

ADAPTATION TO CLIMATE AND CLIMATE CHANGE IN ROCKY MOUNTAIN BUTTERFLIES:
MORPHOLOGY, PHYSIOLOGY, AND BEHAVIOR

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ABSTRACT

Heidi Joan MacLean: ADAPTATION TO CLIMATE AND CLIMATE CHANGE IN ROCKY MOUNTAIN
BUTTERFLIES: MORPHOLOGY, PHYSIOLOGY AND BEHAVIOR
(Under the direction of Joel G. Kingsolver and Lauren B. Buckley)

Understanding the relative contributions of evolution, plasticity and behavior for local adaptation to climate, and for adaptive responses to recent climate change, is a major challenge for global change biologists. My dissertation explores these issues for *Colias* butterflies along an elevation gradient. Adults use thermoregulatory behaviors to achieve optimal body temperatures. Past studies show local adaptation in fixed morphological differences in ventral hind-wing melanin and thoracic setal length and a degree of developmental plasticity associated with melanin. My dissertation makes four major contributions. First, reciprocal-transplant and common-garden experiments documented differences in thermal sensitivity for flight initiation between high (*C. meadii*) and lower (*C. eriphyle*) elevation species. *C. meadii* initiated flight at lower body temperatures than *C. eriphyle*. This result was contrary to expectations that behavior will hinder evolution in fixed physiological differences along climatic gradients. Second, laboratory experiments showed that high-elevation adults had higher survival after heat-shock relative to lower-elevation adults. In contrast, there were no significant differences in upper thermal limits for eggs from different elevations suggesting the lack of movement did not lead to the evolution of fixed physiological differences in this sedentary life stage. Third, I used historic collections of a high-elevation *C. meadii* to quantify changes over the past 60 years. While mean temperatures during the adult flight have increased

significantly, both hind-wing melanin and setal length have also increased. I suggest that this is due in part to developmental plasticity; cooler pupal temperature was associated with increased melanin. I propose that this is non-adaptive because pupal temperatures are poor predictors of adult temperatures. Fourth, I quantified seasonal changes in wing melanin for two populations of *C. eriphyle*. Seasonal change in wing melanin was associated with increases in pupal temperature. In contrast to *C. meadii*, pupal temperatures were a good predictor of adult flight season temperature for *C. eriphyle*. Collectively, my results suggest that behavior does not reduce the importance of physiology and morphology in local adaptation to climate, and that potential for adaptive plasticity in high-elevation ectotherms may be strongly limited by variability and unpredictability in seasonal climate.

To my family, for always believing in me.

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CHAPTER ONE: INTRODUCTION- ADAPTATION TO LOCAL CLIMATE AND CLIMATE CHANGE IN ROCKY MOUNTAIN BUTTERFLIES

Temperature is a driving force in local adaptation, and most ectotherms have a restricted range of body temperatures over which they can achieve high rates of growth, locomotion and other aspects of performance (Andrewartha & Birch 1954; Magnuson, Crowder & Medvick 1979; Huey & Hertz 1984). Having a restricted thermal range allows for optimization of biochemical processes, and organisms with higher thermal optima typically have a higher maximal performance (Hochachka & Somero 2002; Somero 2004). Thermal constraints on activity and locomotion can be important determinants of fitness and population demography (Stanton & Cook 1984; Adolph & Porter 1993a) and can be used to predict responses to climate change (Buckley 2008; Kearney & Porter 2009; Sinervo *et al.* 2010; Buckley & Kingsolver 2012). Terrestrial ectotherms can adapt evolutionarily to local thermal and climate conditions through shifts in morphology and behavior that allow them to achieve preferred body temperatures or through physiological shifts in the thermal range of performance (Angilletta 2009). Local adaptations of ectotherms across environmental gradients pose several questions 1) do trait distributions vary predictably over time? 2) How plastic are thermally important traits? 3) In addition to morphological compensation, do species have different thermal sensitivities to cope with different environments? Understanding these components of local adaptation is not only interesting in the context of evolution but also paramount in predicting species responses to climate warming.

There is evidence that many ectotherms, specifically insects and lizards, adapt to different thermal conditions along latitudinal and elevation gradients through morphological differences in body size, coloration, and insulation (Watt 1968; Berry & Willmer 1986; Gillis & Smeigh 1987; Schultz, Quinlan & Hadley 1992; Ellers & Boggs 2004). For example, darker coloration in high altitude organisms allows them to absorb more solar radiation and achieve body temperatures significantly higher than ambient (reviewed in Mani 1968 and; Hodkinson 2005). In addition to morphological adaptations, another mechanism of local climatic adaptation is variation in thermal sensitivity; or changes in the shape and position of their thermal performance curves for growth, locomotion, survival, and reproduction (Frazier, Dillon & Crane 2006). For example, high-elevation species are capable of activity at lower temperatures than their lower-elevation congeners across frog families (Navas 1996; Navas 1997; Wilson 2001). Thermal adaptations to cool temperatures at high latitudes are broadly observed across ectothermic taxa (Huey 1991; Kingsolver & Woods 1997; Bradshaw, Fujiyama & Holzapfel 2000; Wilson 2001; Liefing, Hoffmann & Ellers 2009; Sunday, Bates & Dulvy 2011). Performance at cooler temperatures may be critical to organisms at high elevations or high latitudes where the duration of time suitable for activity or development is limited (Kingsolver & Watt 1983; Adolph & Porter 1993b; Sinervo & Adolph 1994). High temperatures also limit activity (reviewed in Angilletta 2009) by forcing individuals to seek thermal refuge rather than perform any fitness-enhancing functions. With current and future temperatures rising, these limits may have increasing negative effects on populations.

Temperature plays a major role in shaping adaptation over time, and recent climate change has increased temperatures as well as temperature variability (Field 2014) especially in

montane regions. The ecological responses of organisms to recent climate change have been widely documented. They include changes in seasonal timing, shifts in geographic distributions, alterations in species interactions, and changes in population density or the likelihood of extinction (Parmesan 2006; Chen *et al.* 2011; Diamond *et al.* 2011). Recent studies have also documented evolutionary changes in the timing of diapause (Bradshaw, Fujiyama & Holzapfel 2000), of nesting (Visser *et al.* 1998) and of flowering (Anderson *et al.* 2012); in body size (Sheridan & Bickford 2011); and in coloration relative to background matching (Galeotti *et al.* 2009; Mills *et al.* 2013). While we have this documented evidence of responses to climate change, we do not yet understand the relative roles of plasticity and evolution in species responses to recent climate change. To do this, we need a system that has historical data.

For the past 50 years, *Colias* butterflies have been an important model system for understanding thermal biology and our recent work employs them further to study local adaptation to climate. While members of the genus inhabit environments ranging from agricultural fields to alpine meadows and arctic tundra, previous studies indicate all adult flight occurs in the same narrow thermal window (28-40°C) (Watt 1969). Regardless of habitat, flight is essential for all major activities, including courtship, mating, feeding, and oviposition, and flight time is limited by climate (Nielsen & Watt 1998; Watt *et al.* 2003; Watt 2004). The two species used were *Colias eriphyle* and *Colias meadii*. *Colias eriphyle* is widely distributed throughout western North America across a range of elevations (1,400-2,900m) (Springer & Boggs 1986). They are typically found in irrigated agricultural fields and recently expanded their larval diet to include the agriculturally important alfalfa. *C. eriphyle* exhibits a gradient in wing melanin along their elevation range with lighter individuals being found at lower elevations. *Colias meadii* is confined to subalpine and alpine meadows above 2,500m elevation in the Rocky

Mountains (Watt 1968). Their larvae are host plant generalists feeding on high-alpine legumes. *Colias* species at higher latitudes and higher elevations have increased ventral hindwing melanin and longer thoracic setae to reduce convective heat loss and achieve a body temperature conducive to flight (Watt 1968; Kingsolver & Watt 1983; Ellers & Boggs 2004). In contrast, lower latitude and lower elevation species have less ventral hindwing melanin and shorter thorax setae. *C. eriphyle* has an average absorptivity of 53-60% and thorax setae length of 0.82-1.08mm along an elevation gradient from 1,700m-2,700m, and *C. meadii* has an average absorptivity of 64% and thorax setae length of 1.46mm (Kingsolver 1983b). A biophysical model indicates that darker, more melanic wings allow the butterflies to absorb more solar radiation and longer thorax setae slow heat loss, thereby enabling butterflies to achieve the high body temperatures required for flight (Watt 1968; Kingsolver 1983). Additionally, there is some behavioral data to suggest that *Colias* are able to maintain or cool their body temperatures through flight or avoidance of direct solar radiation (Kingsolver 1983). While some work has been done on the consequences of overheating in the laboratory, little has been done to examine their thermal limits or their overheating behaviors in the field. Because of the importance of thermoregulatory traits to fitness in this system, we suspect that these traits may be responding to climate change.

The relative contributions of geographic (fixed) differences in morphology and physiology, and of developmental plasticity and behavior, to local climatic adaptation, is poorly understood in most systems. Similarly, the roles of morphological and physiological evolution, plasticity and behavior in determining adaptive responses to recent climate change is largely unknown. In the short term, behavior and adaptive plasticity are expected to reduce the evolutionary changes in morphology and physiology in response to environmental change, but this expectation has not been tested in the context of recent climate change. My dissertation

explores these issues for *Colias* butterflies along an elevation gradient in the Rocky Mountains and exploits on the years of research in this model system. Specifically I will quantify the thermal limits of flight activity, the upper thermal limits on survival across life stages, and the patterns of adult phenotypes over time. I will then estimate plastic contributions to the adult phenotype by using field data to estimate the plasticity of wing melanin in response to pupal temperature. In chapter one of my dissertation, I will look at the constraints on initiation of flight activity to evaluate the components of local adaptation of morphology and thermal sensitivity. In chapter two, I look at the upper thermal limits of performance and overheating in both eggs and adult butterflies. In the third chapter, I quantify trait distribution over time in *Colias meadii meadii* using museum specimens from the last 60 years. Finally, in the fourth chapter I use field caught specimens to look at ventral hind wing melanin in response to pupal temperature over the course of the flight season. Both evolutionary changes and phenotypic plasticity can contribute to local adaptation and adaptation to recent climate change.

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CHAPTER TWO: MORPHOLOGICAL AND PHYSIOLOGICAL DETERMINANTS OF LOCAL ADAPTATION TO CLIMATE IN ROCKY MOUNTAIN BUTTERFLIES

Summary

Behavioral thermoregulation can retard the evolution of physiological differences among populations and species of ectotherms that occur in different thermal environments. *Colias* butterflies use thermoregulatory behaviors to achieve body temperatures required for flight and *Colias* species in different climates have similar optimal temperatures for flight. Many *Colias* adapt to local climate conditions through differences in two key morphological traits, wing melanin, and thoracic setal length. However, the relative contributions of differences in morphology and in thermal sensitivity for flight initiation in different climatic conditions have not been determined. We addressed this issue for four populations of two *Colias* species along an elevation gradient: *C. meadii* (3.3-3.6 km elevation) and *C. eriphyle* (1.3-2.8 km). Reciprocal transplants experiments (full solar radiation) showed that morphological differences between species lead to differences in body temperature and those differences contributed strongly to differences in our measures of flight initiation; probability, time and air temperature. Common garden experiments (without direct solar radiation) revealed significant differences in thermal sensitivity for flight initiation between high (*C. meadii*) and lower (*C. eriphyle*) elevation species owed to physiology and not morphology. By taking these studies in concert we find, *C. meadii* initiated flight at lower body temperatures than *C. eriphyle* suggesting that differences in both thermal sensitivity (15%) and in morphology (85%) contributed to the differences in flight initiation between the two species in the field. This finding is counter to expectations that

behavioral flexibility will retard the evolution of fixed physiological differences along climatic gradients.

Introduction

Most ectotherms have a restricted range of body temperatures over which they can achieve high rates of resource acquisition, growth and other aspects of performance (Angilletta, 2009). Locomotion is a key aspect of performance in many ectotherms, and thermal constraints on locomotion can be important determinants of fitness and can be used to predict responses to changing climatic conditions (Buckley & Kingsolver, 2012a, Adolph & Porter, 1993b, Kearney et al., 2009a, Sinervo et al., 2010). Terrestrial ectotherms can adapt evolutionarily to local thermal and climate conditions through shifts in behavior or morphology that allow them to achieve preferred body temperatures, or through physiological shifts in the thermal range of performance (Angilletta, 2009). By understanding the relative contributions on each of these, we may be able to predict more accurately the ecological responses to environmental change.

Many insects adapt to local climates along elevation and latitudinal gradients through morphological differences in body size, coloration, and insulation, allowing them to achieve higher body temperatures in cooler environmental conditions (reviewed in Mani, 1968 and, Hodkinson, 2005). Thermoregulatory behaviors and microhabitat choice can also be used to elevate body temperatures to the preferred thermal range and can lead to conserved thermal limits across thermal environments (Angilletta et al., 2002, Watt, 1968a, Chappell, 1983, Buckley et al., 2015). Conversely, thermal sensitivities can vary across elevation or latitudinal gradients. While upper thermal limits tend to be highly conserved, there is a great deal of observed variability in lower thermal limits across these gradients (Sunday et al., 2011). Performance at cooler temperatures may be particularly important for organisms at higher

elevations or latitudes where time available for activity or development can be strongly limited (Kingsolver & Watt, 1983a, Adolph & Porter, 1993b, Sinervo & Adolph, 1994). The mix of morphological, behavioral, and physiological means of adaptation varies widely among different taxa and systems. This is one of the first field experiments used to quantify the relative contribution of each to local adaptation along environmental gradients.

Here we employ *Colias* butterflies, known for their local adaptation in morphological traits, to examine behavioral and physiological adaptations along an elevation gradient. The body temperatures of *Colias* adults are strongly influenced by morphological traits (Watt, 1968a, Kingsolver, 1983c). Moreover, *Colias* populations and species exhibit local adaptation in morphological traits across their range; with increasing elevation or latitude, populations have more ventral hindwing melanin and thicker thoracic setae (Ellers & Boggs, 2004b, Watt, 1968a, Kingsolver & Watt, 1983a). *Colias* butterflies employ a closed-wing basking posture when their body temperature is below optimum. In this posture, the melanic scales on their ventral hindwings act to absorb solar radiation, and elevate body temperature (Watt, 1968a, Kingsolver, 1983c). While there is local adaptation in thermally important morphological traits across their range, *Colias* species share similar thermal optima for flight between 34-38°C (Ellers & Boggs, 2004b, Watt, 1968a). The time available for flight activity is limited, especially at high elevations and flight activity can strongly limit reproductive success as females lay a single egg on each plant visited. As a result, we expect that there are differences in the thermal sensitivity of flight initiation between high and low elevation species of *Colias*.

In this study, we use field experiments to quantify the relative contributions of morphology and thermal sensitivity in flight initiation for high elevation *Colias meadii* and low elevation *Colias eriphyle*. First, we use reciprocal transplant experiments to compare flight

initiation among *Colias* species. These experiments enable us to determine how morphology, behavior and thermal sensitivity influence body temperatures and flight initiation across elevations. Then, we use a common garden experiment in the absence of direct solar radiation at one elevation (1.7 km) to compare differences in thermal sensitivity of flight initiation between *Colias* species. This experiment isolates physiological differences in the thermal sensitivity of flight initiation from morphological differences that may influence body temperature. By combining information from both experiments, we quantify the contributions of morphological and physiological mechanisms to local adaptation along an elevation gradient.

Materials and Methods

Study system

Colias butterflies have been an important model system for understanding thermal biology and local adaptation to climate because they have both morphological and behavioral mechanisms for thermoregulation. Empirical measurements and biophysical modeling confirm that darker, more melanic, wings allow the butterflies to absorb more radiation to heat faster and thicker thorax setae slow heat loss (Kingsolver, 1983c, Watt, 1968a). While past studies suggest *Colias* species require similar flight temperatures (30-40°C) (Watt, 1968a), field observations suggest that flight may occur at body temperatures below 30°C, especially for *Colias* species at higher elevations (Kingsolver & Watt, 1984).

We used two species along an elevation gradient on the western slope of the Rocky Mountains; *C. eriphyle*, and *C. meadii*. *Colias eriphyle* is widely distributed throughout western North America across a range of elevations (1.4-2.9 km) (Springer & Boggs, 1986). *Colias meadii* is confined to subalpine and alpine meadows above 2.5 km elevation in the Rocky Mountains (Watt, 1968a). The species exhibit substantial variation in thermally important

phenotypes. *Colias eriphyle* has an average solar absorptivity of 53-60% and thorax setae length of 0.82-1.08mm along an elevation gradient from 1.7-2.7km. *Colias meadii* has an average solar absorptivity of 65% and thorax setae length of 1.46mm (Kingsolver, 1983c).

Our field studies involved four sites over a range of elevations in western and central Colorado. We collected *C. eriphyle* from two sites, one near Olathe, Montrose Co., CO (N38.62, W108.02, 1.6km) and one 90km away in Gunnison, Gunnison Co., CO (N38.56, W106.94, 2.3km). We collected *C. meadii* from Cumberland Pass, Gunnison Co. CO (N38.41, W106.29, 3.6km) and Mesa Seco in Hinsdale Co., CO (N37.59, W107.13, 3.3km-3.7km). Past studies conducted at Mesa Seco revealed differences in genotypic frequencies of the PGI locus between the lower and upper part of the mesa, a mere 0.5km apart (Watt et al., 1996) suggesting that there may be physiological differences within this site. As a result, we distinguish individuals sampled from both below tree-line (<3.3 km) and above tree line (>3.4km) meadows within Mesa Seco.

Micrometeorological measurements

To quantify thermal conditions and butterfly temperatures during the experiments, we measured solar radiation, wind speed, and air and soil temperatures during each field study. We used a solar radiation sensor (Pace SRS-100) at plant height; an anemometer (Pace WSD-100) at 1.2 m; and thermistors (Pace PT-907) at 10 cm above the soil surface in the shade and 0.5 cm below the soil surface. An additional thermistor was modified to serve as a physical model in the sheltered environment common garden experiments (see below). The sensor was coated in epoxy, painted yellow, and paper wings were attached to mimic butterfly morphology (Kingsolver & Moffat, 1982). The epoxy models were validated using fresh butterflies with a thermocouple inserted into their thorax and recorded at three minute intervals. The average error

between the epoxy model and the butterfly was $0.6^{\circ}\text{C} \pm 1.1^{\circ}\text{C}$ (mean \pm SD). Measurements were recorded every 10s and averaged values were output every minute using a Pace Scientific X5-SE logger.

