated with latitude and elevation; however, the maximum temperature for physiological performance often is not. This makes performance at temperatures between the optimum and the maximum of particular interest. Temperature can influence long-term performance (growth and development), as well as short-term performance (heat shock protein) responses differentially. In the present study, two populations of the clouded sulphur butterfly *Colias eriphyle* Edwards that differ in elevation, thermal regime and optimal and maximum temperatures are studied to quantify their responses to repeated, sub-lethal heat treatments early in development (second instar). Heat treatments accelerate development during the second to fourth instars in both populations initially, although this effect disappears by pupation. Heat treatment decreases pupal mass in the lower elevation population, suggesting that repeated exposure to high temperatures early in development may reduce final size and fecundity in this population. Heat shock protein gene (*hsp70*) expression levels in the lower elevation (1633 m a.s.l.) population are highest 24 h after the start of the heat treatment and then the fall to pre-exposure levels by 36–72 h, suggesting a rapid response to stressful temperatures. By contrast, heat treatment has no significant effect on pupal mass in the higher elevation (2347 m a.s.l.) population. This population has higher levels of *hsp70* expression overall but constant expression levels, suggesting that the temperature treatments used are insufficient to elicit a heat stress response. Overall, the effects of repeated exposure to sub-lethal high temperatures early in development on growth, final size and gene expression differ between populations that differ in thermal sensitivity.

Key words. *Colias*, heat stress, *hsp70*, thermal performance curve.

Introduction

The range of temperatures over which an organism can operate is often characterized by a thermal performance curve (TPC) (Huey & Stevenson, 1979). Thermal performance curves have a particular shape, with performance increasing gradually to the optimum and then rapidly decreasing at temperatures above the optimum. As the temperature increases, ectothermic organisms reach their critical thermal maximum where the organism stops functioning and prolonged exposure can cause death. Upper thermal limits of terrestrial organisms do not vary consistently with latitude (Addo-Bediako *et al.*, 2000; Sunday *et al.*, 2011), although thermal optima (T_{opt}) generally decrease with increasing latitude (Huey & Kingsolver, 1993; Cunningham & Read, 2003; Sun & Friedmann, 2005). These contrasting environmental patterns for upper thermal limits and thermal optima make an organism's performance at temperatures above the optimum but below the thermal maximum of particular interest.

It is known that there are differences in how TPCs relate to physiological traits between temperate and tropical species and across latitudes (Cunningham & Read, 2003; Deutsch *et al.*, 2008; Sunday *et al.*, 2011; Nilsson-Örtman *et al.*, 2012). However, not much is known about how these differences in TPCs will affect growth and development across elevations or between

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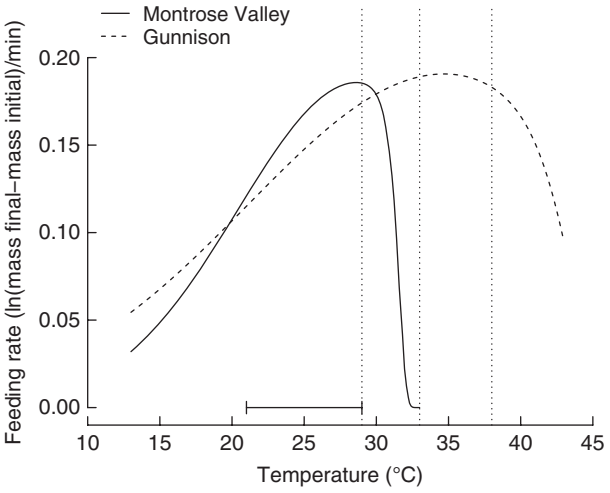

Fig. 1. Short-term feeding rates of fifth-instar *Colias eriphyle* larvae from two geographical sites, Montrose Valley, Colorado and Gunnison, Colorado, that differ in elevation, as well as temperature regime. The feeding rates were used to determine the heat treatment temperatures (dotted vertical lines) used in the subsequent study of larvae derived from females obtained from these sites. The rearing conditions are indicated by the horizontal bar above the *x*-axis. Data are from Higgins *et al.* (2014).

