

Geographic divergence in upper thermal limits across insect life stages: does behavior matter?

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Abstract Insects with complex life cycles vary in size, mobility, and thermal ecology across life stages. We examine how differences in the capacity for thermoregulatory behavior influence geographic differences in physiological heat tolerance among egg and adult *Colias* butterflies. *Colias* adults exhibit differences in morphology (wing melanin and thoracic setal length) along spatial gradients, whereas eggs are morphologically indistinguishable. Here we compare *Colias eriphyle* eggs and adults from two elevations and *Colias meadii* from a high elevation. Hatching success and egg development time of *C. eriphyle* eggs did not differ significantly with the elevation of origin. Egg survival declined in response to heat-shock temperatures above 38–40 °C and egg development time was shortest at intermediate heat-shock temperatures of 33–38 °C. Laboratory experiments with adults showed survival in response to heat shock was significantly greater for *Colias* from higher than from lower elevation sites. Common-garden experiments at the low-elevation field site showed that *C. meadii* adults initiated heat-avoidance and over-heating behaviors significantly earlier in the day than *C. eriphyle*. Our study demonstrates the importance of examining thermal tolerances across life stages. Our findings are inconsistent with the hypothesis that thermoregulatory behavior inhibits the geographic divergence

of physiological traits in mobile stages, and suggest that sessile stages may evolve similar heat tolerances in different environments due to microclimatic variability or evolutionary constraints.

Keywords *Colias* · Wing melanin · Overheating · Heat shock · Thermoregulation

Introduction

Many ectotherms adapt to local climatic conditions through changes in their thermal sensitivity (Angilletta 2009). Variation in environmental temperatures along latitudinal and elevation gradients is strongly associated with differences in optimal temperature, thermal breadth and lower thermal limits for insects and other ectotherms (Sunday et al. 2011). By contrast, upper thermal limits do not exhibit a strong association with these climatic gradients and are largely uncoupled with lower thermal limits (Addo-Bediako et al. 2000; Chown 2001; Diamond et al. 2012; Sunday et al. 2011). Studies variously quantify thermal limits as death, loss of mobility, or loss of fecundity, but trends are similar across metrics. The conservation of upper thermal limits across latitude and elevation may be due to evolutionary constraints on adaptation to high temperature. Alternatively, behavioral thermoregulation may buffer selective differences among environments and retard the evolution of physiological differences along climatic gradients, a hypothesis known as behavioral inertia (Huey et al. 2003). As a result, species- or life stages—that lack the ability to behaviorally mediate body temperature may be more likely to adapt physiologically to local climatic conditions (Hertz 1981; Krebs and Loeschcke 1995; but see Marais et al. 2009; Mitchell et al. 2013).

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Insects and other organisms with complex life cycles may vary in size, mobility, and thermal ecology across life stages. As a result sessile life stages, such as eggs or pupae, may evolve different thermal sensitivities, including differences in lower or upper thermal limits, from their mobile life stages (Kingsolver et al. 2011; Loeschcke et al. 1997; Potter et al. 2011). Insect tolerance of heat and cold varies across ontogeny (often in a complex manner) and declines with age within a life stage (typically measured in adults), but evidence for this variation comes primarily from studies of dipteran species that have not explicitly examined thermoregulatory behavior (reviewed in Bowler and Terblanche 2008). Most forecasts of climate change impacts exclusively consider the adult life stage, but studies in butterflies (Radchuk et al. 2013) as well as lizards (Levy et al. 2015) have shown that thermal sensitivity in juvenile, immobile life stages can reverse predictions that the organisms will benefit from climate change. These studies point to the importance of considering thermal tolerance across the life cycle with an explicit consideration of thermoregulatory behavior.