Reciprocal Transplants- flight initiation with direct solar radiation

To quantify differences in flight initiation across the elevation gradient, we conducted reciprocal transplant experiments at a low and high elevation site. The high elevation site was split between a lower meadow and an upper plateau in order to explore within site variability. We transplanted *C. eriphyle* from Olathe (1.6km) and *C. meadii* collected from 3.3km and 3.6km within Mesa Seco. At each site 24 open-bottom cages were placed on top of vegetation. The cages were 30cm in diameter and 60cm tall cylinders constructed of SeeVue (Pfiser ®) window screen topped with bridal veil and positioned using garden staples. The screen reduced solar radiation by less than 15%. A single animal was placed on the vegetation at the bottom of each enclosure prior to local sunrise. Cages were checked every two minutes and the time of flight initiation was recorded. We also recorded if an individual did not initiate flight. At Mesa Seco, a portable weather station (see above) was placed at the middle of the site to record air and soil temperatures, solar radiation, and wind speed. The experiments conducted on *C. meadii* and *C. eriphyle* were repeated twice at each site on different days in July 2011. The experiments conducted on *C. meadii* from above and below tree-line were repeated five times on different days in July and August 2011.

Predicting butterfly temperatures

We used an established and validated biophysical model for *Colias* to predict steady-state body temperature based on thermally important traits (absorptivity of the ventral hind wings and thoracic fur thickness) and environmental condition at the time of initiation (Kingsolver &

Moffat, 1982, also see Buckley & Kingsolver, 2012a, Kingsolver, 1983a). We used a steady-state (rather than transient) model because the thermal response time (time constant) for *Colias* is typically less than 60s (Kingsolver 1983a). The model is outlined below. Environmental parameters were averaged over six minutes prior to flight initiation.

Steady-state energy balance of a butterfly at rest on vegetation is characterized as

$$Q_s = Q_t + Q_c$$

where Q_s is the total solar radiative heat flux, Q_t the thermal radiative heat flux, and Q_c is the convective heat flux. Conduction of heat between the butterfly and the vegetation occurs through legs and is thus considered negligible as are metabolic heat input and evaporative heat loss (Watt, 1969, Watt, 1968a). Solar radiative heat flux is estimated as:

$$Q_s = Q_{s,dir} + Q_{s,dif} + Q_{s,ref}$$

$$Q_s = \alpha A_{s,dir} H_{s,dir} / \cos(z) + \alpha A_{s,ref} H_{s,dif} + \alpha r_g A_{s,ref} H_{s,tot}$$

where $Q_{s,dir}$, $Q_{s,dif}$, $Q_{s,ref}$, are the direct, diffuse, and reflected solar radiative fluxes, respectively; $H_{s,dir}$, $H_{s,dif}$, $H_{s,tot}$, are the direct, diffuse, and total solar radiative horizontal flux densities in mW/cm^2 , respectively; α is wing solar absorptivity measured for each individual; r_g is substrate solar reflectivity ; z is the zenith angle ; $A_{s,dir}$, $A_{s,ref}$ are the direct and reflected solar radiative heat transfer surface areas, and are assumed to equal to $A_{s,tot}$, total solar radiative heat transfer surface area (cm^2).

Thermal radiative flux including both downward (sky) and upward (ground) radiation is estimated as:

$$Q_t = 0.5 A_t \epsilon \sigma (T_b^4 - T_{sky}^4) + 0.5 A_t \epsilon \sigma (T_b^4 - T_g^4)$$

where A_t is the thermal radiative heat transfer surface area (cm²), T_b is the body temperature (K), T_g is the ground surface temperature (K), T_{sky} is the equivalent black body sky temperature (K), ϵ is butterfly thermal emissivity, and σ is the Stefan-Boltzman constant.

Convective heat flux is calculated by:

$$Q_c = h_T A_c (T_b - T_a)$$

where A_c is the convective heat transfer surface area (cm²), and T_a is the air temperature (K).

Again we assume $A_c = A_t = A_{s,ttl}$.

The total convective heat transfer coefficient, h_T , is calculated as the boundary layer conductance h_c and the fur layer conduction in series:

$$\frac{1}{h_T} = \frac{1}{h_c} + (r_i + \delta) \ln \left(\frac{r_i + \delta}{r_i} \right) k_e$$

Where δ is the thoracic fur thickness (measured on each individual to nearest mm), r_i is the radius of the mesothorax, and k_e is the thermal conductivity of the fur. The boundary layer conductance, h_c , can be estimated using the relationship between two non-dimensional numbers. The Nusselt number, $Nu = h_c D / k_a$, is the ratio of convective to conductive heat transfer, where k_a is the thermal conductivity of air. We used the maximum width of the mesothorax as the characteristic dimension of the butterfly D , which was approximated to 0.35cm. The Reynolds number, $Re = uD / \nu$, is the ratio of inertial forces to viscous forces, where u is wind speed and ν is kinematic viscosity. We used the $Nu-Re$ relation for a cylinder, $Nu = 0.6 Re^{0.5}$, which is a reasonable approximation for a *Colias* butterfly (Kingsolver & Moffat 1982).

Common Garden- Flight initiation in the absence of direct solar radiation

To quantify differences in thermal sensitivity we conducted two sets of common garden experiments in the absence of direct solar radiation: one set between populations of *C. eriphyle* from Olathe (1.6km) and Gunnison (2.3km); and the other between the two species, *C. eriphyle*

from 1.6km (Olathe) and *C. meadii* from 3.3-3.6km (Mesa Seco and Cumberland Pass). We compared body temperatures at flight initiation without the influence of morphological traits. All trials were conducted at a low elevation site in Montrose (N38.46, W107.88, 1.7km), to ensure that temperatures in the tent were high enough to elicit flight. These trials were conducted in three 2.75m x 3.35m nylon enclosures (Kelty® medium portable shelters) that reduced solar radiation at solar noon by 65%. A portable weather station (see above) was placed inside the middle tent and set to record air and soil temperatures, solar radiation, and wind speed at one-minute intervals. Temperature of initiation was estimated by recording temperature of the physical butterfly model (see above) at one-minute intervals. Each set of experiments was repeated five times on different days in July and August of 2012. Individuals were held at $\sim 3^{\circ}\text{C}$ until the start of the assay. They were then placed in the center of the tent before local sunrise with a researcher in the southwest corner. If an individual initiated flight, the decimal hour was recorded. It was also noted if an individual did not initiate flight over the course of the trial.

Statistical analyses

All statistical analyses were conducted in R (version 2.15.2). For the reciprocal transplant experiments, the probability of flight and the time of flight metrics were analyzed with population or species and elevation as fixed effects and Julian date nested within site as a random effect. Predicted body temperature of flight initiation was analyzed with collection site or species and elevation as fixed effects and with Julian date as a random effect. We used a heteroscedastic variance model to compare the distributions of model temperatures at initiation between the two populations or species. This comparison allowed us to look at the difference in the shape of the distributions while accounting for the structure of our experimental design. We compared a

model with fixed variance to a model with variance fit independently for each population or species.

For the common garden experiments, probability of flight initiation was modeled as a binomial response (flight or no flight) using a generalized linear model with population or species and, because external environmental factors determined the rate of heating in the tent, we used initial morning temperature (mean air temperature inside the tent from 07:30 to 07:45 MDT) as fixed effects; tent was nested within Julian date as a random effect to account for between-tent and between-day variance. We then tested for the significance by comparing simpler models to the full model (including an interaction term) using Chi-Square tests. The simpler models consisted of a single predictor variable or both predictor variables without an interaction. Time of flight initiation and temperature of flight initiation were analyzed using linear mixed-effects models with the same fixed and random effect, and tested using ANOVAs.

Results

Reciprocal Transplants- flight initiation with solar radiation

In the reciprocal transplant, the proportion of *C. eriphyle* that initiated flight decreased with increasing elevation and the proportion of *C. meadii* that initiated flight remained constant across elevations, producing an interactive effect (species: $\chi^2_{(2, N= 83)} = 17.39$, $p < 0.001$; trial

elevation: $\chi^2_{(2, N= 83)} = 11.83$, $p=0.02$; interaction: $\chi^2_{(1, N= 83)} = 17.39$, $p= 0.005$) (Fig 3).

Moreover, the *C. meadii* initiated earlier than *C. eriphyle* ($F_{(1,54)} = 7.99$ $p < 0.05$) regardless of trial elevation ($F_{(1,54)} = 42.57$, $p=0.09$) (Fig 3d). This confirms that high-elevation *C. meadii* are more likely to initiate flight and to fly earlier than low-elevation *C. eriphyle* independent of ambient temperature or trial elevation.

When we compared *C. meadii* collected from the bottom and the top of Mesa Seco (3.3km and 3.6km) we saw no difference in proportion that initiated (site: $\chi^2_{(2, N=116)}=3.91$, $p=0.14$; trial elevation: $\chi^2_{(2, N=116)}=4.35$, $p=0.11$; interaction: $\chi^2_{(1, N=116)}=1.09$, $p=0.29$, Figure 3a) and no effect of collection site ($F_{(1,82)}=0.15$, $p=0.87$). There was, however, a lag in time of initiation at the higher trial elevation regardless of collection site ($F_{(1,82)}=4.46$, $p=0.03$ for elevation; $F_{(1,82)}=0.03$, $p=0.85$ for the interaction, Fig 3b).

We calculated a predicted body temperature at flight initiation for the high elevation trials. We only included animals that were able to initiate flight so the number of *C. eriphyle* included was smaller. Predicted body temperature was calculated for the high elevation transplant using the average phenotype for the *C. meadii*: $69.1\% \pm 3.3\%$ (mean + SD) absorptivity and $1.27\text{mm} \pm 0.23\text{mm}$ thorax setae length and the average phenotype for *C. eriphyle*: $51.8\% \pm 7.0\%$ absorptivity and $0.63\text{mm} \pm 0.18\text{mm}$ thorax setae. Of the butterflies that were able to initiate flight, there was no species difference in the body temperature at initiation ($F_{(1,23)}=0.22$, $p=0.64$, Fig 4). Butterflies initiated flight at cooler air temperatures at the highest elevation ($F_{(1,23)}=6.48$, $p=0.02$), but there was no interaction between species and elevation ($F_{(1,23)}=1.38$, $p=0.25$).

Common Garden- Flight initiation in the absence of direct solar radiation

The common garden experiments allowed us to look at flight initiation in a controlled environment. When we looked at two populations of *C. eriphyle*; one from 1.6km (Olathe) and one from 2.3km (Gunnison), differences in thermal sensitivity of flight initiation between the *C. eriphyle* populations from varying elevations were limited. *C. eriphyle* from the Gunnison population were more likely to initiate active flight ($\chi^2_{(2, N=178)}=9.05$, $p=0.01$) independent of morning air temperature ($\chi^2_{(2, N=178)}=3.7$, $p=0.15$, Figure 1a). Of those that did initiate, there

was no difference in population ($F_{(1,138)}=1.41$, $p=0.23$), initial morning air temperature ($F_{(1,138)}=0.16$, $p=0.71$), or the interaction ($F_{(1,138)}=0.47$, $p=0.49$, Fig 1b). The butterfly model temperature at initiation showed no significant effect of population ($F_{(1,138)}=2.57$, $p=0.11$) or the interaction of population and initial morning air temperature ($F_{(1,138)}=0.88$, $p=0.34$, Fig 1c).

When we compare *C. eriphyle* from 1.6km and *C. meadii* from 3.6km in low solar radiation, we see that *C. meadii* initiates at slightly cooler temperatures relative to the low elevation *C. eriphyle*. While we detected no significant difference in the probability of flight initiation (figure 2a, $\chi^2_{(2, N= 249)} = 4.06$, $p= 0.13$ for species , $\chi^2_{(2, N= 249)} = 3.01$, $p= 0.22$ for collection site, $\chi^2_{(1, N= 249)} = 1.95$, $p= 0.16$ for the interaction), *C. meadii* initiated flight earlier than *C. eriphyle* ($F_{(1,220)}= 4.21$, $p=0.04$). All butterflies initiated later on cooler mornings ($F_{(1,220)}= 373.64$, $p<0.05$). *C. meadii* also initiated flight at cooler model temperatures than *C. eriphyle* ($F_{(1,220)}= 4.64$, $p=0.03$) regardless of initial morning air temperatures ($F_{(1,220)}= 5.54$, $p=0.07$), and there was no interaction between species and initial morning temperature ($F_{(1,220)}= 1.95$, $p=0.16$). *C. meadii* initiates flight earlier and at slightly lower body temperatures than *C. eriphyle*.

Discussion

Thermal limits on activity and fitness

The duration of time available for activity is a central determinant of survival and reproduction in many ectotherms, and thermal limits on activity time have been widely reported (Adolph & Porter, 1993b, Sears, 2005). The temperature dependence of insect flight may have greater fitness implications because there is a minimum body temperature threshold for active flight to occur, and this threshold can be well above mean ambient environmental temperatures. In butterflies, flight is essential for key fitness-related activities including predator-avoidance,

courtship, mating, nectar-feeding and egg-laying. The importance of flight is intensified by the short adult life span of *Colias* in the field (6-10 days Watt, 1977). For *Colias* at higher elevations, the average time available for flight activity can be less than 3 hours per day (Kingsolver, 1983a, Kingsolver, 1983c). As a result, flight time can strongly limit lifetime reproductive success for *Colias* females, especially at elevations above 2.5km (Kingsolver, 1983a, Ellers & Boggs, 2004b).

In *Colias* and many other ectotherms, populations and species are locally adapted to enable activity in different environmental conditions. Previous work done with *C. eriphyle* along an elevation gradient showed *C. eriphyle* from higher elevations are able to initiate flight earlier than those from lower elevations, due to differences in wing melanin among populations (Ellers & Boggs, 2004b). These effects are even more striking when differences between species are considered. Specifically, our reciprocal transplants with *C. eriphyle* and *C. meadii* at high elevation (3.6km) reveal that less than 10% of *C. eriphyle* are able to initiate flight at all under these cool environmental conditions, in contrast to resident *C. meadii* (91%). Failure to fly not only reduces potential mating and reproductive success, but can have immediate fitness consequences: individuals in our field experiments that were not able to achieve flight were often subject to predation by ants and wasps (MacLean, personal observation). For montane and alpine *Colias*, low environmental temperatures put a premium on achieving flight at cooler temperatures to maximize the time available for activity. In both experiments, *C. meadii* initiates flight at cooler air temperatures and a higher proportion at high elevation sites relative to low elevation sites demonstrating local adaptation to their high elevation environment. These results affirm the importance of local adaptation for flight activity and fitness in this system. As

discussed below, our experiments demonstrate that both physiological and morphological differences among species contribute to this local adaptation.

Physiological determinants of performance

Numerous studies have documented physiological differences in thermal performance curves for ectotherms from different climatic regions and thermal environments. For example, differences in optimal temperatures and thermal breadth along latitudinal gradients have been reported for lizards (Vanberkum, 1986, Vanhooydonck & Van Damme, 2003), frogs (Johnalder et al., 1988, Navas, 1997), and insects (Frazier et al 2006; Deutsch et al 2009). Similarly, latitudinal gradients in critical lower thermal limits (T_{\min}) occur in multiple groups of ectotherms (Diamond et al., 2012a, Andrew et al., 2013, Sunday et al., 2011). However, most of these data and patterns are based on laboratory measurements of performance. Using these laboratory estimates to determine performance and activity in field conditions can be problematic, especially for lower and upper thermal limits (Kingsolver et al., 2013, Kearney et al., 2009b). In addition, studies predicting activity duration for ectotherms across large geographic scales often use data on thermal optima to predict thermal performance breadth (Adolph & Porter, 1993b, Buckley & Kingsolver, 2012a, Kearney & Porter, 2009), adding uncertainty about predictions for lower and upper thermal limits (Kingsolver et al., 2013). To avoid these issues, we used field experiments to determine the lower thermal limits for flight initiation between *Colias* populations and species

Previous studies with *Colias* using tethered butterflies in the field show similar thermal optima for flight across species between 34-38°C and rarely occurred at body temperatures below 30°C (Watt, 1969). Observations of free-flying *Colias* indicate that flight activity increases when basking body temperatures are above 30°C, but there is some flight activity for

C. meadii at higher elevations occurring at basking temperatures of 28-30°C (Kingsolver 1983b). However, these observations were not designed to assess lower thermal limits for flight. Our common garden experiments yield two important results about thermal biology of *Colias*. First, the average body temperature for flight initiation for *Colias* was between 24-26°C in our experiments, 4°C lower than observed in previous studies. This highlights the importance of measuring temperatures for initiating activity in addition to thermal optima.

Second, and in contrast to previous studies, our results also indicate a small but significant difference in the thermal sensitivity of flight initiation between high elevation *C. meadii* and lower elevation *C. meadii*. Our common garden experiments (in the absence of direct solar radiation) show that *C. meadii* initiate flight at lower body temperatures (0.8°C on average) than *C. eriphyle*. High elevation species occupy cool environments and have reduced their lower thermal limits for flight initiation to increase the time available for flight.

These differences in lower thermal limits for flight may seem modest. Recent biophysical and demographic modeling in this system show that the assumption of lower thermal limits for flight can have major effects on predictions of activity time, reproduction, and fitness at high elevation sites (Buckley & Kingsolver, 2012a). For example, using the previous information on thermal limits for *Colias*, these models predicted *C. meadii* at alpine sites in Colorado (elevation 3.5km) would be unable to maintain populations at these sites (i.e the predicted mean fitness was below replacement rate). Incorporating the values for lower thermal limits reported here into these models increases the predicted mean fitness for *C. meadii* at these high elevation sites (Buckley & Kingsolver, 2012a). This will allow more realistic assessment of the consequences of recent and future climate change for geographical range and evolutionary responses (Buckley & Kingsolver, 2012b).