populations (Berven, 1982). In the present study, experiments are conducted using the clouded sulphur butterfly *Colias eriphyle* Edwards from two geographical sites in Colorado, U.S.A. (Montrose Valley, 1633 m; Gunnison, 2347 m), that differ in elevation, as well as environmental temperature regimes. The *C. eriphyle* larvae from these populations are known to differ in their TPCs for feeding rate (Fig. 1) (Higgins *et al.*, 2014). The present study investigates how different TPCs for short-term feeding rates can influence long-term growth and development and whether sub-lethal temperatures are sufficient to elicit heat stress responses.

The heat shock response often determines the maximum temperatures of TPCs (Feder & Hofmann, 1999), although it is not known at what temperatures this response initiates for many species, including *C. eriphyle*. Heat shock proteins are small chaperones that prevent protein misfolding during stress; however, their production comes at the cost of growth and other cellular processes (Krebs & Feder, 1997). In the present study, the expression level of heat shock protein 70 (*hsp70*) is measured before, during and after the heat treatment to understand how expression changes with time. The present study also examines whether expression levels of *hsp70* differ between the two populations. Previous studies with other organisms show differences in gene expression or protein levels for heat shock genes, including *hsp70*, across geographical gradients (White *et al.*, 1994; Tomanek & Somero, 1999; Sagarin & Somero, 2006).

The feeding rate for larvae from the high elevation population (Gunnison, Colorado) is maximal at approximately 35 °C and declines above 38 °C, whereas the feeding rate for the lower elevation population (Montrose Valley, Colorado) is maximal at approximately 28 °C and declines above 33 °C. It is predicted that the larvae from the lower elevation population will not be able to feed during the hottest parts of the 3-day experimental heat treatments, whereas larvae from the higher elevation population should be able to continue feeding. Therefore, an overall decrease in the growth rate of larvae from the lower elevation population is predicted. It is predicted that the heat treatments should be more stressful for the larvae from the lower elevation population because of their lower optimal temperatures and upper thermal limits. Specifically, it is expected that both of the experimental heat treatments (high 33 °C and high 38 °C) would be stressful for the lower elevation larvae because these temperatures correspond to a feeding rate of zero.

The time scale used to measure the critical thermal maximum and upper thermal limits varies between studies and, typically, an experimental organism only experiences a single exposure to high temperature during development. In the present study, repeated exposure to high nonlethal temperatures is used aiming to understand both the short- and long-term effects of potentially stressful temperatures during development. This simulates the effect of heat waves that occur with extreme highs over a series of days. To examine the long-term effects, body mass increase and development time at each instar and to pupation are measured. Based on short-term TPCs for feeding rate, it is also possible to estimate and predict growth rate to pupation and to compare these predictions with the findings for the two populations with different TPCs for feeding. To study the short-term effects of heat exposure, expression levels of *hsp70* are measured before, during and after the treatments. The fitness costs associated with the heat shock response may provide a partial explanation for some of the long-term fitness effects.

Materials and methods

Study system

The clouded sulphur butterfly *C. eriphyle* occurs in open habitats in the western U.S.A. and occupies elevations between 1400 and 2900 m a.s.l. in western Colorado. The larvae have five larval instars and undergo a facultative winter diapause during the third instar. The larvae feed on plants in the *Fabaceace* family, including *Medicago sativa* (alfalfa), *Vicia* (vetch) spp. and *Trifolium* (clover) spp. *Colias eriphyle* females were sampled from alfalfa (*M. sativa*) fields located in the Montrose Valley, Colorado (38.62°N, 108.02°W, 1633 m) and from a county park with meadows including vetch (*Vicia*) and clover (*Trifolium*) near Gunnison, Colorado (38.56°N, 106.94°W, 2347 m). These two study sites have different growing seasons, accounting for variations in larval development, adult flight time and the number of generations per year (voltinism). In the Montrose Valley, the growing season can start as early as April and continue through October, resulting in three to five generations of *C. eriphyle* per year, whereas, in Gunnison, the growing season starts in June and continues through September, resulting in two generations of *C. eriphyle* per year (Higgins *et al.*, 2014).