Here we explore upper thermal limits of *Colias* butterflies for two life stages—eggs and adults—that differ in their capacity for movement and behavioral thermoregulation. We use survival as our metric of thermal tolerance. We compare *Colias* populations and species along an elevation and climatic gradient, to examine whether there are geographic differences in physiological heat tolerance. *Colias* adults use behavior, including wing and body postures and microhabitat selection, to regulate body temperatures for flight and other activities (Watt 1968). *Colias* populations and species from different climatic regions vary in morphological traits (including wing melanin) but have similar optimal and preferred body temperatures for basking and flight (Ellers and Boggs 2004; Watt 1968). In contrast, *Colias* eggs are sessile and exhibit little morphological variation among populations and species. Geographic variation in the thermal tolerances of *Colias* eggs is unknown, and upper thermal limits of insect eggs have received little attention, with the exception of those found in some moth species, e.g., *Manduca sexta* and *Plutella xylostella* (Potter et al. 2011; Shirai 2000). Previous studies with *Colias* larvae show that populations and species from different elevations differ significantly in their thermal sensitivity for feeding (Higgins et al. 2014; Sherman and Watt 1973), suggesting that local adaptation of upper thermal limits can occur over relatively small geographic distances in this system (Higgins et al. 2014).

The behavioral inertia hypothesis (Huey et al. 2003) predicts more divergence in upper thermal limits along climatic gradients for sessile eggs than for mobile adults. To test these expectations, we use laboratory studies of heat tolerance of eggs and adults, and field studies of heat stress

and thermoregulatory behavior in adults, along an elevation gradient in two populations of *Colias eriphyle* as well as two populations of *Colias meadii* from sub-alpine habitats.

Materials and methods

Study populations

We studied two *Colias* species found in western North America. *C. eriphyle* is widely distributed across elevations (1.4–2.9 km) (Springer and Boggs 1986). *C. meadii* is confined to subalpine and alpine meadows above 2500 m in the Rocky Mountains (Watt 1968). Because *Colias* eggs are small (0.13 mm³) (García-Barros 2000) and are deposited singly on host-plant leaves, they are subject to leaf microclimatic conditions that vary widely across time and space but typically range from 15 to 25 °C in the temperate zone (Pincebourde and Woods 2012). As adults, *Colias* butterflies employ a closed-wing basking posture. The melanic scales on their ventral hindwings act to absorb solar radiation, and can elevate body temperature up to 15 °C above ambient. By avoiding solar radiation or orienting their closed wings parallel to the sun, they can maintain or even lower their body temperature (Rawlins 1980; Watt 1968). Along the elevation gradient, adults exhibit substantial differences in two thermally important phenotypes: solar absorptivity of the posterior ventral hindwing and length of the ventral thoracic setae. We examined two populations of *C. eriphyle*, one from a low elevation near Olathe, Montrose County, Colorado (38.62°N, 108.02°W, 1.6 km) and one from a middle elevation near Gunnison, Gunnison County, Colorado (38.56°N, 106.94°W, 2.3 km). We also examined the high-elevation species of *C. meadii* collected from two sites at Cumberland Pass, Gunnison County, Colorado (38.41°N, 106.29°W, 3.6 km) and Mesa Seco in Hinsdale County, Colorado (37.59°N, 107.13°W, 3.3–3.7 km, labeled as 3.5 km).

Laboratory assays of heat tolerance

To explore physiological heat tolerance of eggs along the elevation gradient we used the two populations of *C. eriphyle* (1.7 and 2.3 km) and one population of *C. meadii* (3.5 km). We exposed freshly laid eggs (24 h old) to a ramping heat shock following a similar approach to that of Potter et al. (2009). We first shipped adult females overnight from Colorado back to the laboratory at the University of North Carolina, Chapel Hill within 24 h of capture. Females were then placed in oviposition cages (1 m³) with a pot of vetch (*Vicia villosa*) and a dish of 10 % honey-water. Each day, the eggs from each female were liberated from their plant and loaded into a lane of a 96-well