Morphological and physiological contributions to local adaptation

Although many studies have documented local adaptation to climate via differences in the thermal sensitivity of performance (Hertz et al., 1983, Navas, 1996, Angilletta Jr et al., 2002, Stevenson, 1985) or morphological differences (Berry & Willmer, 1986, Ellers & Boggs, 2004b), few studies have explored these two mechanisms simultaneously (Frazier et al., 2008). To our knowledge, no studies have attempted to quantify their relative contributions.

Our reciprocal transplant experiments, performed in the presence of direction solar radiation, allow us to distinguish the contributions of morphological and physiological mechanisms of local climatic adaptation of *Colias* species along the elevation gradient. The effects on flight initiation are greatest for transplants between the lighter low-elevation species and the darker high-elevation species (as seen in Figure 3). For example at the highest elevation site (3.6km), greater than 90% of the resident *C. meadii* are able to initiate flight, whereas less than 10% of low-elevation (1.6km) *C. eriphyle* ever achieved flight in our experiments (Fig 3c). Moreover, the high-elevation individuals actually initiated at a higher proportion at the highest elevation site, relative to their performance at the lower elevation sites, creating an interaction. For individuals that did initiate flight, initiation occurred earlier (on average 35 minutes) for *C. meadii* than for *C. eriphyle* at all sites (Fig 3d). These results confirm the findings of previous work in documenting the importance of morphological differences in wing melanin and thoracic insulation for local adaptation in *Colias* (Watt, 1968a, Kingsolver, 1983c, Ellers & Boggs, 2004b).

By combining the results of our common garden and reciprocal transplant experiments, we can quantify the relative contributions of these two mechanisms to differences in flight initiation at the low elevation site. Differences in flight initiation reflect both physiological and

morphological differences in the reciprocal transplants (direct solar radiation present), but only physiological differences in thermal sensitivity in the common garden experiment (direct solar radiation absent). At the low elevation site, the higher elevation species initiates flight 5 minutes earlier without direct solar radiation and 35 minutes earlier with direct solar radiation. Thus both differences in thermal sensitivity (15%) and in morphology (85%) contributed to the differences in flight performance between the two species in the field. The morphological differences that provide high elevation butterflies with darker wings, thicker thorax setae, and ultimately higher body temperatures help butterflies achieve faster flight and ultimately greater fitness. However, because of environmental variability and the short window during which these animals are adults, it seems that the high elevation butterflies also have to adapt, to a lesser degree, on a physiological level. Thus both morphology and thermal sensitivity contribute to local adaptation.

Considering the contributions of local adaptation in morphology, physiology, and behavior may be crucial to accurately forecasting responses to future climates. The type and strength of selection imposed by rapid climate change is likely to vary among populations (Hoffmann & Sgrò, 2011). Populations can respond to climate change by shifting their distribution, evolving higher thermal tolerance, or adapting to greater environmental variability and it is likely that successful populations will use a combination of these tactics (Parmesan, 2006, Bradshaw & Holzapfel, 2006). Differences in the strategies by which populations and species respond may account for the individualistic abundance, phenology, and distribution shifts observed in response to recent climate change (Williams et al., 2007). Our study highlights the importance of measuring aspects of local adaptation in phenotypes in the field that can be directly translated into fitness and demography in models such as an ecological thermal minimum in addition to the optima of performance curves.

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Figure Legends

Figure 1: Results from the common garden between two populations of *C. eriphyle*. a) The higher elevation population exhibits a higher probability of initiation (mean \pm 95% confidence intervals) b) Both populations initiate at a similar time (decimal hours, means \pm se). c) Body temperatures at initiation (means \pm se) are warmer when air temperatures are warmer. The flight metrics are plotted as a function of air temperature (means \pm se). d) We depict the distributions of initiation temperatures binned by 0.5°C for the two populations.

Figure 2: Results from the common garden between *C. eriphyle* (open symbols) and *C. meadii* (closed symbols). a) The two species do not differ significantly in their probability of initiation (mean \pm 95% confidence intervals). b) Cooler initial temperatures lead to later initiation times (h, means \pm se) in both species. c) On warmer mornings, *C. meadii* are initiating at cooler temperatures (C, means \pm se). d) We depict the distributions of initiation temperatures binned by 0.5°C for the two species.

Figure 3: Results from reciprocal transplants. We depict the probability of initiation (left column, mean \pm se) and the time at initiation (right column, hour, mean \pm se) for each population as a function of the elevation (m) of the observation site. (h). a &b) *C. meadii* from the two sub-populations at Mesa Seco show little difference in probability of initiation or in time of initiation between sites. c&d) *C. eriphyle* have a lower probability of initiation relative to *C. meadii* at the high elevation sites and *C. meadii* are initiating 35 minutes earlier than *C. eriphyle*.

Figure 4: Body temperatures of *C. eriphyle* and *C. meadii* at Mesa Seco are predicted to be elevated above air temperatures, but do not differ significantly between species when we account for morphological difference. The larger symbol depicts the mean and standard error for both the air temperature and the predicted body temperature of individuals of each species.

Figure 1

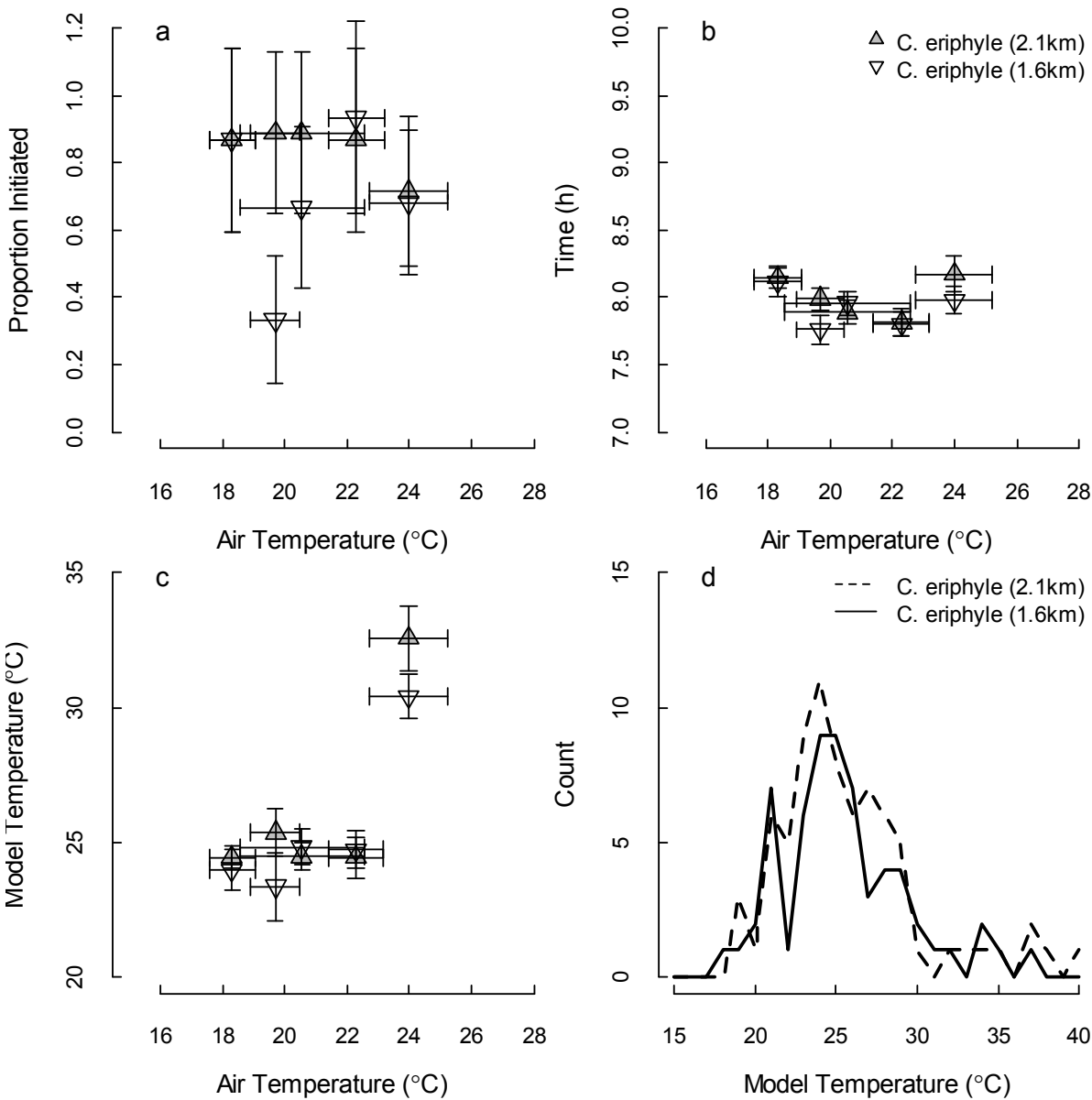


Figure 2

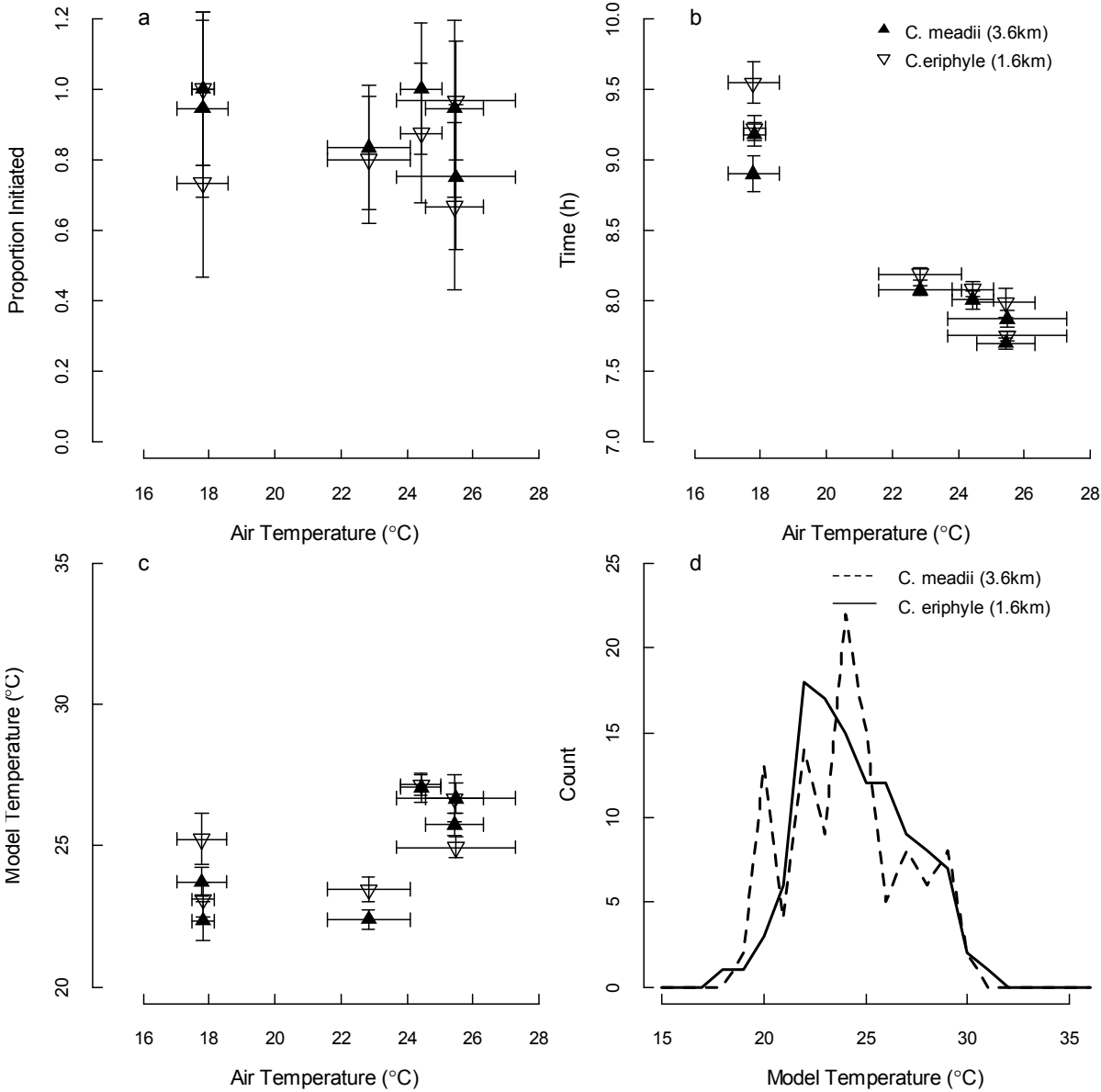


Figure 3

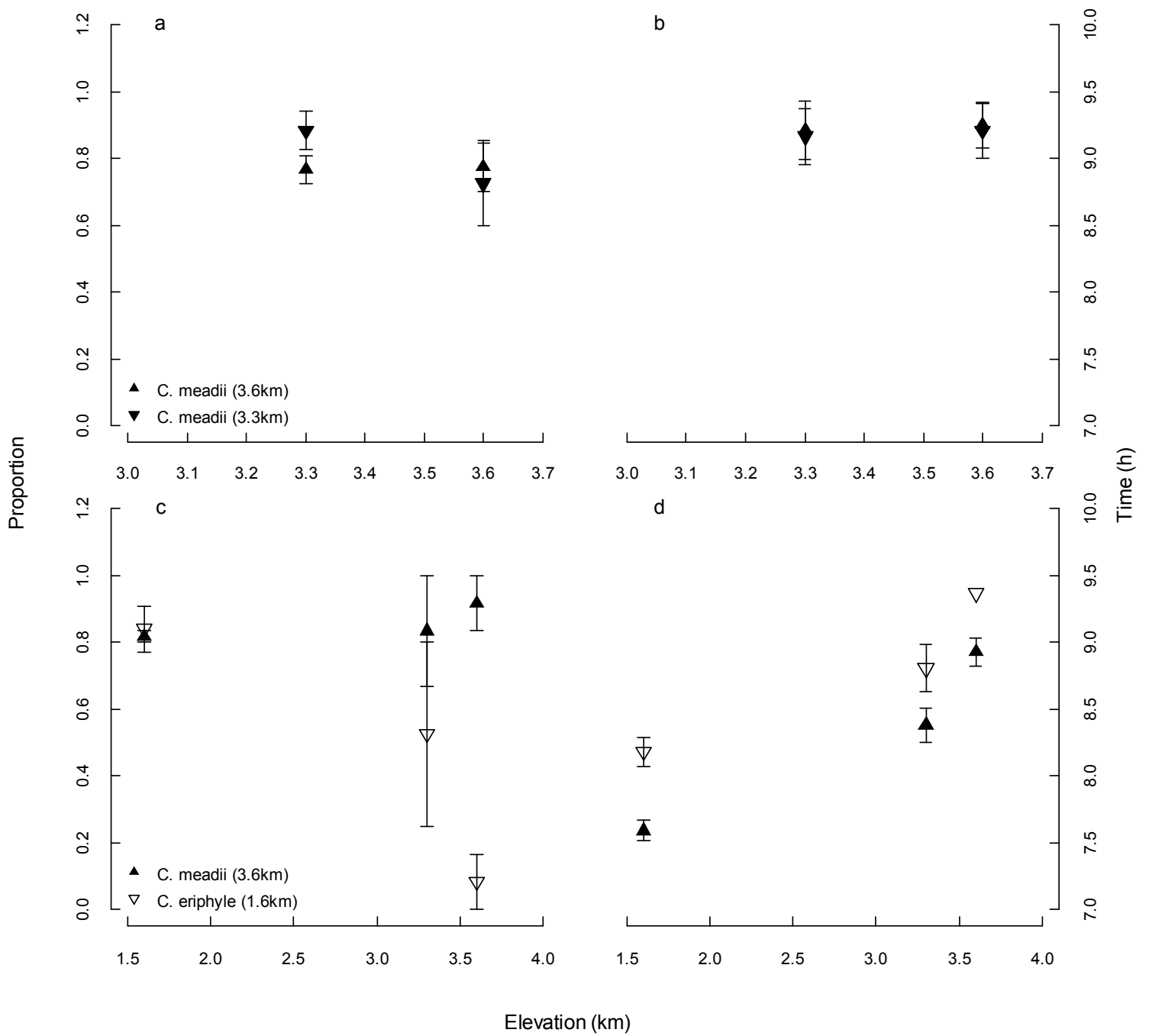
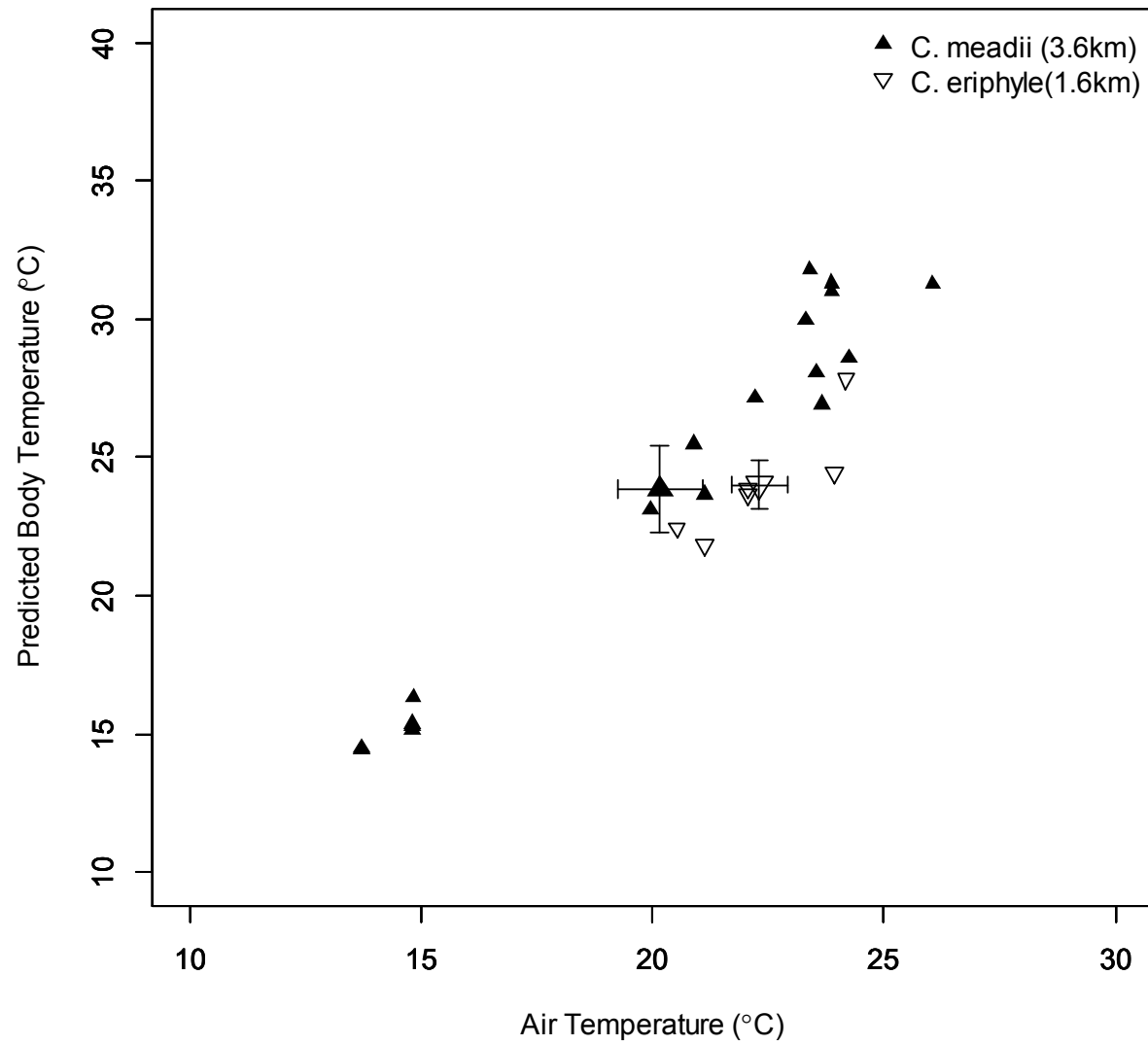


Figure 4



CHAPTER THREE: GEOGRAPHIC DIVERGENCE IN UPPER THERMAL LIMITS ACROSS INSECT LIFE STAGES - DOES BEHAVIOR MATTER?