Growth and development experiments

Adult female butterflies were collected from each site and shipped overnight to the University of North Carolina at Chapel Hill, North Carolina. The butterflies were kept in cages under greenhouse conditions (approximately 26 °C and natural light). Females were fed 10% (w/v) honey water solution by a moistened sponge that was changed daily and were allowed to oviposit on potted *Vicia villosa* in the greenhouse. Eggs were removed each day and placed in environmental chambers (Percival 36VL; Geneva Scientific, Wisconsin) maintained under an LD 14 : 10 h photocycle at 21–29 °C (mean 25 °C). Larvae were reared individually were provided with leaves of *V. villosa ad libitum* as food source. The developing larvae were scored daily for age and instar. Upon entering the second instar, approximately 60 larvae from each population (180 larvae total from each population) were placed randomly into one of three temperature treatments: a medium heat treatment, 21–33 °C, ramping from 21 °C at 03.00 h to 33 °C at 15.00 h, holding at 33 °C for 1 h, and then ramping steadily back to 21 °C at 03.00 h the next day; a high heat treatment, 21–38 °C ramping from 21 °C at 03.00 h to 38 °C at 15.00 h, holding at 38 °C for 1 h, and then ramping steadily back to 21 °C at 03.00 h the next day; or the control group, which went back into the rearing chamber, ramping from 21 °C at 03.00 h to 29 °C at 15.00 h, holding at 29 °C for 1 h, and then ramping steadily back to 21 °C at 03.00 h the next day. These temperatures were chosen based on the differences in mean TPCs for larval feeding rates between the two populations (Higgins *et al.*, 2014): in particular, the mean optimal temperature for feeding is lower for the Montrose Valley population ($T_{opt} = 28$ °C) than for the Gunnison population ($T_{opt} = 35$ °C) (Fig. 1). Because of these differences, it is predicted that the treatments at both 33 and 38 °C should be stressful for Montrose Valley larvae. By comparison, the Gunnison larvae should not be stressed at 33 °C and should face mild stress at 38 °C. However, neither treatment should be lethal or cause permanent damage to larvae derived from either population.

Each larva was kept in its respective temperature treatment for 3 days and then returned to the control rearing conditions for the duration of the experiment. Age at each instar, mass at each instar (beginning at the third instar), overall growth rate to pupation and development time were recorded. Development time, pupal mass and growth rate were analyzed with population and temperature as fixed effects and sib-family (female parent) as a random effect in linear mixed effects models using the *nlme* package (Pinheiro *et al.*, 2015).

Expression of hsp70

RNA was extracted using Qiagen RNeasy kits (Qiagen, Valencia, California) from whole larvae at four time points (82, 106, 154 and 178 h after hatching) during the 3-day heat treatments (10 biological replicates, with three technical replicates each) (Fig. 2). Extracted RNA concentrations for each sample were quantified using a NanoDrop 1000 (ThermoScientific, Waltham, Massachusetts) and measured for RNA purity. Extracted RNA (1 µg) was reverse transcribed using the High Capacity cDNA

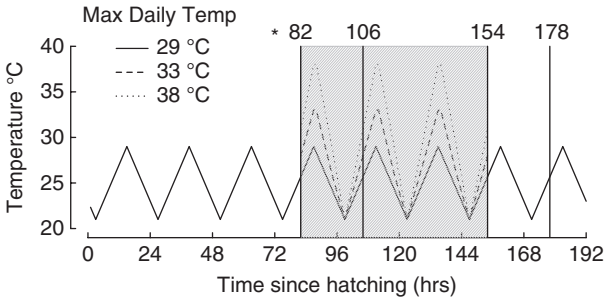

Fig. 2. Temperature regimes for the control (29 °C) and heat (33 and 38 °C) treatments during the study of larval development rates for *larvae* derived from the Montrose Valley and Gunnison populations of the sulphur butterfly *Colias eriphyle*. The heat treatments (shaded box) began at the onset of the second instar (indicated by star), which starts at approximately 3 days after hatching. The vertical lines indicate when larvae were killed for measurement of heat-shock protein *hsp70* expression.