plexiglass plate (one lane per female). From here, they were transferred onto an aluminum plate (described in Woods and Bonneau 2006) with eight lanes, each comprising 12 evenly spaced columns. Each column experienced a unique daily thermal cycle driven by connecting circulating water baths to either end of the plate. One side maintained 26 °C. The other side ramped up to and down from 48 °C over 24 h and held the maximum temperature for 2 h daily. Because leaf temperatures in the temperate zone do not regularly exceed 40 °C based on estimates in Pincebourde and Woods (2012), we felt 48 °C would be sufficiently stressful. Eggs were then transferred by hand to a hatching box lined with wet construction paper and placed under a camera in a 25 °C incubator with 24 h light. The camera took a single snapshot every 5 min over the next 96 h. The binary hatched/unhatched data were recorded once each day for 5 days and the precise time to hatching after eggs had been placed on the gradient was determined with camera images. This approach allowed us to evaluate a fine time scale to look for potential differences between the *C. eriphyle* populations. Despite best efforts, *C. meadii* females did not lay a sufficient number of eggs (less than 50 eggs from only three females) to be assayed in a rigorous way.

To explore physiological differences in heat tolerance in adults among populations and species, we used a fixed treatment of 60 min exposure to high temperatures as adults cannot be confined to a gradient. We exposed 20 freshly caught males from each of the four populations to one of five temperatures (25, 40, 42, 45, and 47 °C). We recorded survival both immediately after the heat exposure and after 24 h. Following morning collections during July 2012 and 2013, butterflies were transported at 3 °C, measured, weighed, photographed and scored for wing wear. Each butterfly was placed in a clear plastic cup furnished with a wooden craft stick and covered with netting. Butterflies were allowed to acclimate for 1–4 h before being randomly placed in a temperature chamber (TriTech Research DigiTherm DTM-MP-38). Butterflies were given a 10 % honey–water solution immediately following the shock. Because we only had two chambers, the experimental temperatures were randomized among times and days.

Field studies of thermoregulatory behavior and heat stress in adults

High temperatures in the field can constrain activity by inducing solar-avoidance behavior or causing overheating. We used common garden experiments at Montrose (1.6 km) to evaluate how exposure to high temperatures affects behavior and survival. Individuals were collected from each site the morning before each experiment. They were placed in glassine envelopes and stored at 3 °C. Later that day, individuals were measured and scored for

wing wear before being returned to 3 °C to preserve the integrity of the wings and lower the metabolic rate of the butterfly overnight. While we are unaware of any evidence of this practice changing heat tolerance in *Colias*, we acknowledge that this may have influenced the observed thermal limits. As a result all animals were treated in the same way to ensure the relative comparisons were meaningful. Individuals were placed in a closed tent, which warmed due to ambient conditions, until 10 a.m. Each individual was then transferred to a 0.3-m × 0.6-m cage made of SeeVue window screening in the city of Montrose, Montrose County (38.46°N, 107.88°W, 1.6 km). We observed behavior and survival at 2-min increments over 2 h. Flying consisted of a flight bout lasting more than 10 s and overheating consisted of wing flailing or death. Two types of behavioral posturing were characterized by the angle of ventral hind wing orientation to the sun: 90° ± 10° indicated basking and 180° ± 10° indicated shade seeking. For each trial, from seven to 12 transplanted males were compared to from seven to 12 *C. eriphyle* males from the Olathe (1.7 km) population. We repeated the trial three times for each of the Mesa Seco (3.5 km) and Cumberland Pass (3.6 km) *C. meadii* populations and five times for the Gunnison (2.3 km) *C. eriphyle* population.

For each day of experimentation, we measured solar radiation (Pace SRS-100) at plant height, wind speed (Pace WSD-100) at 1.2 m, and temperatures (Pace PT-907 thermistors) at 10 cm in the shade and at 0.5 cm below the soil surface. We also estimated butterfly body temperatures using physical models consisting of a thermistor coated in epoxy painted yellow with paper wings attached to match the color (solar absorptivity) of each of the two species (Kingsolver and Moffat 1982). The physical models were validated using fresh butterflies with a thermocouple inserted into their thorax. Measurements at 10-s intervals were averaged to minute intervals using a Pace Scientific X5-SE logger. All field trials were carried out during July 2012.