Summary

Insects with complex life cycles vary in size, mobility, and thermal ecology across life stages. The lack of thermoregulatory behaviors in sessile life stages can lead to evolved difference in thermal sensitivities. Building on the work done on the thermal ecology of *Colias* butterflies across life stages, we examine potential local adaptation to high temperatures in sessile eggs and thermoregulating adults. *Colias* adults exhibit differences in morphology (wing melanin and thoracic setal length) along spatial gradients, whereas eggs and larvae are morphologically indistinguishable. Here we compare the physiological tolerance of *Colias eriphyle* eggs from two elevations and the physiological tolerance and the behavioral thermoregulation of adults in *C. eriphyle* populations from two elevations and *Colias meadii* from high elevation. We find that hatching success and egg development time of *C. eriphyle* eggs did not differ with the elevation of origin. Egg survival declined in response to heat-shocks above 38-40°C and development time was shortest at intermediate heat-shock temperatures of 33-38°C. Laboratory experiments showed that *Colias* adults from higher elevation sites had higher upper thermal limits (survival after heat shock) than those from lower elevations. 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*. This suggests that the limited capacity for movement or behavior by eggs did not lead

to the evolution of fixed physiological differences in this life stage but thermoregulatory behaviors did not extinguish physiological differences in adults.

Introduction

Many ectotherms adapt to local climatic conditions through changes in their physiological 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 strong association with these climatic gradients, and are largely uncoupled with lower thermal limits (Sunday et al., 2011, Addo-Bediako et al., 2000, Chown, 2001, Diamond et al., 2012b). The conservation of upper thermal limits across latitude and elevation suggest evolutionary constraints or that behavioral thermoregulation may dampen selection for high-temperature tolerance (Huey et al., 2003, Buckley et al., 2015). The efficacy of thermoregulatory behavior is limited by available microhabitats (Sunday et al., 2014).

Species or life stages that lack the ability to behaviorally mediate body temperature are more likely to adapt physiologically to local climatic conditions (Hertz, 1981, Krebs & Loeschcke, 1995). Insects with complex life cycles 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 from their mobile life stages (Kingsolver et al., 2011, Loeschcke et al., 1997). Understanding the determinants and consequences of upper thermal limits across life stages is important for understanding the biological responses to recent and future climate changes (Deutsch et al., 2008, Kingsolver et al., 2013).

The *Colias* butterfly system provides an excellent system for exploring differences in upper thermal limits because of their complex life history and known behavioral thermoregulation as adults. There is little variation in morphology of the eggs or larvae of *Colias* species or populations. In southwestern Colorado, *Colias* utilize a mixture of weedy legume species as host plants, including clover (*Trifolium gymnocarpon* and *T. dasyphyllum*), vetch (*Vicia Americana*, *Vicia villosa*, and *Astragalus alpinus*), and alfalfa (*Medicago sativa*) (Higgins et al., 2014, Tabashnik, 1983). Their small (0.13 mm^3) eggs are deposited singly on host leaves (García-Barros, 2000) and are, therefore, subject to leaf microclimatic conditions (Pincebourde & Woods, 2012). *Colias* larvae are cryptically colored (green) and show a limited capacity for behavioral thermoregulation, although at stressfully high body temperatures they will drop off of the host plant to the ground (Sherman, 1973). Populations and species of *Colias* larvae differ significantly in their thermal sensitivity for feeding (Sherman, 1973, Higgins et al., 2014). For example, *C. eriphyle* larvae from different elevations in western Colorado differ in their optimal and maximum feeding temperatures, suggesting that local adaptation of upper thermal limits can occur over relatively small geographic distances (Higgins et al., 2014). Little is known about geographic variation in the thermal tolerances of eggs for *Colias* and Lepidoptera (but see Shirai, 2000, Potter et al., 2011).

In contrast to eggs, the body temperatures of *Colias* adults are strongly influenced by both morphological traits and thermoregulatory behavior (Watt, 1968a, Kingsolver, 1983c). *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 wing parallel to the sun, they can maintain or even lower their body temperature (Rawlins, 1980, Watt, 1968a). While there is local

adaptation in thermally important morphological traits across their range, *Colias* species share similar thermal optima for flight of ~35°C (Ellers & Boggs, 2004b, Watt, 1968a). Recent studies indicate elevation differences in lower thermal limits (H. MacLean, unpublished) but differences in upper thermal limits in adults have not been explored.

Because *Colias* eggs lack the ability to thermoregulate and are subject to the thermal ecology of their host plant, we expect differences in upper thermal limits for eggs along elevation and climatic gradients. Here we compare the physiological tolerance of *C. eriphyle* eggs from two elevations. In contrast, we expect little difference along the elevation gradient in upper thermal limits for *Colias* adults because of their capacity for movement and behavioral thermoregulation. To test this, we compare both the physiological tolerance and behavioral thermoregulation along an elevation gradient in two populations of *C. eriphyle* as well as two populations of *C. meadii* from similar sub-alpine alpine habitats

Materials and Methods

Study Populations

We studied two *Colias* species found in western North America. *Colias eriphyle* is widely distributed across a range of elevations (1,400-2,900m) (Springer & Boggs, 1986). *Colias meadii* is confined to subalpine and alpine meadows above 2,500m in the Rocky Mountains (Watt, 1968a). Adults of these species 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 Co., CO (N38.62, W108.02, 1,600m) and one from a middle elevation Gunnison, Gunnison Co., CO (N38.56, W106.94, 2,300m). We studied two populations of *C. meadii* from higher elevations that are approximately 135 km apart, at Cumberland Pass, Gunnison Co. CO

(N38.41, W106. 29, 3,600m) and Mesa Seco in Hinsdale Co., CO (N37.59, W107.13, 3,300m-3,700m).

Laboratory assays of heat tolerance

To explore physiological heat tolerance of eggs in the two populations of *C. eriphyle*, we exposed freshly laid eggs to a ramping heat-shock. Following the approach of Potter et al (2009), we placed eggs on a thermal gradient for 24 hours with heat-shock (maximum) temperatures for the ramping treatments ranging from 26-48°C. Adult females were shipped overnight from Colorado back to the laboratory at the University of North Carolina, Chapel Hill within 24 hours of capture. Females were placed in oviposition cages (1m³) with a pot of vetch (*V. villosa*) and a dish of 10% honey-water. Each day, the eggs from each female were liberated from their plant and loaded into one lane of a 96 well plexiglass plate. From here, they were transferred onto an aluminum plate (described in Woods & Bonnecaze, 2006) with twelve evenly-spaced columns with eight rows each. The thermal gradient was established by connecting two circulating water baths to either end of the plate with one side maintained at 26°C and the other side to ramping up to 48°C over 4 hours and holding the maximum temperature for 2 hours before ramping back down to 26°C and holding in a 24 hour period. 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. The camera took a single snapshot every five minutes over the next 96 hours. The binary hatched/unhatched data was recorded once each day for five days and the precise time of hatching was determined with the data captured by the cameras. We tried to measure thermal limits of *C. meadii* eggs but were unable to obtain a sufficient number of viable eggs from females to do this.

To explore physiological differences in heat tolerance in adults among populations and species, we exposed 20 freshly caught males from each of the four populations to one of five temperatures (25°C, 40°C, 42°C, 45°C, and 47°C) for 60 minutes. We recorded survival both immediately after the heat exposure and after 24 hours. 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 plastic cup furnished with a wooden craft stick and covered with netting. Butterflies were allowed to acclimate for 1-4 hours before being randomly placed in a temperature chamber (TriTech Research DigiTherm DTM-MP-38). Because we only had two chambers, the experimental temperatures were set randomly and assayed at different times and on different 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 the low elevation site to evaluate how exposure to high temperatures in the field 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, scored for wing wear before being returned to 3°C until the start of the experiment. Individuals were placed in a closed tent, which warmed due to ambient conditions, until 10am. Each individual was then transferred to a 0.3mx 0.6m cage made of SeeVue® window screening in the city of Montrose, Montrose Co., CO (N38.46, W107.88, 1, 700m). We observed behavior and survival over two hours. We classified the behavior of each individual every two minutes. Flying consisted of a flight bout lasting more than ten seconds 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: $90^{\circ} \pm 10^{\circ}$ indicated basking and $180^{\circ} \pm 10^{\circ}$ indicated shade seeking. For each trial, 7-12 transplanted males were compared to 7-12 *C. eriphyle* males from the Olathe population. We repeated the trial three times for each of the Mesa Seco and Cumberland Pass *C. meadii* populations and five times for the Gunnison *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 air and soil temperatures (Pace PT-907 thermistors) at 10 cm in the shade and at 0.5 cm below the soil surface, respectively. 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 & Moffat, 1982). The physical models were validated using fresh butterflies with a thermocouple inserted into their thorax. Measurements at 10s were averaged to minute intervals using a Pace Scientific X5-SE logger. All field trials occurred during July 2012.

Statistical Analysis

All statistical analyses were performed in R (v. 2.15.3) (RCoreTeam, 2013) using the survival and nlme libraries (Grambsch, 2014). For the egg survival and development time experiment, we first modeled hatching as a binomial response using a nlme model with population and heat-shock (maximum) temperature as a continuous first and second order variable and with 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 as a first and second order variable continuous explanatory variable with the day of trial as a random intercept.

For the adult survival experiment, we modeled survival following exposure as a binomial response. We used glm (Pinheiro, 2014) with species, sex, heat-shock temperature as continuous

variables, 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 who exhibited noticeable wing wear but whose wings were not yet fraying or tearing resulting 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 for the solar avoidance and overheating to determine if model temperature could account for differences in behavior.

Results

Eggs were most likely to hatch at intermediate heat-shock temperatures (optimum between 30-38°C), regardless of population. Both low and high elevation populations hatched at similar rates (Figure 1 A). Heat-shock temperature, or the highest temperature reached during the ramp, had strong first- ($d_{(1,395)}=71.40$, $p<0.001$) and second- ($d_{(1,393)}=42.84$, $p<0.001$) order effects on hatching success. Not only was there no main effect of population ($d_{(1,394)}=0.15$, $p=0.69$), there was no interactive effect of population with either temperature term ($d_{(1,392)}=1.48$, $p=0.22$; $d_{(1,390)}=0.373$, $p=0.54$). Most eggs are able to withstand substantial thermal stress before succumbing (between 46-47°C). High heat-shock temperatures did lead to a longer time to hatch, which did not differ among populations ($F_{(1,299)}=0.12$, $p=0.72$). Hatching occurred significantly faster at intermediate heat-shock temperatures, (Fig 1 B, temperature: $F_{(1,299)}=9.41$,

$p=0.002$; temperature²: $F_{(1,299)}=x$, $p<0.001$). Here again, there were no interactive effects of population with heat-shock temperature (first-order $F_{(1,299)}=0.30$, $p=0.58$; second-order $F_{(1,299)}=1.05$, $p=0.30$).

As in eggs, heat-shock temperatures above 40°C reduced adult survival. Adult survival immediately following the heat-shock treatment declined significantly with temperature ($d_{(1,504)}=87.73$, $p<<0.001$) regardless of population (Fig. 2). However, a greater proportion of *C. meadii* adults survived heat-shock temperatures above 42°C relative to *C. eriphyle* ($d_{(1,503)}=3.861$, $p=0.04$) (Fig 2) indicating that *C. meadii* has a greater thermal tolerance than either *C. eriphyle* population.

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 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 $X^2_1=1.33$, $p=0.249$) or overheating behaviors (Wald $X^2_1=1.13$, $p=0.288$). In contrast *C. meadii* adults from both populations, initiated shade-seeking (Wald $X^2_1=10.95$, $p=0.009$) and overheating behaviors (Wald $X^2_1=8.73$, $p=0.003$) 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 $X^2_2=69.52$, $p<<0.001$) (Fig 4 A) and overheating (Wald $X^2_2=43.75$, $p<<0.001$) (Fig 4 B).

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 temperatures (Sunday et al., 2014, Diamond et al., 2012b); whereas differences in behavior and mobility throughout ontogeny can lead to the evolution of different thermal sensitivities at different life stages (Kingsolver et al., 2011). While there are studies that look at thermal tolerances throughout ontogeny (reviewed in Bowler & Terblanche, 2008) and studies that look at geographic variation in thermal tolerances (reviewed in Sunday et al., 2011), there are far fewer studies that quantify local adaptation of thermal tolerances across populations and across life stages (but see Hammond & Hofmann, 2010, Krebs & Loeschcke, 1995). Here we compared upper thermal limits in sessile eggs and thermoregulating adults of *Colias* butterflies across an elevation gradient. We expected that eggs would demonstrate local adaptation similar to that observed in the larvae because eggs cannot thermoregulate and because mean air temperature varies between the two populations (Higgins et al., 2014). In contrast, we expected to see no difference in the upper thermal limits of adults between two lowland and sup-alpine species of *Colias* in southwestern Colorado because adults are able to employ thermoregulatory behavior and exhibit a highly conserved thermal optima.

Despite differences in ambient temperatures between populations, we observed no difference in the thermal tolerances of *C. eriphyle* eggs. The mean air temperature in Olathe, CO is approximately 5°C warmer on average than that of Gunnison, CO. These two populations demonstrate a dramatic difference in thermal sensitivity of larval feeding rates, with the Gunnison population feeding more at higher temperatures relative to Olathe (Higgins et al 2014).

We offer two alternative hypotheses to explain our results. First, the 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 & Woods, 2012). Both populations reside in grassy irrigated meadows with microclimate heterogeneity. The variation in available microclimatic leaf temperatures (-5°C to $+15^{\circ}\text{C}$ from ambient) is greater than the temperature differences between habitats. 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). Alternatively, selection for increased egg temperature may be up against an evolutionary constraint. Strong selection imposed by abnormally warm years may have driven both populations to the highest possible level given their standing genetic variation, as is suggested in other ectotherm species (Angilletta et al., 2013). Without further data, it is difficult to say which of these is more likely.

Thermoregulatory behavior is important in adult *Colias*. As thermoregulatory behavior leads to the conservation of upper thermal limits (Sunday et al., 2014, Buckley et al., 2015), we expected to see no difference across populations or species of *Colias* butterflies. 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 *C. eurytheme* (Kingsolver 1983b). What we found is rather surprising: High-elevation *C. meadii* are better able to survive body temperatures above 42°C than either population of *C. eriphyle* from lower elevations. We offer two hypotheses to explain this pattern.

First, although high-elevation sites experience relatively lower temperatures on average, they also experience greater temperature variability and increased solar radiation. The efficacy of thermoregulatory behavior is limited by the microhabitat in the environments, and alpine vegetation is shorter than 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). We observed that high-elevation *C. meadii* initiates shade seeking behavior and overheating sooner than the low elevation *C. eriphyle*. As a result, *C. meadii* realized decreased activity time in high temperature environments relative to *C. eriphyle*.

Alternatively, this might be explained by life history strategy. 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. Surviving the winter as a diapausing larva can be difficult, with an estimated 98% mortality rate for an intermediate elevation species (Hayes, 1980). Moreover, it has been shown in a number of ectotherms that there is a life history trade-off between adult survival and reproduction under stressful conditions (Stearns, 1992); for example, in adverse climates adults may enter a reproductive diapause and resorb resources from reproductive tracts in order to prolong their life to survive until climate becomes more favorable. For *C. eriphyle*, if an adult has mated in the first flight, chances are high that their offspring will emerge for a second flight and produce larvae that will overwinter. *Colias meadii* only have one opportunity to reproduce as much as possible to secure offspring that will survive the winter diapause. Thus, there may be a greater fitness advantage for *C. meadii* who are able to survive high temperatures over short periods than there would be for *C. eriphyle*.