Reverse Transcription Kit (Applied Biosystems, Carlsbad, California) with random hexamer primers (10-µL reaction volume). *Manduca sexta* primers for the target gene *hsp70* (GenBank: AY220911.1) (forward: 5′-GTGCTGACCAAGATGAAGGA-3′, reverse: 5′-CGCTGTGAGTTGAAGTA-3′) and for the reference gene 18S rRNA (GenBank: U88190.1) (forward: 5′-CAGCACATCTTAGGGCATCAC-3′, reverse: 5′-CAACTC ACTGGCGACGTATTA-3′) were used in the polymerase chain reaction (PCR) of the cDNA. The PCR product was sequenced and *Colias* specific *hsp70* primers (forward: 5′-CCAGTAACAACCTTGGCAAAC-3′, reverse: 5′-CTGTGA GTCGTTGAAGTACG-3′) and 18S rRNA (forward: 5′-CTAT CTCGTGCGGCT -3′, reverse: 5′-GTAATCAACTCACTGGC GA-3′) were designed and used for quantitative PCR. The specificity for each primer pair was first checked by the melting curve profile and then confirmed by agarose gel electrophoresis at 100 V for 30 min (data not shown). Primer efficiency was almost 100% in all experiments. The quantitative PCR was conducted with 0.4 ng of cDNA in a 15-µL reaction volume using the SYBR Green FastMix (Quanta Biosciences, Gaithersburg, Maryland) in 96-well plates (Bio-Rad, Hercules, California) on a Bio-Rad CFX96 thermocycler. PCR was initiated with a Taq activation step performed at 95 °C for 10 min followed by 40 amplification cycles of a 95 °C denaturation step for 2 s and a 72 °C combined annealing/elongation step for 10 s. In every PCR plate, nontemplate controls were included to confirm the absence of contamination. The data were analyzed using the method of Pfaffl (2001). Expression of the target *hsp70* gene was normalized relative to the reference18S rRNA gene and calibrated to the control (high 29 °C) larvae from Montrose Valley. Linear mixed effects models were used to analyze differences in relative expression ratio among treatments and time points with sib-family as a random effect, and time, temperature and population as fixed effects. RNA from the Gunnison larvae was only collected at 82 and 106 h because of a laboratory accident that destroyed many of the samples. All data were analyzed in R, version 3.1.1 (R Foundation for Statistical Computing, Austria).

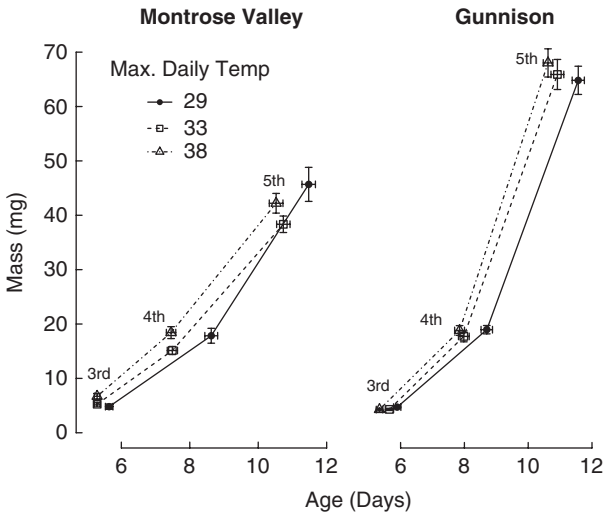

Fig. 3. Mean ± SE mass and age of third- to fifth-instar *Colias eriphyle* larvae during larval development in the control (high 29 °C) and heat treatments (high 33 or 38 °C). Heat treatments occurred for 3 days (72 h) after the onset of the second instar.