Statistical analysis

All statistical analyses were performed in R (version 2.15.3) (R Core Team 2013) using the survival and nlme libraries (Grambsch 2014). For the egg survival and development time experiment, we first used a nlme model with egg hatching as a binomial response, population as a categorical predictor, heat-shock (maximum) temperature as a continuous predictor, and the day of trial as a random intercept. Then, for the individuals that hatched, we modeled time to hatching as a continuous response variable with population and heat-shock temperature and with the day of trial as a random intercept. In both cases we included

first-order (linear) and second-order (quadratic) continuous terms for temperature to account for curvature.

For the adult survival experiment, we modeled survival following exposure as a binomial response. We used glm (Pinheiro et al. 2014) with species and sex as categorical variables, heat-shock temperature as a continuous variable, the interaction of species and heat-shock temperature as fixed effects, and population (within species) as a random intercept. Preliminary analyses showed that wing wear, a proxy for age, had a significant effect on the probability of survival. Because we did not have an even distribution of all ages across treatments, we subset the data to only include individuals that exhibited noticeable wing wear but whose wings were not yet fraying or tearing. This resulted in a dataset of 506 males [a score of ≤ 3 (Watt et al. 1977)].

For the field transplant experiment, we fit Cox proportional hazards regression models (function `coxph` in the R survival library) for each behavior: solar avoidance and overheating. This allowed us to compare the initiation time of each behavior between *C. meadii* and *C. eriphyle* and the high and low populations of *C. eriphyle*. Then, we included model temperature as a time-dependent covariate to determine if model temperature could account for differences in behavior.

Results

In both populations of *C. eriphyle*, egg hatching success was high at heat-shock temperatures between 28 and 38 °C, but declined significantly at higher temperatures [temperature, $d_{(1,395)} = 71.40$, $p < 0.001$; temperature², $d_{(1,393)} = 42.84$, $p < 0.001$] (Fig. 1a). There was no significant effect of population [$d_{(1,394)} = 0.15$, $p = 0.69$] nor any interactive effect of population with either temperature term [temperature, $d_{(1,392)} = 1.48$, $p = 0.22$; temperature², $d_{(1,390)} = 0.373$, $p = 0.54$]. Mean time to hatching measured from the start of the experiment was shortest at intermediate heat-shock temperatures (30–38 °C; Fig. 1b). Time to hatching was longer at both low and high shock temperatures [temperature, $F_{(1,299)} = 9.41$, $p = 0.002$; temperature², $F_{(1,299)} = 47.388$, $p < 0.001$]. Hatching time did not differ significantly between populations [$F_{(1,299)} = 0.12$, $p = 0.72$], nor were there any significant interactions of population with heat-shock temperature [temperature, $F_{(1,299)} = 0.30$, $p = 0.58$; temperature² $F_{(1,299)} = 1.05$, $p = 0.30$].

As in eggs, adult survival declined significantly with increasing heat-shock temperatures above 40 °C [$d_{(1,504)} = 87.73$, $p \ll 0.001$; Fig. 2]. Adult survival was significantly greater in *C. meadii* than in *C. eriphyle* [$d_{(1,503)} = 3.861$, $p = 0.04$], especially at heat-shock temperatures above 42 °C (Fig. 2).