This study highlights how available thermal environments may limit or drive the evolution of upper thermal limits in insects, particularly ones with complex 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 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 (Williams et al., 2007, Field, 2014). 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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Figure Legends

Figure 1: A) Hatching probability and B) hatching time for both populations of *C. eriphyle* as a function of maximum heat-shock temperature over a 24 hour period. The filled triangle symbols with the solid line represent the higher elevation population from Gunnison, CO (2.3km) and the upside-down open triangle symbol with the dashed line represents the lower elevation population from Olathe, CO (1.6km). While intermediate temperatures increase hatching probability (panel A), they decrease hatching time (panel B) regardless of population of origin.

Figure 2: Proportion of adults surviving a 1 hour heat-shock as a function of heat-shock temperature. All individuals survived 1 hour at 25°C (not shown). A greater proportion of adults from the higher elevation populations survived exposure to hotter temperatures. The filled triangle symbols with the solid line represent the higher elevation population of *C. eriphyle* from Gunnison, CO (2.3km) and the upside-down open triangle symbol with the dashed line represents the lower elevation population of *C. eriphyle* from Olathe, CO (1.6km). The two populations of *C. meadii* are represented circle symbols, the filled circle with the solid line represents the Cumberland Pass population and the open circle with the dashed line represents the Mesa Seco population.

Figure 3: A representative day from the common garden trial for *C. meadii* from Cumberland Pass (dashed line) and *C. eriphyle* from Olathe (solid line) occurring on July 23, 2012. A) Model temperature and the proportion of each population (B) shade seeking, (C) overheating, and (D) dead as a function of time.

Figure 4: A representative common garden trial for *C. meadii* from Cumberland Pass (filled circle) and *C. eriphyle* from Olathe (open upside-down triangle) occurring on July 23, 2012. Plots illustrate (A) the proportion of individuals engaged in shade seeking and (B) the proportion of individuals engaged in overheating as a function of physical model temperature.

Figure 1

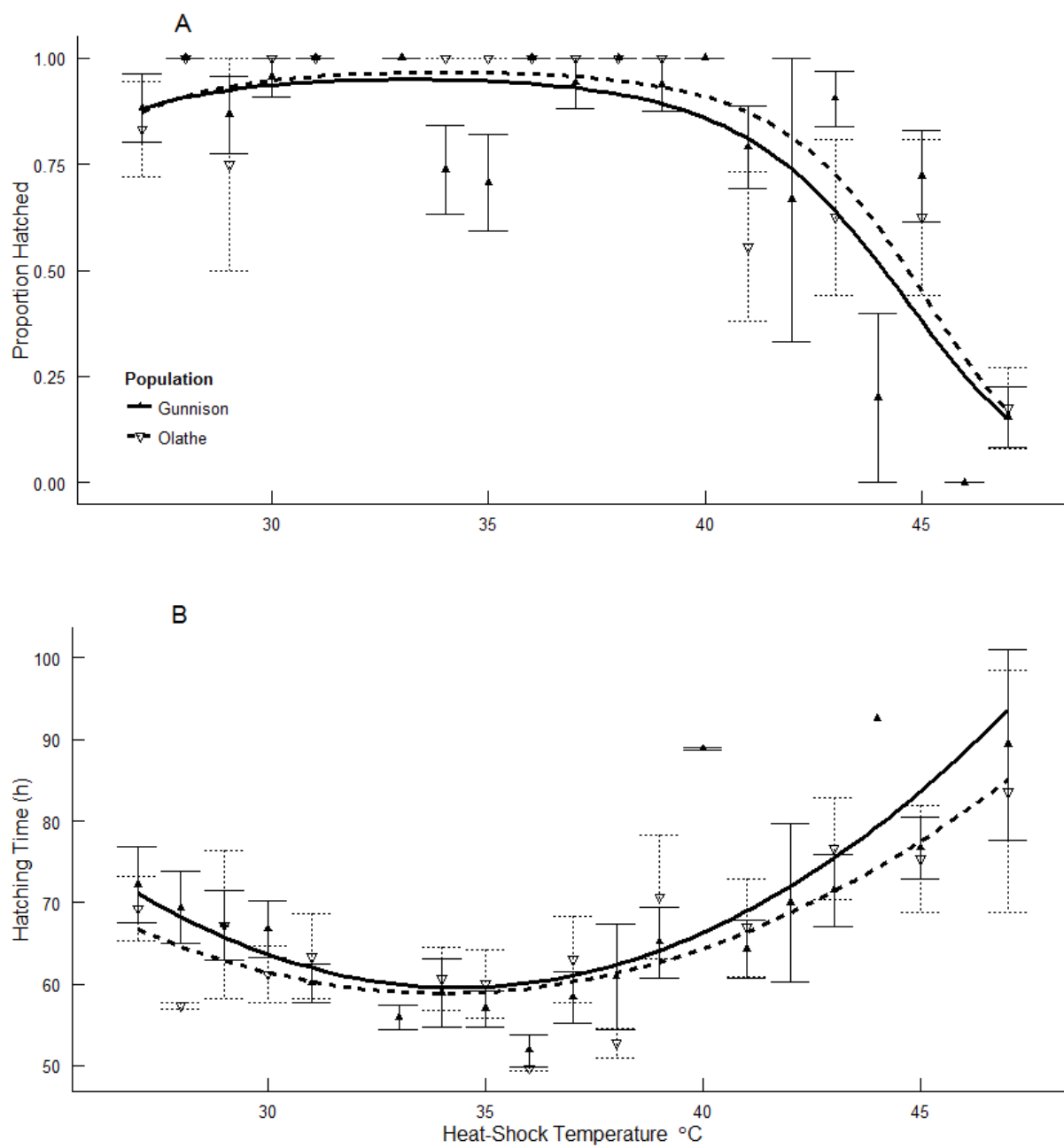


Figure 2

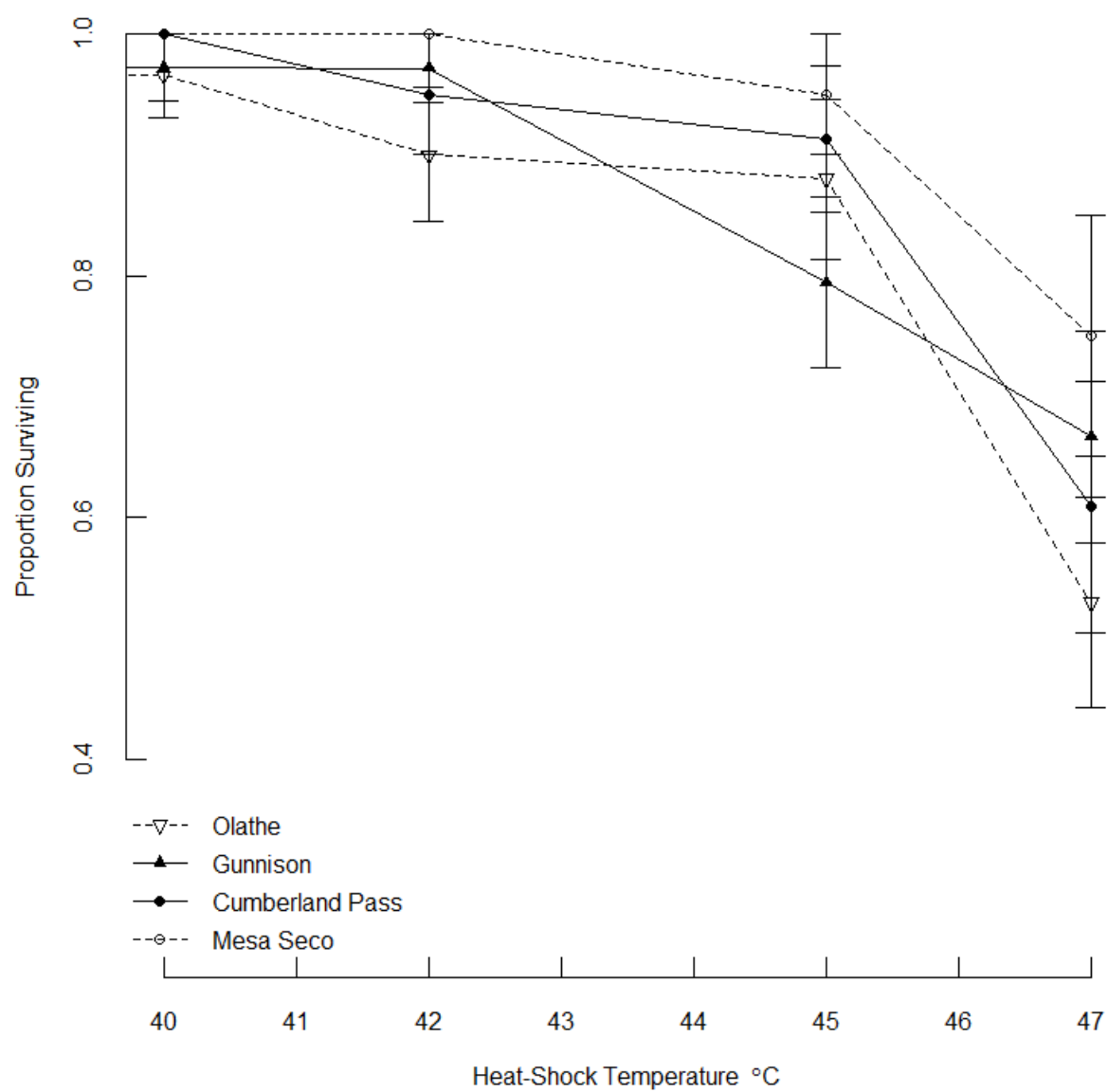


Figure 3

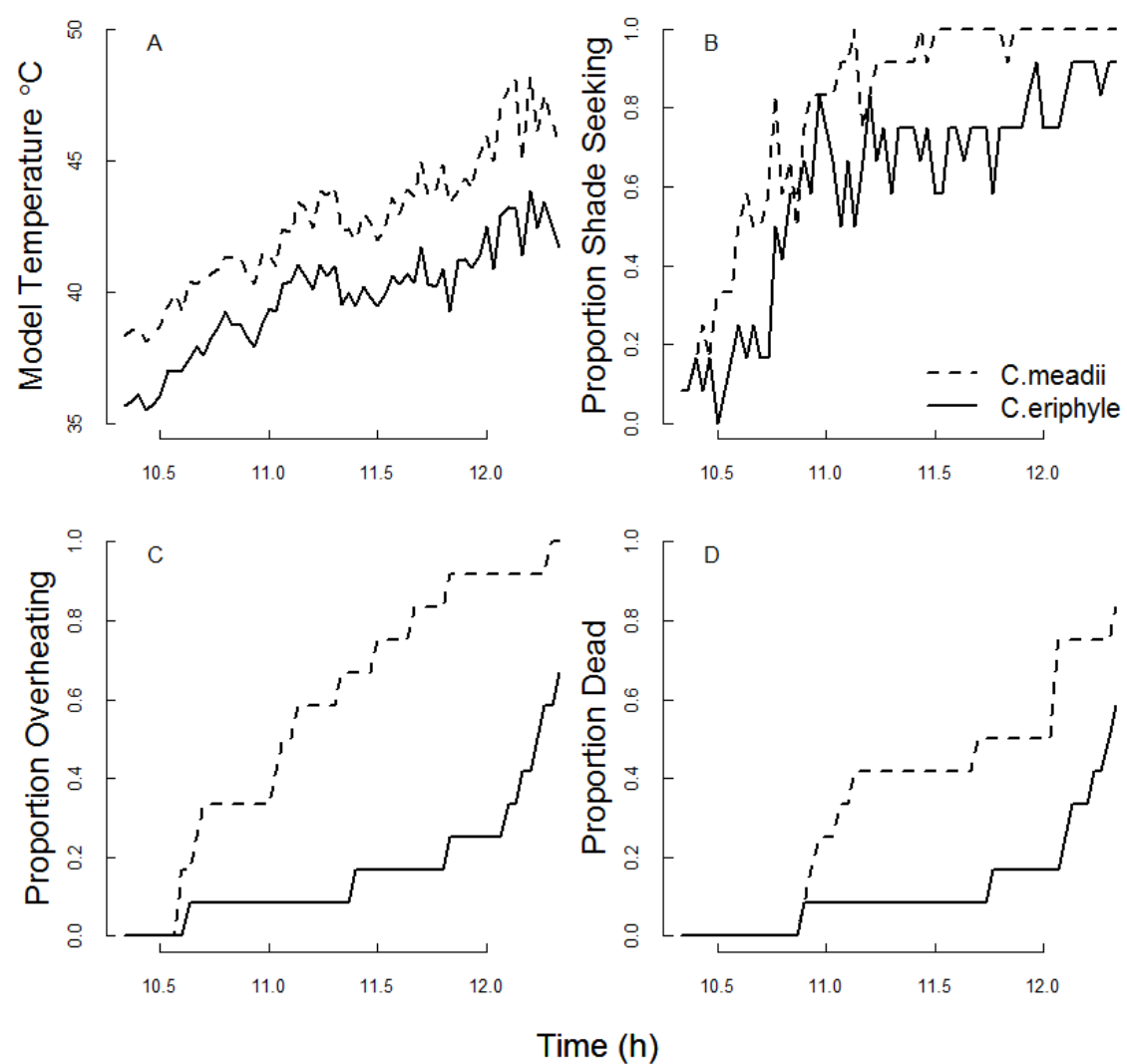
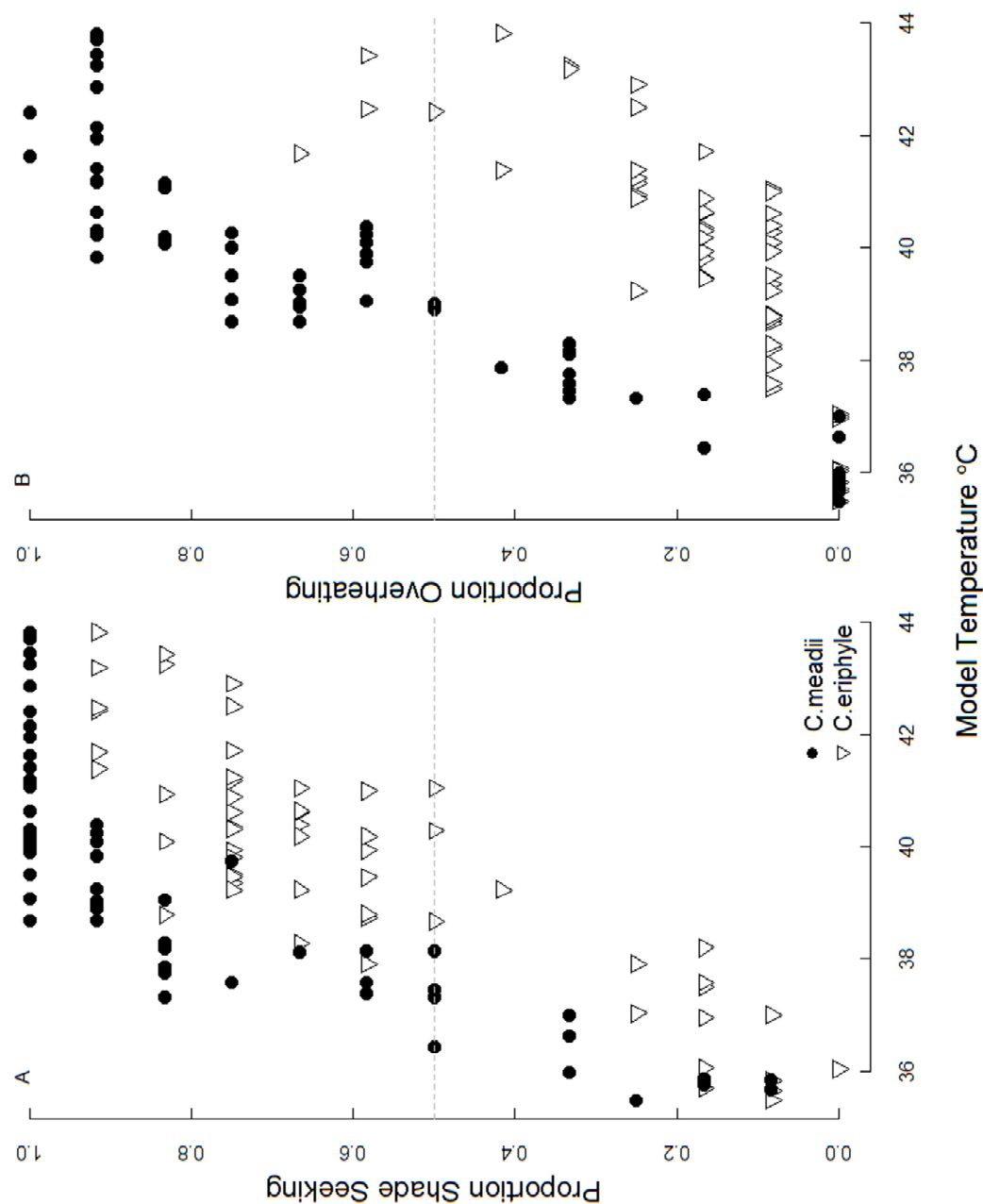


Figure 4



CHAPTER FOUR: HISTORICAL CHANGES IN THERMOREGULATORY TRAITS OF AN ALPINE BUTTERFLY IN RELATION TO CLIMATE CHANGE

Summary

Have natural populations responded adaptively to recent climate changes? Are these responses due to evolutionary changes or to phenotypic plasticity? We explore these questions by considering two key thermoregulatory traits-- ventral hindwing melanin, and thoracic setal length—in an alpine butterfly in the Rocky Mountains, *Colias meadii*. Past studies demonstrate adaptive differences in wing melanin and setal length along elevation and climatic gradients, as well as phenotypic plasticity in wing melanin in response to pupal temperatures in some *Colias* species. We predicted that climate warming in the southern Rocky Mountains in recent decades would lead to decreases in mean wing melanin and setal length, and we used historical collections of *C. meadii* from the last 60 years to quantify historical changes to test this prediction. Mean temperatures during the adult flight summer (July-August) have increased significantly during the past 60 years in this region. However, contrary to adaptive expectations, both wing melanin and setal length have significantly increased during this period. Our analyses suggest that this historical pattern is in part due to developmental plasticity: cooler temperatures during pupal development (June) were associated with significantly greater adult wing melanin. We propose that plasticity in this high-elevation species does not result in an adaptive response to climate variation and climate change, because temperatures during pupal development are poor predictors of thermal conditions during the adult flight season. Our results illustrate how

unpredictable variation in seasonal and annual climate can limit the adaptive plastic and evolutionary responses of populations to increasing temperatures.