Results

Growth and development

For both populations, the heat treatments at 33 and 38 °C decreased mean development time to third ($F_{2,344} = 10.5$, $P < 0.0001$), fourth ($F_{2,342} = 26.8$, $P < 0.0001$) and fifth ($F_{2,345} = 15.3$, $P < 0.0001$) larval instars (Fig. 3). However, there were no significant effects of heat treatment ($F_{2,348} = 1.76$, $P = 0.17$) and population ($F_{1,23} = 0.37$, $P = 0.55$) or their interaction on mean development time to pupation ($F_{2,348} = 0.41$, $P = 0.66$) (Fig. 4). This suggests that the heat treatments accelerated development during the middle (second to fourth) larval instars, although these effects have disappeared by the end of larval development.

For pupal mass, there were significant effects of treatment ($F_{2,330} = 6.34$, $P = 0.04$) and population ($F_{1,22} = 105.6$, $P < 0.0001$), as well as an interaction between treatment and population ($F_{2,330} = 4.8$, $P = 0.009$) (Fig. 4). Increasing treatment temperatures reduced mean pupal mass for the Montrose Valley population but not for the Gunnison population (Fig. 4).

Significant effects were also detected in the growth rate to pupation and effects of population ($F_{1,22} = 19.9$, $P = 0.0002$) and of the interaction between treatment and population ($F_{2,329} = 1.0$, $P = 0.37$), although treatment alone was not significant ($F_{2,329} = 1.0$, $P = 0.35$) (Fig. 4).

hsp70 expression

The data for the Montrose Valley larvae show that the relative expression levels of *hsp70* were not significantly affected by temperature ($F_{1,53} = 3.36$, $P = 0.072$) but did vary significantly over time ($F_{1,53} = 7.35$, $P = 0.01$) and there was no significant interaction of time and temperature treatment ($F_{1,53} = 2.20$, $P = 0.144$) (Fig. 5). The expression levels were highest 24 h after being placed into the treatment (106 h after hatching) and then went back down to pre-exposure levels.

When comparing the relative expression levels of *hsp70* between both populations, only at 72 h after hatching and 106 h after hatching (24 h into the heat treatment) was there a significant effect of population ($F_{1,8} = 6.9$, $P = 0.03$), such that the expression levels of *hsp70* at 82 and 106 h were higher in the larvae from Gunnison compared with those from Montrose Valley. There was no significant effect of temperature treatment ($F_{1,19} = 1.11$, $P = 0.31$), nor was there an interaction between the two temperature and population ($F_{1,19} = 0.67$, $P = 0.42$).

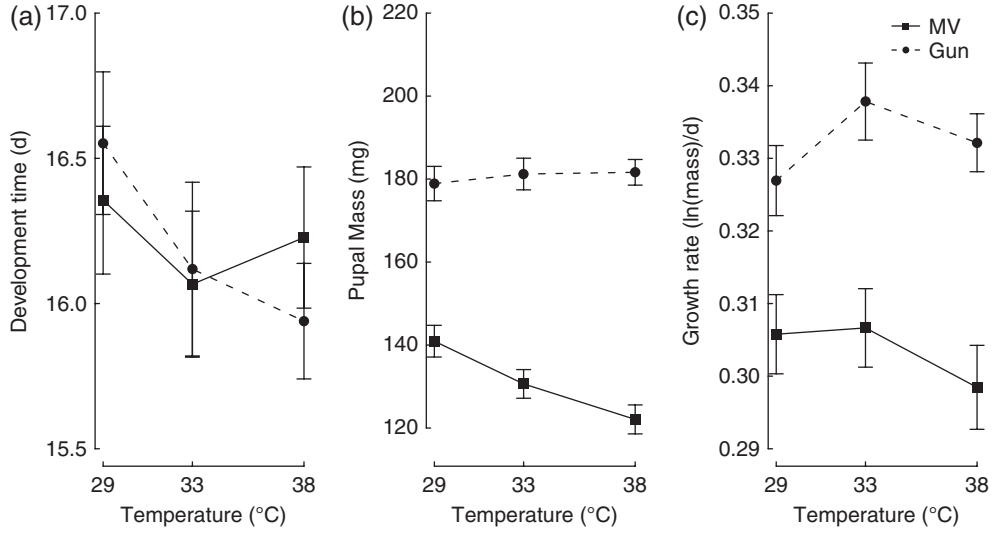

Fig. 4. Mean ± SE (a) development time to pupation, (b) pupal mass and (c) growth rate for *Colias eriphyle* larvae from Montrose Valley (squares, dashed line) and Gunnison (circles, solid line) populations.