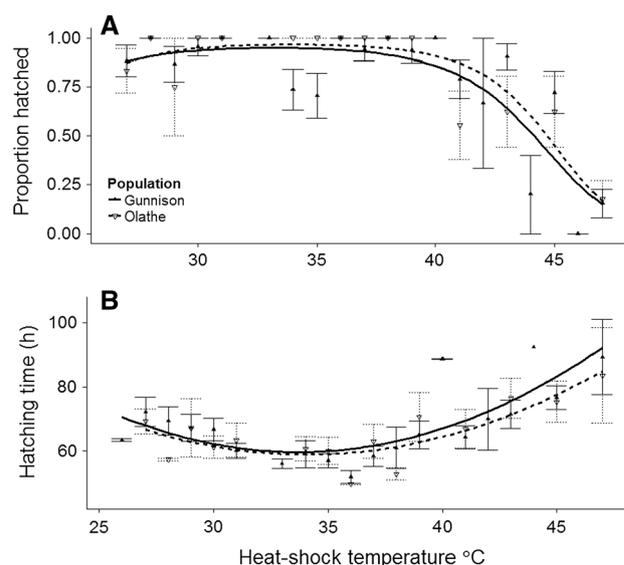


Fig. 1 **a** Hatching probability and **b** hatching time for both populations of *Colias eriphyle* as a function of maximum heat-shock temperature over a 24-h period. Filled triangle with dashed line represents the higher elevation population from Gunnison, Colorado (2.3 km); inverted open triangle with solid line represents the lower elevation population from Olathe, Colorado (1.6 km). While intermediate temperatures increase hatching probability (**a**), they decrease hatching time (**b**) regardless of population of origin. Lines represent second-order polynomial fits for each population

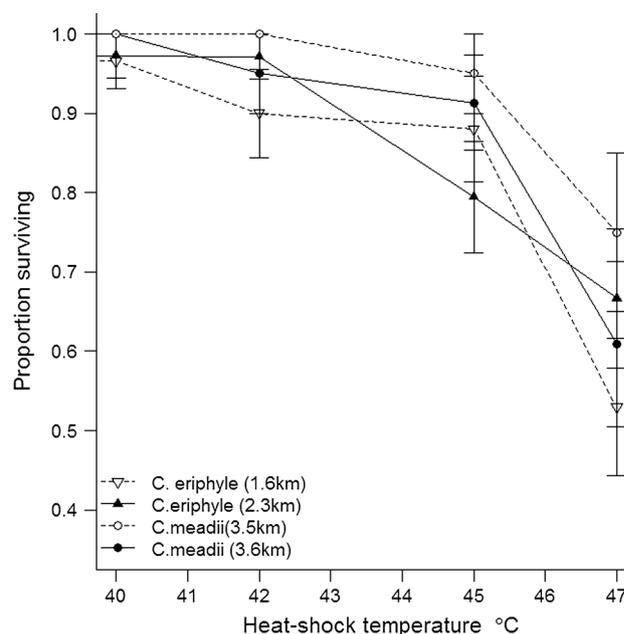


Fig. 2 Proportion of adults surviving a 1-h heat shock as a function of heat-shock temperature. All individuals survived 1 h at 25 °C (not shown). A greater proportion of adults from the higher elevation populations survived exposure to hotter temperatures

Fig. 3 A representative day from the common garden trial for *Colias meadii* from 3.6 km (dashed line) and *Colias eriphyle* from 1.6 km (solid line) on 23 July 2012. **a** Model temperature, and the proportion of each population **b** seeking shade, **c** overheating, and **d** dead, as a function of time