Introduction

The ecological responses of organisms to recent climate change have been widely documented: they include changes in seasonal timing, shifts in geographic distributions, alterations in species interactions, and changes in population density or the likelihood of extinction (Parmesan, 2006, Diamond et al., 2011, Chen et al., 2011). The evidence of potentially adaptive responses is heartening. Recent studies have documented evolutionary changes in the timing of diapause (Bradshaw et al., 2000), of nesting (Visser et al., 1998) and of flowering (Anderson et al., 2012); in body size (Sheridan & Bickford, 2011); and in coloration relative to background matching (Galeotti et al., 2009, Mills et al., 2013). However, evidence for evolutionary or historical changes in traits relevant to thermal physiology is largely lacking (but see Higgins et al., 2014, Zeuss et al., 2014). Museum collections and other historical collections offer the opportunity to measure morphological traits over long time frames (Anderson et al., 2008), but these resources have not been used to study morphological traits that are thermally important.

As in many other regions, annual mean temperatures across the Rocky Mountains in the western United States have been increasing in recent decades (Coumou & Rahmstorf, 2012). Across Colorado, for example, mean air temperatures in July have increased about 0.5°C per decade for the past fifty years (<http://climatetrends.colostate.edu/>). Increases in mean temperatures and earlier snow melt have caused important ecological changes in many montane and alpine organisms, including plants (Inouye, 2008, Anderson et al., 2012), mammals (Ozgul et al., 2010), and insects (Boggs & Inouye, 2012, Nufio et al., 2010). Studies that have

documented these temporal changes in phenological timing, size, and body condition have not considered trends in thermoregulatory traits.

Colias butterflies are an important model for understanding how meteorological conditions, thermoregulatory behaviors, and morphological traits combine to determine patterns of body temperature, flight, and fitness in the field (Boggs et al., 2003, Buckley & Kingsolver, 2012a, Kingsolver, 1983c, Kingsolver & Moffat, 1982). *Colias* rely on flying for all major activities, including courtship, mating, feeding, and oviposition, but they are only able to fly over a narrow range of body temperatures (28°- 40°C) (Watt, 1968a, Kingsolver, 1983c). Thus their flight time is often limited by climate (Nielsen & Watt, 1998, Watt et al., 2003, Watt, 2004). Two key morphological traits are important for thermoregulation: 1) increased melanin on the posterior ventral hindwings allows the butterflies to absorb more solar radiation and thus achieve higher body temperatures during basking, and 2) longer setae on the thorax reduce convective heat loss and increase body temperatures above ambient temperature (Kingsolver, 1983b, Watt, 1968b). Wing melanin is both heritable (Ellers & Boggs, 2002) and phenotypically plastic in response to rearing conditions (Watt, 1969, Hoffman, 1972), and thus can contribute to local adaptation.

Colias performance and fitness is strongly temperature dependent. Cool ambient temperatures can limit activity and constrain reproductive success, in part because females lay eggs on plants singly rather than in clusters on leaves (Buckley & Kingsolver, 2012a, Kingsolver, 1983b). Warm ambient temperatures may result in overheating, which not only limits flight time, but can also directly reduce the number of viable eggs produced by each female (Kingsolver & Watt, 1983a). This suggests that ventral hind wing melanin and thorax setae length should be under selection from climate warming. Specifically, we predict that

increased mean daytime temperatures resulting from climate warming should either cause selection to favor reduced wing melanin and setal length or there will be evidence of phenotypic plasticity where in climatic variables explain the variability of thermoregulatory traits. In this study we measured morphological traits affecting thermoregulation in specimens of a sub-alpine and alpine butterfly, *Colias meadii*, from museum and private collections. We then related these changes in these morphological traits affecting thermoregulation to temperatures during the past 60 years (1953-2013) for the southern part of the range.

Materials and Methods

Colias meadii occurs in subalpine and alpine meadows above 2.5 km elevation throughout the Rocky Mountains, from northern New Mexico, USA to southern Alberta, Canada (Watt, 1968b). They are univoltine butterflies, and their flight season lasts four to six weeks. *Colias meadii* enter diapause and overwinter as third instar larvae. The subspecies *C. meadii meadii* is found largely in the southern part of the range at elevations between 3000 and 4000m (Region 1 in Appendix Fig 1). It is genetically distinct from more northern populations of the species in the Wyoming Basin (DeChaine & Martin, 2005). In this study we focus on *C. m. meadii* in the southern part of the range, where summer temperatures have increased significantly over the last 60 years (Buckley & Kingsolver, 2012a). Our findings for temporal and elevation trends in thermoregulatory traits across the region can be found in the appendix to this chapter. In this region, *C. m. meadii* individuals pupate in early to mid-June, and adult butterflies emerge in early July.

We processed specimen from museums in United States with the largest holdings of *C. meadii* from Colorado: the Yale Peabody and the McGuire Center for Lepidoptera and Biodiversity; as well as museums located in Colorado: the C.P. Gillette Museum of Arthropod

Diversity at Colorado State University, the University of Colorado Museum; and museums with holdings from time periods absent from other museums: the Smithsonian Museum of Natural History, the Milwaukee Public Museum, and the California Academy of Science. Because museum holdings of *C. m. meadii* are sparse for the past 25 years, we also solicited samples from private collectors via the Yale Lepidoptera listserv and measured samples from one private collector. Finally, we used specimens we collected in 2011 and 2012 from two sites in Colorado. Because the collections contained few females after 1990, we restricted our analyses to males (N=385, from 20 counties). Setae of some specimens were in poor condition and could not be used in the analysis of setal length (N=277).

Pinned specimens were measured by removing labels with forceps, transcribing the locality data, and then placing the head of the pin in a lump of modeling clay to expose the ventral hindwing. The specimen was then photographed through a 100-mm macro lens in RAW format with a Canon Rebel XSi mounted on a copy-stand. Each image included a black and white standard. Because the height of the animal on the pin was variable, we used auto focusing to allow for the clearest image. We measured the length of the thorax setal length with an ocular micrometer on a Wild M5 microscope measuring the longest setae between the first and second leg. All measurements were taken by MacLean and specimens were prepared for measurements in groups of five so that the precise metadata associated with each specimen at the time of measurement was not obvious to the researcher. Measurements were later converted into millimeters. Specimens were distributed randomly throughout collection boxes and thus were measured randomly within each collection.

We photographically assessed the degree of wing melanin on the posterior ventral hindwing. First, we selected a triangulated region between the eyespot, hind wing insertion, and the

wing margin (Stamberger, 2006, Ellers & Boggs, 2004a). Using a MatLab program (T. Hedrick, unpublished), we digitized the region of interest and the black and white standard for each sample. This provided a standardized, grey-level value between 0 and 1 for each specimen. In order to verify if our measure of grey-level was a good proxy for absorptivity, samples collected in the field 2012 and 2013 were photographed, and the absorption spectrum was measured from 350-1050nm in a spectrophotometer with an optical integrating sphere (FieldSpecPro FEFR 7501, ASD Inc) for the same wing region. Previous studies used absorption at 650nm as a measure of melanin and solar absorptivity for *Colias* wings (Watt, 1968a, Kingsolver, 1983b). Grey level and absorption at 650nm were highly correlated ($n=60$, $R^2 = 0.78$), confirming that grey level is an appropriate measure of melanin and solar absorptivity in this system.

To evaluate temporal changes in climate, we selected a representative NOAA Cooperative weather station at 3500m in Climax, CO (39.37N, 106.18W, , 2013). This weather station was selected for its relevant elevation, its central location among sampling sites, and the completeness of its climate records throughout the time period of interest. We chose to use temperature data from one, representative weather station because long-term data are lacking for most stations above 3km in Colorado and, while interpolated data sets offered more complete coverage, they perform poorly at high elevation sites in the Rocky Mountains (McGuire et al., 2012). We used the mean of daily maximum temperatures for July as an index of average daytime conditions during the flight season of *C. meadii* (Buckley & Kingsolver, 2012a). While the start of the flight may change with year, the majority of the flight season remains in July in the southern part of the range. Because adult wing melanin is influenced by pupal rearing temperatures in some montane *Colias* species in Colorado (Hoffman, 1978), we also considered whether annual variation in wing melanin or setal length relates to temperature regardless of

year. *Colias meadii meadii* are typically in pupation during June in the southern region, so we used a mean of daily mean June temperature as an index of pupal conditions that may cause plastic changes in thermoregulatory traits.

We are interested in how the two thermoregulatory traits, grey level and setal length vary over time and with temperature. To account for trait similarity due to geographic proximity, we used spatial autoregressive (SAR) models accounting for spatial covariation in the error term (SAR_{err}) (Kissling & Carl, 2008). Spatial weights matrix with a neighborhood distance of 40 km for grey level and 20km for setal length were the best models for the data (Table S1). For each trait we estimated three different SAR_{err} models with different predictors. To evaluate temporal trends in each trait, we included year and elevation as predictors. Second, to evaluate the relationships between adult traits and environmental temperatures during the flight season, we modeled each trait as a function of the mean of daily maximum temperatures for July and elevation. The mean daily maximum temperature was chosen as an index of average daytime conditions during the flight season. Finally, to evaluate whether pupal temperatures may affect adult traits due to plasticity, we modeled the two traits as a function of elevation and of monthly mean temperatures for June (when *C. m. meadii* in this region are pupating). Analyses were conducted in R using library spdep (Bivand et al., 2011). For each model we report estimated model coefficients (and standard errors) and associated significance tests (based on Z values). We used a Moran's I test on the model residuals to confirm that the spatial error models fully accounted for spatial autocorrelation in the data (Kissling & Carl, 2008) (Table 1). We compared the SAR model output to linear models with the same predictors, and location as a random intercept (Table 2). There was no difference in the significance, or direction of the slope for any result.

Results

Mean July maximum temperature increased significantly from 1953 to 2013, ($p < 0.001$; slope = 0.03, $se = 0.008^\circ\text{C}$), resulting in a total increase of approximately 1.8°C over 60 years (Figure 1). However there is substantial variability among years around this trend: the standard deviation between years was 0.7°C , and all years spanned 2.9°C . By contrast, mean June temperature did not increase significantly during this time period ($p = 0.17$). For the 60 years in which we have samples (1953-2013), mean June temperature actually declined over time ($p < 0.001$, slope = -0.018). Thus, over the past 60 years, relevant environmental temperatures have increased during the flight season but not during pupal development. While short periods of unseasonably warm temperatures may result in earlier snow melt, the mean temperature in June has been decreasing for the years in which we have data.

Grey level increased significantly with year ($p < .0001$) (Figure 3a), suggesting that mean wing melanin of *C. m. meadii* has increased during this 60-year period. Based on the estimated slope (slope $b = 0.001 \pm 0.0003$), the mean grey level increased by 17%. However, grey level was independent of elevation ($p = 0.913$). Mean daily maximum temperature for July was not a good predictor of ventral hind wing melanin ($b = 0.0006 \pm 0.003$; $p = 0.857$). However, grey level decreased significantly with increasing mean June daily mean temperatures ($b = -0.009 \pm 0.003$; $p = 0.003$) (Figure 4a). There was no significant effect of elevation in this model ($p = 0.350$).

Mean setal length also increased significantly by 20% over the observation period ($b = 0.015 \pm 0.003$; $p < 0.001$) (Figure 3b). Setal length increased with elevation ($b = 0.0004 \pm 0.0001$; $p < 0.001$). Setal length decreased significantly with increasing July temperature ($b = -0.061 \pm 0.025$, $p = 0.015$). However, there was no significant relationship between thorax setae length and mean June temperature ($b = 0.016 \pm 0.025$; $p = 0.508$). Setal length increased with

increasing elevation in this model ($b=0.0005 \pm 0.0001$, $p < 0.001$). These results suggest that phenotypic plasticity may have contributed to temporal changes in grey level and wing melanin, but not in setal length.

Discussion

We predicted that recent climate warming could lead to selection for lower values of two key traits that affect heat gain. Our analyses suggest the opposite trend: namely, grey level and setal length increased significantly during the past 60 years in populations of *C. m. meadii* in the southern Rocky Mountains (Fig. 3). We suggest two possible explanations for these unexpected findings: selection for increased ventral hind wing melanin and setal length over the past 60 years or developmental plasticity in response to pupal temperatures producing darker morphs in years with cooler Junes.

It seems unlikely that selection has led to darker wings with longer setae. Recent modeling of wing melanin for *C. m. meadii* in Colorado suggests that recent warming has increased overheating risk at lower (3000m), but not higher (3500m) elevations (Kingsolver & Buckley, 2015a). Indeed, their analyses predict consistent selection for increased wing melanism at higher elevations during the past 60 years, but that directional selection for decreased wing melanin has become more common and stronger in recent decades at lower elevations. However, seasonal and annual variation in climate generates substantial variation in the strength and direction of predicted selection, maintaining phenotypic variation and reducing evolutionary responses in wing melanin (Kingsolver & Buckley, 2015a). The large variation in wing melanin we report here (Figs. 3-5) makes it difficult to detect whether evolution has occurred in this system without genetic data.

Our analyses show that years with cooler June temperatures produce adults with darker wings (Fig. 5). Lower temperatures experienced during pupal development increase adult wing melanin in montane *Colias* (Hoffman, 1978, Higgins, 2014, Watt, 1969). This result suggests that annual variation in wing melanin is in part the result of phenotypic plasticity. Whether such plasticity is adaptive depends on whether environmental cues during pupal development (here, mean June temperatures) are a good predictor of environmental conditions experienced by adults (mean July maximum temperatures) (Huey & Kingsolver, 1993, Moran, 1992, Padilla & Adolph, 1996). June and July temperatures in this region were only weakly correlated during the past 60 years ($r=0.19$, $p=0.18$), suggesting that pupal temperature in June was a poor predictor of temperatures during the flight season (Fig. 2). Because July temperatures were not significantly correlated with mean wing melanin (Fig. 4), phenotypic plasticity was not necessarily adaptive for *C. m. meadii* during this time period. This result highlights how environmental unpredictability can strongly limit adaptive responses, including adaptive plasticity, to climate change (Kingsolver & Buckley, 2015b).

Several other studies have explored changes in insect wing melanism in response to recent warming. In her PhD dissertation, Stamberger (Stamberger, 2006) measured wing melanin and setae length in male *C. meadii* from eight sites in southwestern and central Colorado collected between 1980 and 2003. Her analyses did not detect a significant temporal trend in either trait during this time period, but did indicate a significant positive correlation between mean monthly July temperature and mean wing melanin. A recent analysis of images in field guides indicated a significant decrease in wing melanism in many European butterflies and body melanism in European dragonflies between 1988 and 2006 (Zeuss et al., 2014). Whether this trend is the result of evolutionary or plastic response is unclear. Increasing melanin in response

to decreasing developmental temperatures has been widely reported in insects, including many cases in which melanin plays no role in thermoregulation (Gilbert, 2001, Stoeckh & Goux, 2008, Kemp & Jones, 2001, Green et al., 2012). The thermoregulatory role of wing melanin (if any) has not been determined for most butterflies, and will depend on basking posture, body size, habitat, and climate. In fact, melanin and color for most areas of the wing surfaces have little or no effect on body temperature (Willmer, 1982, Dennis, 1993).

For any collection based study, there is a bias towards collecting individuals in flight. Like many butterflies, *Colias* are cryptic when at rest, and are easier to locate when in flight. Unfortunately, the action of the butterfly when caught is not recorded for any of the museum specimens. As a result individuals caught at a given site in a given year are not necessarily reflective of the average individual at that site in a given year. However, because active flight is essential for feeding, mating and oviposition, we are more likely to consistently collect individuals with trait values associated with higher fitness.

These patterns must be understood in the context of overall variation in these traits (Figs. 2-3). The predictor variables of year and mean June temperature only explain 1% of the variability in grey level and 24% of the variability in setal length; these effects are dwarfed by phenotypic variation within and between sites and among years. *Colias meadii* persist in sub-alpine environments that have tremendous inter-annual variability in temperature, precipitation, and length of growing season. In addition, the sub-alpine and alpine habitats of *C. meadii* have high topographic relief and thus will have high local variation in microclimatic temperatures even in a given season, and this may also maintain trait variability within each site (Sears et al., 2011). Despite a changing environment, *C. meadii* demonstrate high amounts of variability in

thermoregulatory traits which may ultimately garner their persistence under novel climatic regimes.

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Tables and Figures

Table 1: Model output for the different SAR models. The bolded variable in the model is the one being reported. The p-value associated with a Moran's I test on the residuals of each SAR_{err} model. We test whether variation in the model residuals can be accounted for by the spatial weights matrix. Here an insignificant p-value means that no significant spatial autocorrelation remains in the model residuals.

Table 2: Linear Model Results with location as a random effect. These models include the exact same terms as the SAR_{err} models (Table 1) but deal with spatial autocorrelation by location (defined by a 7km area) as a random effect. Here we used a Mantel Statistic as an assessment of spatial autocorrelation. The Mantel Statistic compares the observed Mantel correlation to a randomly generated Mantel correlation matrix (based on 1000 permutations). Here a p-value > 0.05 indicates not significant difference between the observed and null matrices.

Figure 1: Air temperature trends from 1953 to 2013 at the weather station in Climax, CO (39.37N, 106.18W, , 2013) a) Temperature trends for Mean July Maximum Temperature (solid line) and for Mean June Mean Temperature (dashed line) over time of study for all years. b) Temperature data and linear regressions for Mean July Maximum Temperature (solid line, closed circles) and June Mean Temperature (dashed line, open circles) only for the years for which we have specimens.