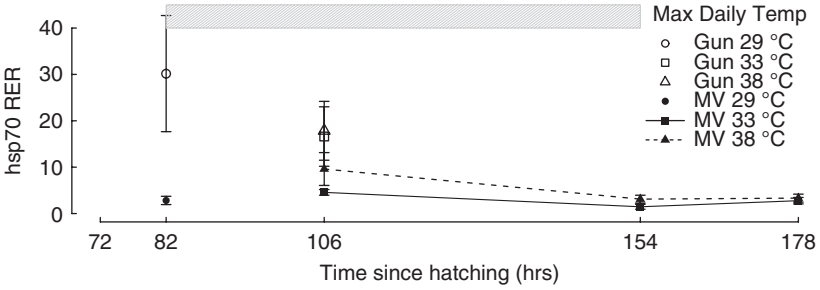

Fig. 5. Mean ± SE relative expression levels (RER) of *hsp70* for larvae from Montrose Valley (solid shapes) and Gunnison (open shapes) exposed to heat treatments of 33 °C (solid line) and 38 °C (dashed line). The shaded rectangle indicates the duration of the heat treatments. All expression levels were calibrated to the Montrose Valley control (high 29 °C) levels of *hsp70* expression.

Discussion

The present study shows that the effects of high sub-lethal temperatures on larval growth and development differ between two populations of *C. eriphyle* from different geographical elevations, such that the lower elevation Montrose Valley population is more susceptible to the stress of experimental heat treatment than the higher elevation Gunnison population. This may be a result of the fact that Gunnison, Colorado has a more variable climate overall, and therefore the larvae are able to deal with more stressful temperatures.

Growth and development

Previous studies investigating ramping of experimental heat treatments during the egg stage of the tobacco hornworm *Manduca sexta* show that high temperatures slow down development in the early instars, although that the effect is no longer apparent in the later instars and at pupation (Potter *et al.*, 2011). Conversely, the present study, using a different lepidopteran species, shows that heat treatments decrease the age at each instar in the second to fourth instars (speeding up development), although they have no effect on the overall development time to pupation. The substantially higher temperatures used in the previous study (Potter *et al.*, 2011) may explain the delayed development associated with a stress response in *M. sexta*, whereas the present study finds accelerated development associated with heat stress for C. *eriphyle*.

In general, exposure to higher environmental temperatures during an organism's development results in rapid growth but a smaller adult body size. This is commonly known as the temperature size rule (TSR) (Atkinson, 1994), as shown for many ectothermic species (Sibly & Atkinson, 1994; Atkinson & Sibly, 1997; Angilletta & Dunham, 2003; Kingsolver & Huey, 2008; Forster *et al.*, 2011). During the experimental heat treatments in the present study, larval development time increases for both populations, although this effect disappears by pupation. However, the Montrose Valley larvae are smaller at the third instar after exposure to the heat treatments relative to the controls ($F_{2,169} = 5.0$, $P = 0.008$) (Fig. 3). This could be simply interpreted as obeying the TSR, in that faster development correlates with smaller size; however, the smaller body size at each instar is not seen in Gunnison population when exposed to an identical heat stress. It is conceivable that the Montrose Valley larvae may have experienced stressful periods when the larvae did little to no feeding (or gaining of mass). The ontogenetic growth model suggests that there are trade-offs between growth of new tissues and maintenance of existing tissues (West *et al.*, 2001). Additionally, larval development during the first to fourth stadia involves both the growth and moulting processes, whereas the fifth stadium involves the final development to pupation. It is likely that the temperature sensitivity of the earlier instars is different from that of the fifth instar. This may explain why the heat treatments do not have an overall effect on time to pupation.