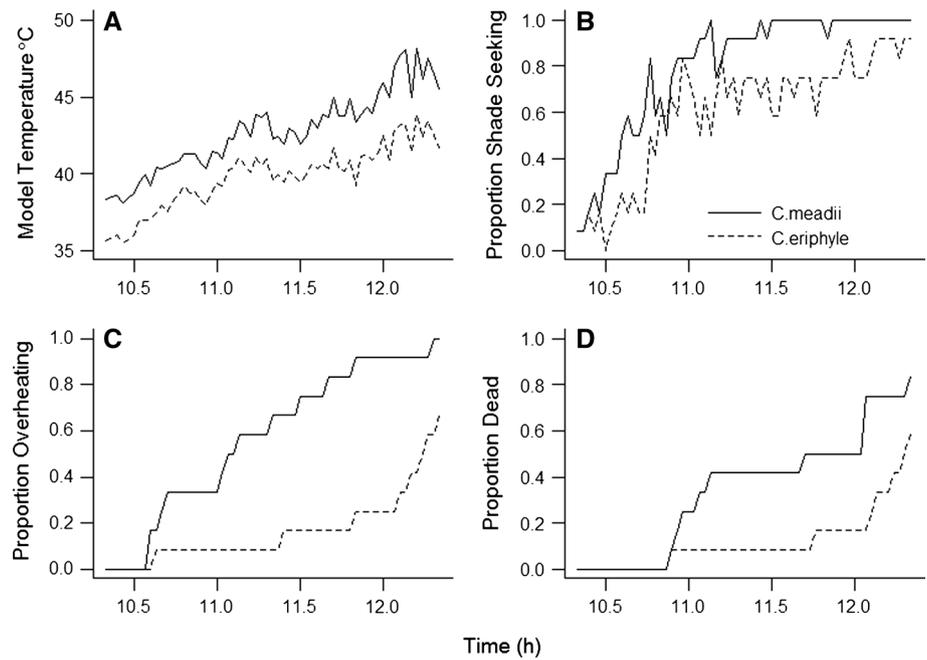
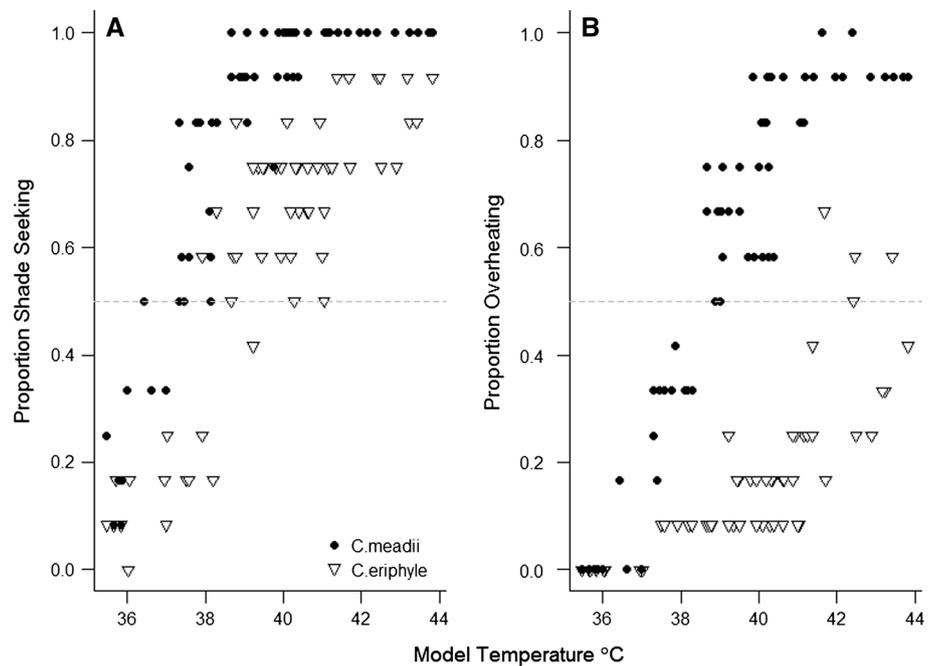


Fig. 4 A representative common garden trial for *C. meadii* from 3.6 km (filled circle) and *C. eriphyle* from 1.6 km (inverted open triangle) on 23 July 2012. **a** Proportion of individuals seeking shade, **b** proportion of individuals overheating as a function of physical model temperature



We used common garden experiments at the lower elevation site to quantify the differences in adult behavior and body temperature among *Colias* populations and species as warming occurred through the course of the mornings (Fig. 3). Comparing the low- and high-elevation populations of *C. eriphyle*, we found no significant difference in the timing of shade-seeking (Wald $X_1^2 = 1.33$, $p = 0.249$) or overheating behaviors (Wald $X_1^2 = 1.13$, $p = 0.288$). In contrast, *C. meadii* adults from both populations, initiated

shade-seeking (Wald $X_1^2 = 10.95$, $p = 0.009$) and overheating behaviors (Wald $X_1^2 = 8.73$, $p = 0.003$) earlier in the morning than *C. eriphyle* adults from the low-elevation population.