Figure 2: A) Grey level and B) setal length have increased over recent decades. Grey level is a proxy for wing melanin ranging from 0 to 1 with higher values indicating a higher degree of melanism. Linear regression and standard error are indicated

Figure 3: A) Grey level shows no significant relationship to July temperature whereas B) setal length has decreased with increasing July temperature. Grey level is a proxy for wing melanin ranging from 0 to 1 with higher values indicating a higher degree of melanism. Linear regression and standard error are indicated

Figure 4: A) Grey level decreases with increasing June temperature whereas B) setal length has no significant relationship with June temperature. Grey level is a proxy for wing melanin ranging from 0 to 1 with higher values indicating a higher degree of melanism. Linear regression and standard error are indicated

Table 1

| <i>Grey-Level (40km)</i> | df | z | p | slope \pm se | AIC | LR Test | Moran's I |
|---|-----------|----------|----------|----------------------------------|------------|-----------------|------------------|
| Grey ~ Year + Elevation | 5 | 4.349 | <0.001 | 0.001 \pm 0.0003 | -850.32 | 20.57, p<<0.001 | p = 0.166 |
| Grey ~ July Max + Elevation | 5 | 0.179 | 0.858 | 0.0006 \pm 0.003 | -831.29 | 24.59, p<<0.001 | p = 0.173 |
| Grey ~ June Mean + Elevation | 5 | -2.999 | 0.002 | -0.009 \pm 0.003 | -840.75 | 22.32, p<<0.001 | p = 0.246 |
| <i>Setal Length (20km)</i> | | | | | | | |
| Setal Length ~ Year + Elevation | 5 | 4.814 | <0.001 | 0.015 \pm 0.003 | -185.94 | 0.068, p=0.79 | p = 0.228 |
| Setal Length ~ July Max + Elevation | 5 | -2.418 | 0.015 | -0.061 \pm 0.025 | -194.18 | 1.56, p=0.21 | p = 0.214 |
| Setal Length ~ June Mean + Elevation | 5 | 0.661 | 0.508 | 0.016 \pm 0.025 | -196.79 | 0.345, p=0.55 | p = 0.219 |

Table 2

| Linear Model | F statistic | p | Mantel Statistic Significance |
|--------------------------------------|--------------------|----------|--------------------------------------|
| Grey Level ~ Year + Elevation | 4.47 | 0.03 | 0.279 |
| Grey Level ~ July Max + Elevation | 1.02 | 0.31 | 0.948 |
| Grey Level ~ June Mean + Elevation | 4.95 | 0.02 | 0.825 |
| Setal Length ~ Year + Elevation | 14.7662 | 0.0002 | 0.838 |
| Setal Length ~ July Max + Elevation | 9.7106 | 0.002 | 0.852 |
| Setal Length ~ June Mean + Elevation | 0.1725 | 0.6782 | 0.861 |

Figure 1

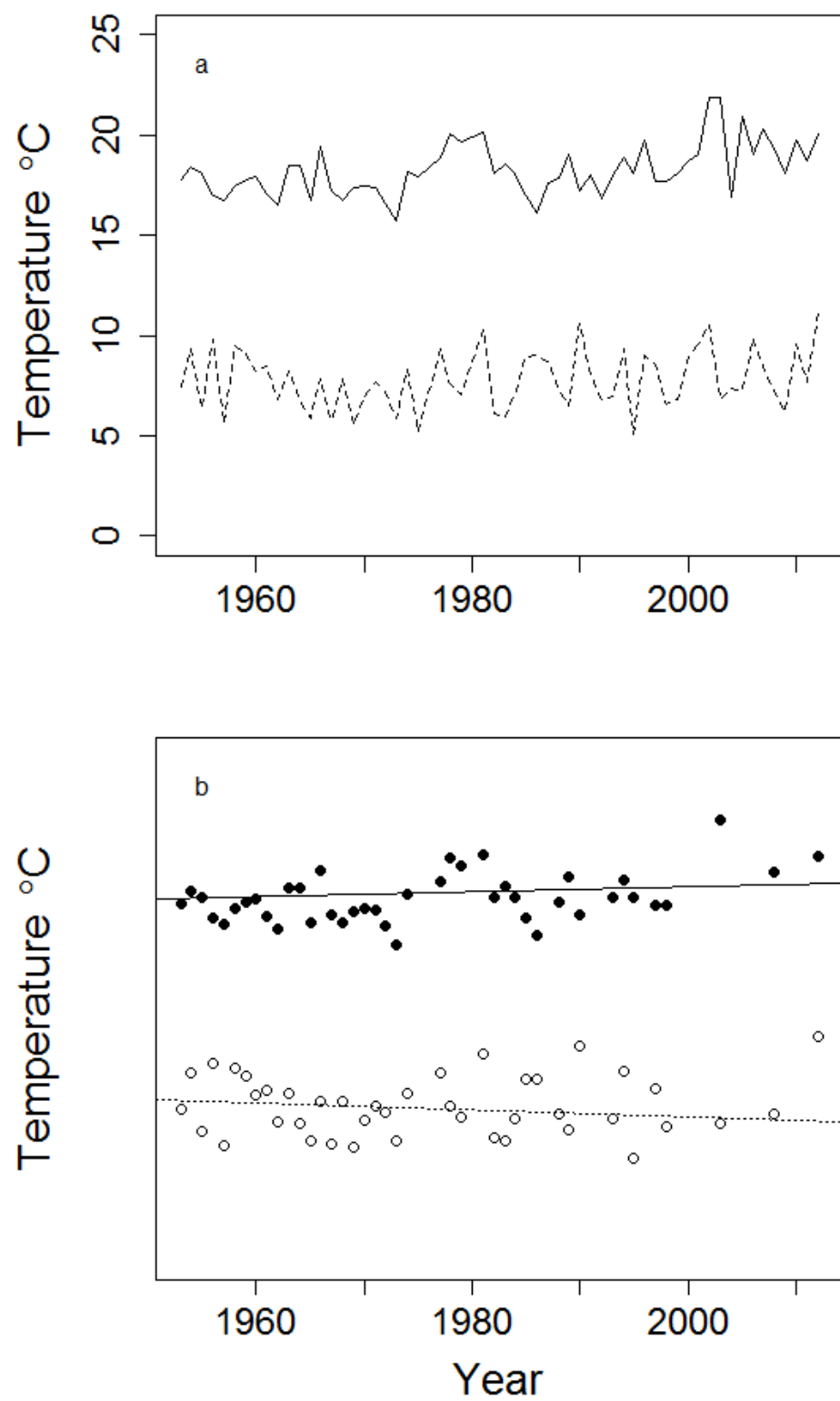


Figure 2

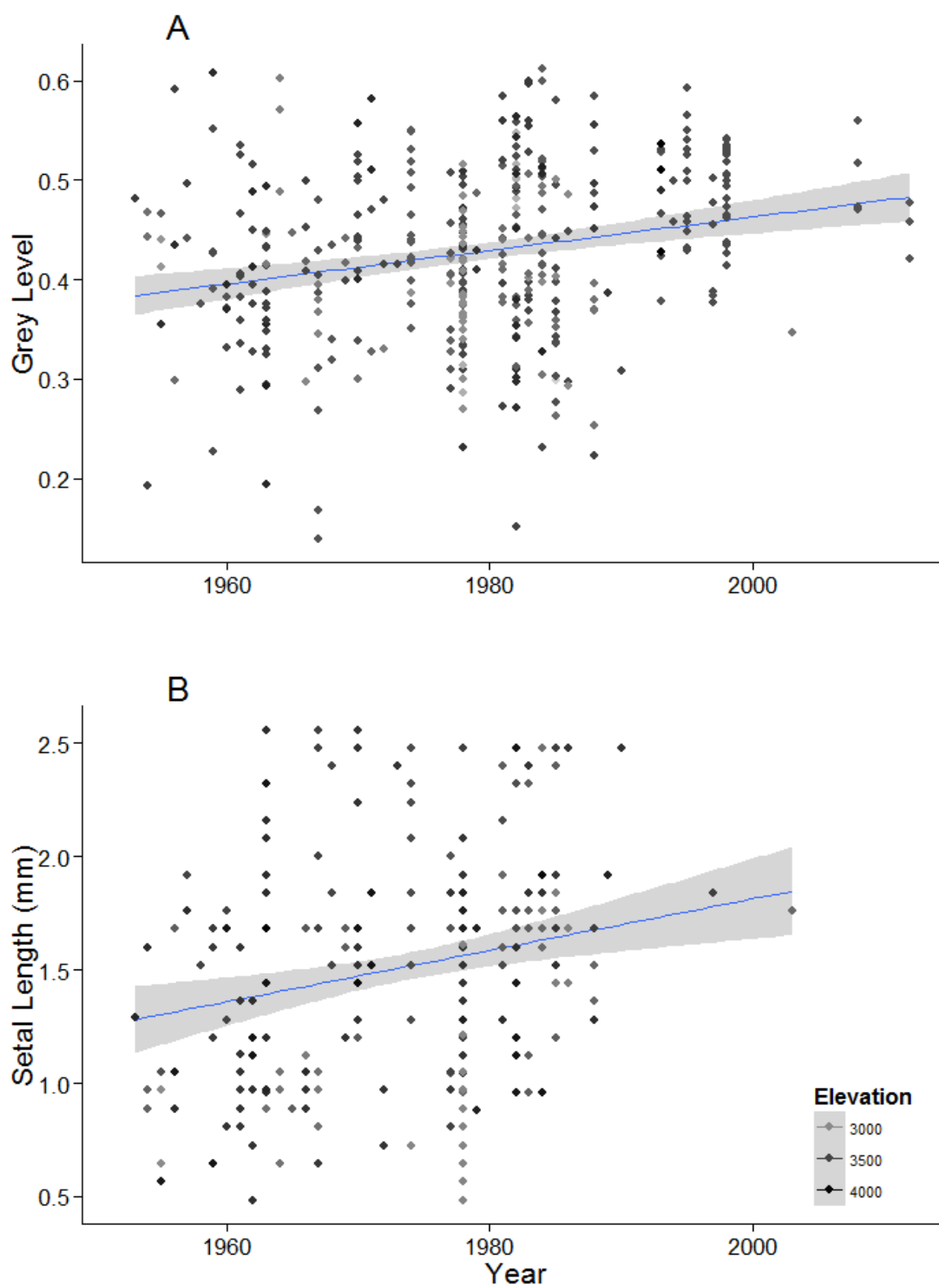


Figure 3

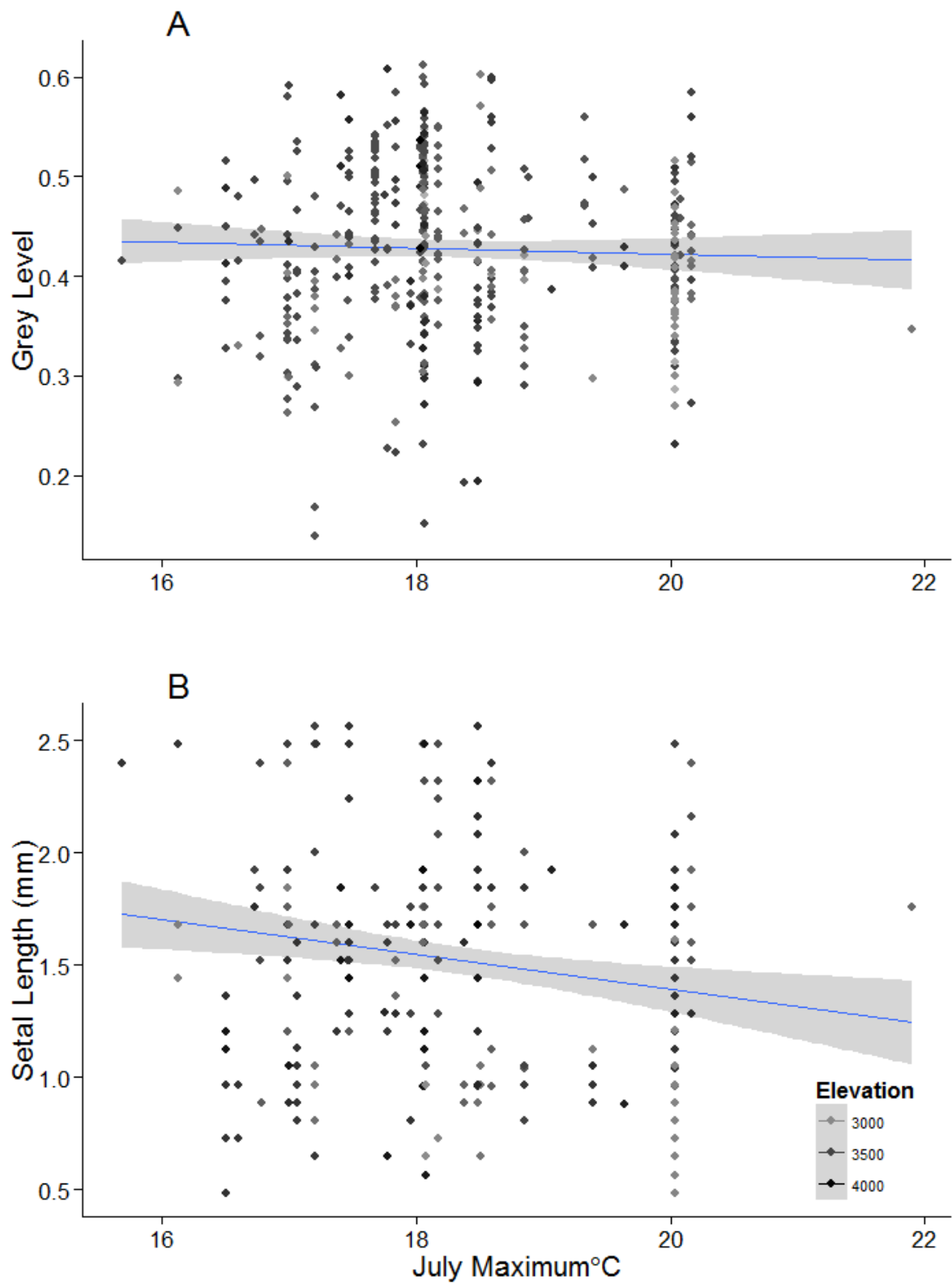
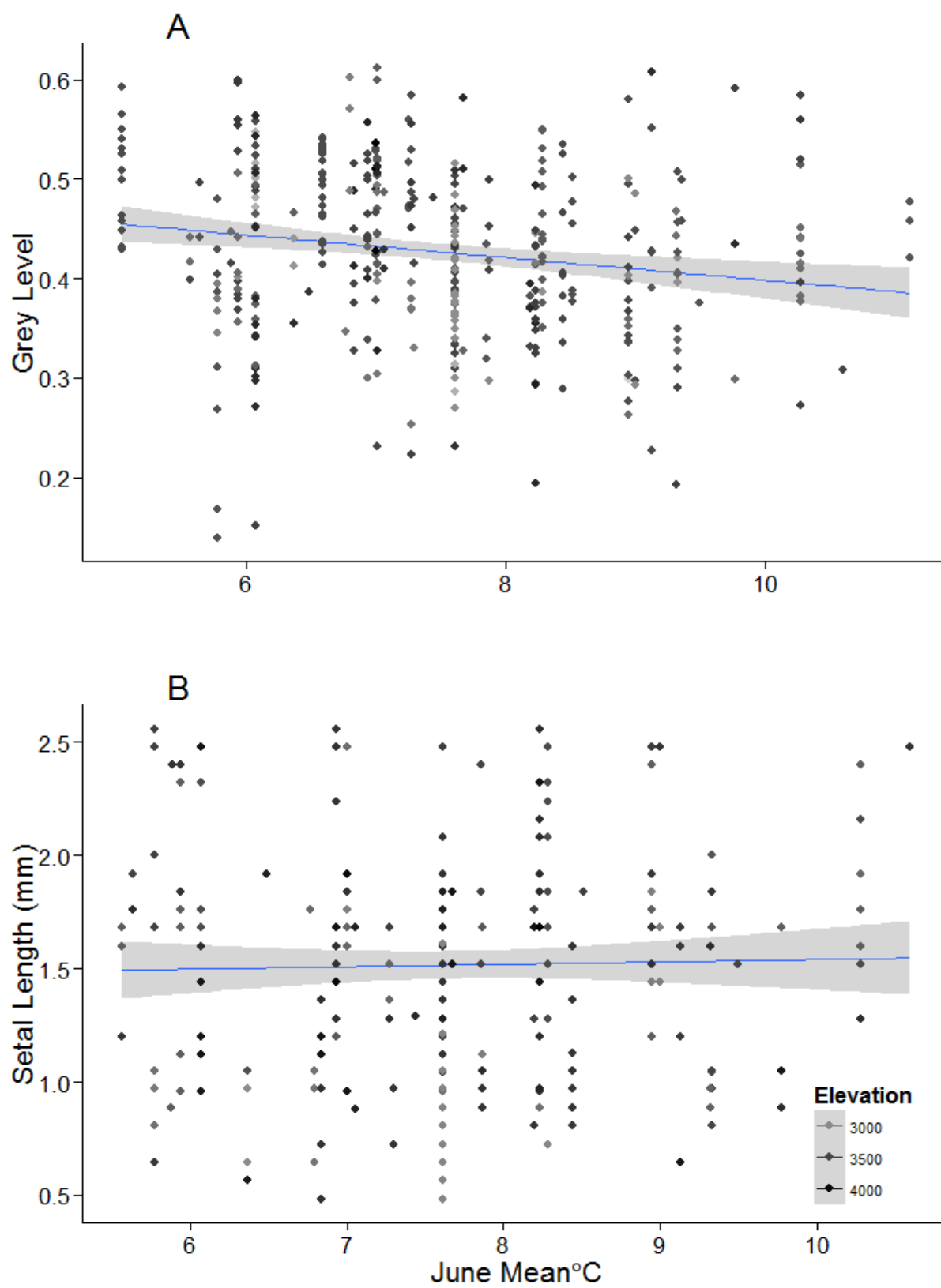


Figure 4



CHAPTER FOUR APPENDIX

Figures and Tables

Table 1: Linear models output for the entire range of *C. meadii*. This encompasses three subspecies (*Colias meadii meadii*, *Colias meadii lemhiniensis*, and *Colias meadii edwardsi*).

Figure 1: Map of all sample locations separated by region.