As predicted, the Montrose Valley *C. eriphyle* are significantly smaller as pupae after exposure of the developing larvae to heat treatments relative to their control counterparts. Under the experimental regimes, larvae experience similar overall mean temperatures despite the 3 days of variable heat treatment. In the high 29 °C treatment, the mean temperature throughout development is 25 °C; in the high 33 °C treatment, the mean temperature is 25.4 °C; and, in the high 38 °C treatment, the mean temperature is 25.9 °C. These differences in mean temperatures are insufficient to account for the large differences in pupal mass observed. This suggests that it is stress, and not simply the TSR, that constrains pupal mass in Montrose Valley. Additionally, stress associated with three experimental heat treatment days also has long-term effects by reducing pupal size in stoneflies (Taylor *et al.*, 1998).

hsp70 expression

Overall *hsp70* expression is found to be higher in larvae of the Gunnison population compared with Montrose Valley, even at the 29 °C high (experimental control) temperature. Gunnison, Colorado, is higher in elevation and, overall, experiences generally cooler yet more variable temperatures than Montrose Valley, Colorado. The maximum, minimum and mean temperatures during the growing season are 25.1, 7.9 and 16.5 °C in Montrose Valley and 22.7, 4.2 and 13.5 °C in Gunnison (Higgins *et al.*, 2014). In other species studied, *hsp70* levels are reported to decrease (Dahlhoff & Rank, 2000; Garbuz *et al.*, 2003) or remain constant (Karl *et al.*, 2009) with environmental elevation. However, Healy *et al.* (2010) report that the cooler, northern populations of killifish have higher levels of expression of *hsp70-2* and *hsp90* than the warmer southern populations (Healy *et al.*, 2010).

Heat treatment does not alter *hsp70* expression in larvae of the Gunnison population of *C. eriphyle*. The Gunnison larvae may have already reached their maximum levels of expression for *hsp70*; however, this is unlikely because they do not exhibit decreased growth and development, which can be symptoms of

reaching maximum expression levels (Krebs & Feder, 1997). The larvae are able to continue feeding well past the temperatures used in the heat treatments (Higgins *et al.*, 2014). Alternatively, the levels of *hsp70* expression measured in the Gunnison larvae may represent basal levels if the experimental heat treatments used in the present study are insufficient to cause stress. It may be that the overall variability of the temperatures experienced in the wild leads to a higher performance at extremes. Measurement of *hsp70* expression levels after exposure of *C. eriphyle* larvae to much higher temperatures, specifically above 38 °C, at which larvae begin to decrease feeding, would help to resolve these findings. In the Montrose Valley larvae, the *hsp70* expression levels increase at 24 h during the heat treatment and then decrease to their pre-heat treatment levels. This is consistent with the findings of previous studies (Dahlgaard *et al.*, 1998; Tomanek & Somero, 1999; Tomanek & Sanford, 2003) highlighting the rapid induction of *hsp70* during heat stress and then a decrease of expression back to normal levels.

Exposure of *C. eriphyle* larvae to high sub-lethal temperatures early in development does not affect overall development time to pupation but does result in differences in pupal mass for the Montrose Valley population and growth rate during early (third to fifth) larval stadia for both populations. The Gunnison population has higher levels of *hsp70* expression overall compared with the Montrose Valley population, although the expression levels do not change before or during the heat treatment. This may signify that the experimental heat treatments used in the present study are not sufficiently stressful to elicit a response, which correlates with the TPC showing that Gunnison continues feeding past 38 °C. Overall, the present study shows that exposure to sub-lethal high temperatures can have many varied effects on growth and development of an insect species, both in the short term and into adulthood, and that the effects can depend on the population. Thus, the present study demonstrates the importance of considering responses to thermal stress at multiple time scales and throughout the life cycle.

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