To evaluate whether these species differences in behavior were the result of differences in body temperature, we examined the incidence of behavior as a function of basking model temperature (Fig. 4). Basking model temperature across all trial days explained the difference in behavior

better than species for both shade-seeking behavior (Wald $X^2_2 = 69.52$, $p \ll 0.001$; Fig. 4a) and overheating (Wald $X^2_2 = 43.75$, $p \ll 0.001$; Fig. 4b).

Discussion

The thermal ecology of an organism can be dictated by large-scale geographic patterns and by small-scale differences in microhabitat (Potter et al. 2013). Broad geographic patterns can lead to differences in thermal sensitivities, typically at lower thermal limits (Diamond et al. 2012; Sunday et al. 2014). Differences in behavior and mobility throughout ontogeny can lead to the evolution of different thermal sensitivities at different life stages (Kingsolver et al. 2011). We found that both egg and adult stages of all populations are able to tolerate hot temperatures (>40 °C) despite their living in high-elevation environments with cool mean temperatures. This points to the importance of tolerating thermal extremes, which occur at high elevations with variable temperatures and intense radiation. The proportion of eggs hatching declines and the time to hatching increases considerably at temperatures beyond 40 °C. However, some populations maintained a relatively high hatching rate (~25 %) at our highest heat-shock temperatures (48 °C). While our experiment covered the range of temperatures we expect *Colias* to regularly encounter, population differences may have emerged at even warmer temperatures. The threshold for adult survival of heat shock is similar to that of eggs: adult survival begins to decline for most populations above 40 °C. Adult survival was considerably lower, but still fairly high at our highest test temperature (47 °C). We expect that repeated or prolonged exposure to our hottest test temperatures would have further reduced survival. We found that the onset of thermoregulatory behavior aligns with deleterious temperatures (>40 °C).

Adults thus seem capable of effectively using thermoregulatory behavior to buffer effects of thermal extremes. We expected that sessile eggs would show greater thermal tolerance, corresponding to conditions at each population's elevation. Previous studies of insects have found little support for behavioral inertia based on larvae to adult comparisons (Marais et al. 2009; Mitchell et al. 2013). Maximal and optimal temperatures for *Colias* larval feeding differ by more than 6 °C between these populations (Higgins et al. 2014). Thus we expected that eggs would demonstrate similar local adaptation because the sessile eggs experience mean and maximal air temperatures that differ by 5–10 °C between the two populations during the growing season (Higgins 2014). Contrary to our expectations, we observed no significant differences in the thermal tolerances of eggs between *C. eriphyle* populations.

One possible explanation is that microclimate variability may be greater than the differences between sites during the growing season. Eggs are small and reside within leaf boundary layers. As a result, they are influenced by both ambient temperatures and, to a greater extent, by leaf temperatures governed by stomatal behaviors (Pincebourde and Woods 2012). Both populations reside in grassy irrigated meadows with microclimate heterogeneity. The variation in available microclimatic leaf temperatures (−5 to +15 °C from ambient) (Pincebourde and Woods 2012) is greater than the temperature differences between sites. However, maternal choice in egg placement may minimize microclimate differences both within and between sites (Bonebrake et al. 2010). The egg stage is considerably shorter than the larval or pupal stages, increasing the variation among eggs in the thermal conditions they experience. Larval feeding time and adult activity time may be stronger fitness determinants than egg temperature. Increased temperature may speed development initially, but the influence may be ameliorated over the course of development (Potter et al. 2011). It may also be possible that there is an evolutionary constraint regards selection for eggs to tolerate increased temperatures. Strong selection imposed by abnormally warm years may have driven both populations to the highest possible level given their standing genetic variation, as has been suggested in other ectotherm species (Angilletta et al. 2013). We find further support for this hypothesis in the other sessile life stage; there is no difference in survival between these populations at a stressful, constant 30 °C pupal environment (Higgins 2014). However, it is difficult to say which of these is more likely, with data for only two populations per species. Data from a continuous latitude or elevation gradient could help decipher the mechanism.

The conservation of thermal limits across geographic ranges can be explained, in part, by thermoregulatory behavior (Buckley et al. 2015; Sunday et al. 2014). Because thermoregulatory behavior is effective in adult *Colias* and many other insects, we expected to see no difference at a low-elevation site in populations from an elevation gradient in *Colias* butterflies (Ellers and Boggs 2004). High body temperatures can decrease fitness in *Colias* in two ways: it limits the duration of flight time by forcing solar-avoidance behaviors and reduces egg viability in the closely related *Colias eurytheme* (Kingsolver 1983). What we found is rather surprising: high-elevation *C. meadii* are better able to survive body temperatures above 42 °C than populations of *C. eriphyle* from lower elevations. This is contrary to the behavioral inertia hypothesis (Huey et al. 2003). High-elevation sites experience relatively lower temperatures on average, but they also experience greater temperature variability and increased solar radiation (Buckley and Kingsolver 2012; Kingsolver and Buckley Kingsolver and Buckley 2015). The efficacy of thermoregulatory behavior

is limited by the microhabitat in the environments, and alpine vegetation is shorter than that of the meadows and agricultural fields at the lower elevation sites. As a result, high-elevation butterflies may experience short, rapid burst of high body temperatures regardless of thermoregulatory behaviors (Kingsolver and Watt 1984). The expression of heat-shock proteins may contribute to tolerance of thermal extremes. Our finding is consistent with species from cooler environments constitutively expressing lower levels of heat shock proteins but having a greater capacity for induction when facing thermal extremes. Such responses have been observed along latitudinal clines in non-thermoregulating insects (Sarup et al. 2006).

Alternatively, this might be explained by life history. At low elevation *C. eriphyle* is multivoltine or bivoltine throughout its range with a facultative 3rd instar diapause whereas *C. meadii* is univoltine with an obligate 3rd instar diapause. Thus, all *C. meadii* but only a few *C. eriphyle* we sampled mid-summer had overwintered. The few *C. meadii* that survive overwintering may be particularly robust [given an estimated 98 % mortality rate for an intermediate elevation *Colias* species (Hayes 1980)]. Cross-tolerance to cold and heat is often observed in insects (Hodkinson 2005; Sinclair et al. 2013), which may enable *C. meadii* that survived diapause to better survive heat shocks.

This study highlights how available thermal environments may limit or drive the evolution of upper thermal limits in insects and other ectotherms with multiple life stages. The evolution of higher upper thermal limits may be dampened in sessile life stages if environmental heterogeneity is high. If thermal refuges are limited in availability, the evolution of higher upper thermal limits may be accelerated, even in thermoregulating life stages. With climate change increasing mean temperatures, the ability to find thermal refuges might be increasingly difficult across elevation gradients and the ability to withstand high temperatures for brief periods of time may be critical to survival (IPCC 2014; Williams et al. 2007). This study highlights the need to investigate thermal limits across life stages and across populations of ectotherms. Assumptions based on ambient temperatures or observed behaviors may be inadequate in predicting population responses to climate warming.

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Author contribution statement H. J. M., L. B. B., and J. G. K. conceived and designed the experiments. H. J. M. and J. G. K. performed the experiments. H. J. M. and J. G. K. analyzed the data. H. J. M. and J. G. K. wrote the manuscript; other authors provided editorial advice.

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