Figure 2: a) Grey level and b) setal length have increased over recent decades. Grey level is a proxy for wing melanin ranging from 0 to 1 with higher values indicating a higher degree of melanism. Linear regression and standard error are indicated

Figure 3: a) Grey level increases with increasing latitude and b) setal length has no apparent relationship with latitude. See Table 1

Table 1

| Linear Model | F statistic | p-value | Mantel Statistic Significance |
|--|-------------|------------------|-------------------------------|
| Grey Level ~Lat * Year + Elevation +Sex Random Intercept = 12km Location | | | 1 |
| Year | 0.853 | 0.3559 | |
| Latitude | 4.627 | 0.0316 | |
| Elevation | 1.222 | 0.2691 | |
| Sex | 12.117 | 0.0005 | |
| Year * Latitude | 3.035 | 0.0817 | |
| Thorax Length ~Lat * Year + Elevation +Sex Random Intercept = 12km Location | | | 1 |
| Year | 66.1347 | <.0001 | |
| Latitude | 0.3519 | 0.5531 | |
| Elevation | 8.3013 | 0.0040 | |
| Sex | 25.5683 | <.0001 | |
| Year * Latitude | 0.1320 | 0.7164 | |

Males are lighter than females over the whole range. As latitude increases, so does grey level. Trait values over the whole range are not related to Year collected. Thorax fur appears to be increasing over time and elevation. Males appear to have longer thorax fur in the museum samples.

Figure 1

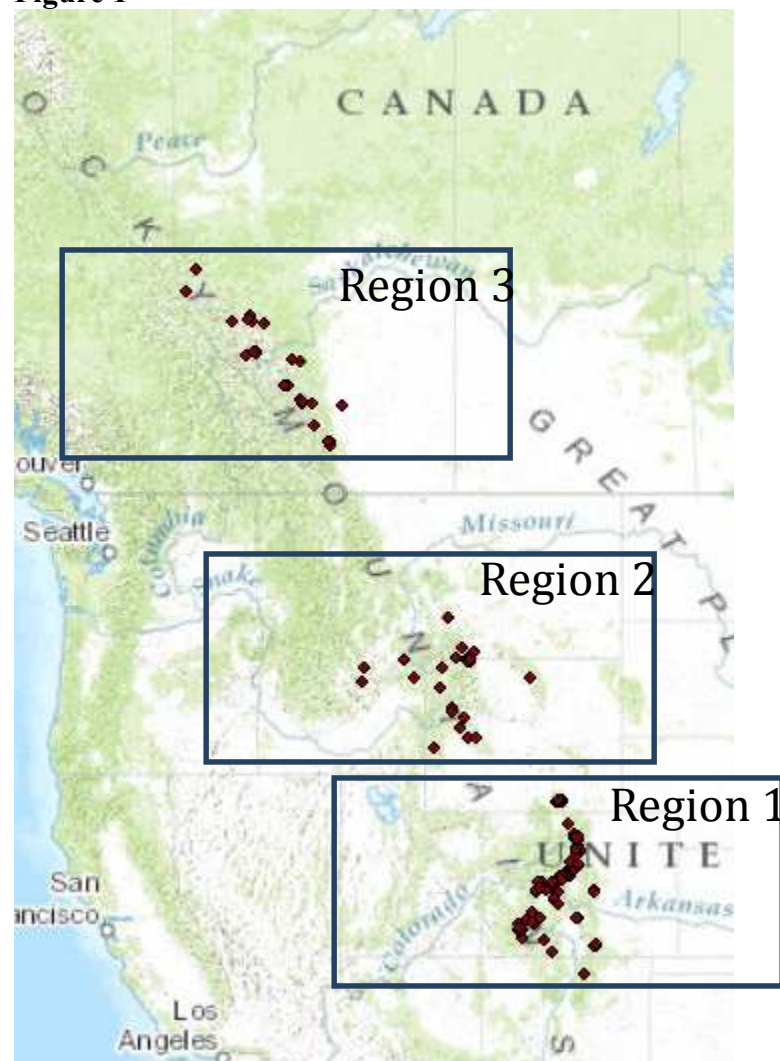


Figure 2

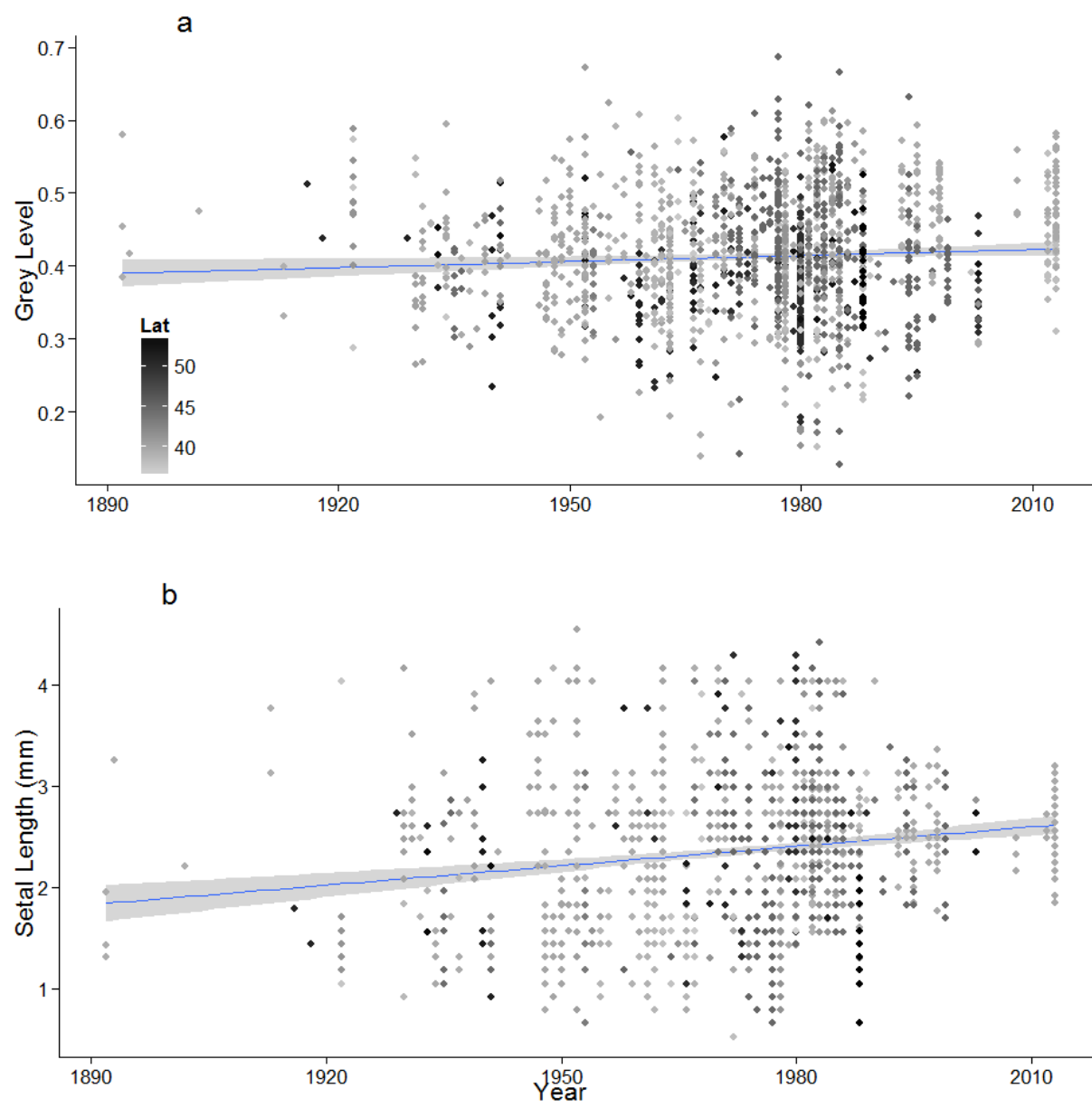
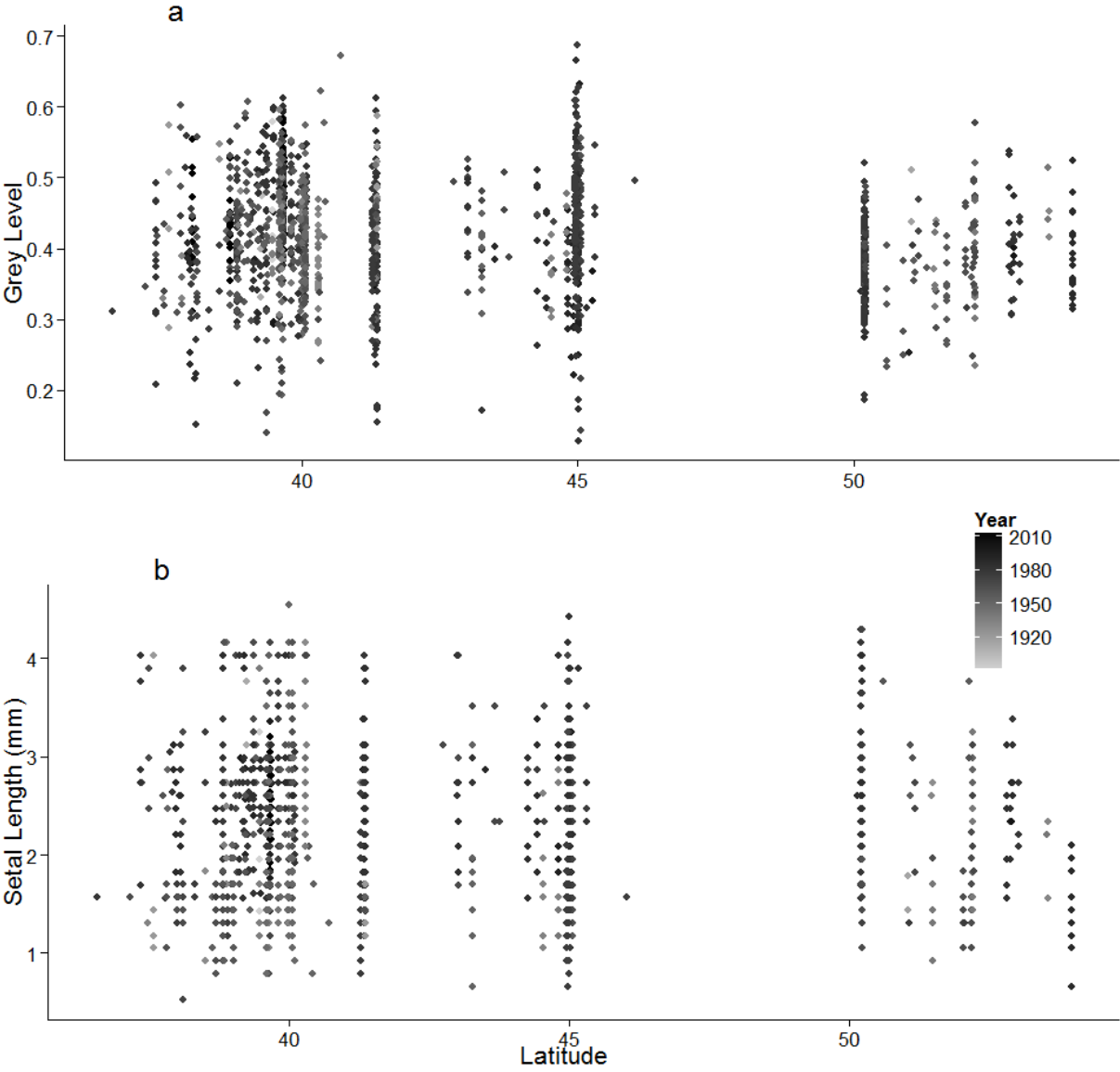


Figure 3



CHAPTER FIVE: SEASONAL VARIATION AND PHENOTYPIC PLASTICITY IN THERMALLY IMPORTANT TRAITS IN *COLIAS* BUTTERFLIES

Summary

Climate and other environmental factors vary seasonally, and many organisms exhibit developmental plasticity that results in seasonal variation for adaptive traits. For example, seasonal plasticity in melanin can help ectotherms achieve optimal body temperatures in cooler seasons and avoid overheating in warmer seasons. Many organisms use photoperiodic cues to assess seasonality, but those cues are less useful in tropical or mountainous regions. In these regions, developmental temperature may serve as an environment cue, but such plasticity is only adaptive if developmental temperatures reliably predict subsequent thermal conditions. Using *Colias* butterflies in the Rocky Mountains as a model system, we quantified seasonal variation in a key thermoregulatory trait—melanin on the ventral hindwings—and whether temperatures during pupal development are reliable predictors of conditions experienced during the adult flight seasons. *Colias eriphyle* butterflies adapt to local climatic conditions along elevational gradients via differences in wing melanin, and increased pupal temperature can reduce wing melanin. We considered seasonal variation for two populations of *C. eriphyle* at low (1.6 km) and middle (2.3 km) elevation sites that have multiple generations (adult flight seasons) each year. Wing melanin significantly decreased with collection date during the season in both populations; these seasonal changes in wing melanin were strongly associated with increases in temperature during pupal development during the season. We used a moving average to infer temperatures in the pupal stage (fixed as the 14 days prior to a collection day), and temperatures

during the adult stage (fixed as the 5 days after a collection day). Pupal temperatures were a good predictor ($r^2 > 0.71$) of temperatures during the adult flight season in both populations, confirming a key condition for adaptive seasonal plasticity in this system.

Introduction

Seasonal polyphenism, or variation in phenotype as a direct response to seasonal environmental conditions, is a specific type of phenotypic plasticity (Weismann 1875). Adaptive seasonal plasticity produces a phenotype that matches the organisms' environment. For example, butterflies and other insects that have multiple generations per year exhibit seasonal polyphenism in color patterns. Some of these seasonal morphs enhance crypsis such as the larval morphs of *Nemoria arizonaria* (Greene 1989) and the adults of *Bicyclus* species (Brakefield & Larsen 1984; Windig *et al.* 1994). Other seasonal morphs contribute to seasonal energy balance, such as the adults of *Pontia occidentalis* (Kingsolver 1995), *Pieris rapae* (Stoehr & Goux 2008) and *Colias eurythyme*, by facilitating more effective thermoregulation through the thermal year (Hoffman 1972). In each of these cases, the cue used to produce the phenotype was reliable and the resultant phenotype produces a survival or fitness advantage. Typically, seasonal polyphenism is governed by highly reliable cues such as photoperiod or larval diet, or by a combination of cues, such as photoperiod and temperature or photoperiod and precipitation (Shapiro 1976). Photoperiod is a highly reliable, celestially regular, abiotic indicator of seasonality for organisms in the temperate zone (Bradshaw & Holzapfel 2007).

In the tropics where photoperiod remains constant, or in mountainous regions where growing seasons are short and environments can be highly variable, organisms may rely on temperature or precipitation as indicators of seasonality instead of photo cues. Provided temperature during development is a good predictor of the adult environment, they may be adaptive. There are

regular annual patterns in both temperature and precipitation; however, there is also a great deal of variability in these cues. Moreover, climate change threatens to increase variability and reduce the reliability of seasonal patterns in temperature and precipitation (Williams, Jackson & Kutzbach 2007; Barros 2014). Adaptive plasticity, particularly irreversible developmental plasticity, can be limited by the lag time between the developmental cue and the environment experienced by the adult (discussed in DeWitt, Sih & Wilson 1998)

Here we use the *Colias* butterfly system to look ask how the reliability of the cues affect the patterns of seasonal trait variation. Many *Colias* use photocues but Rocky Mountain *Colias eriphyle* have evolved to use temperature cues as indicators of season (Hoffman 1978). *Colias* butterflies bask with their wings closed and their ventral wing surfaces oriented perpendicular to the sun. More melanic scales on their ventral hindwings can absorb solar radiation and elevate body temperature during basking. When ambient temperatures are high, *Colias* orient their wings and body to the sun, reducing radiative heating and body temperature (Watt 1969), but this behavior limits the time available for flight activity. Thus, there is a direct fitness cost of having more melanin later in the season. Early in the season, individuals will encounter cooler temperatures and should be more absorbent whereas later in the season they will encounter warmer temperatures and should be less absorbent. Ventral hindwing melanin in *C. eriphyle* is determined through both genetic ($h^2 = 0.43 \pm 0.21$ in males) (Ellers & Boggs 2002) and plastic mechanisms: increasing temperature during pupal development decreases melanin on the ventral hindwings (Hoffman 1978; Higgins 2014). By measuring ventral hind wing melanin in *C. eriphyle*, we can look at within season patterns of plasticity controlled by temperature rather than photoperiod. This allows us to quantify the reliability of the temperature cue over the course of the flight season.

The final chapter of my dissertation aims to explore seasonal variation in *C. eriphyle* wing melanin. I quantify seasonal patterns of variation in wing melanin and relate these patterns to both developmental and adult environmental temperatures in two populations along an elevation and climatic gradient in Southwestern Colorado.

Materials and Methods

We collected specimens from two populations of *C. eriphyle*; one near Olathe, Montrose Co., CO (N38.62, W108.02, 1,600m elevation) and one in Gunnison, Gunnison Co., CO (N38.56, W106.94, 2,300m). Each of these populations was sampled over June through August in 2011, 2012, and 2013 and during the first flight in Olathe in 2014 to capture seasonal differences in larval experiences.

Adults were collected via netting. They were extracted from the net with forceps and placed in a glassine envelope. These individuals were then frozen and returned to the lab at UNC where the spectral absorption of the basal region of the left ventral hind wing was measured at 650 nm with a FieldSpecPro FEFR 7501, ASD Inc with an optical integrating sphere. A 2mm square region of the wing triangulated between the eyespot, wing edge, and wing insertion was measured for reflectance at 650nm (Watt 1968; Hoffman 1978). The region was isolated by cutting a 2mm² window in black flocking paper. To account for the background reflectance of the flocking paper, we took repeated measures of flocking paper and of pure white powder through the window to build a correction factor for the measurements. Three reflectance measurements were collected and averaged for each individual. Reflectance was then converted to absorbance (1-reflectance).

To quantify seasonal patterns of temperature variation, we retrieved NOAA Cooperative Observer Program weather station data for daily minimum and maximum temperatures for both

Gunnison (USC00053662) and Montrose (USW00093013) (downloaded from <http://www.ncdc.noaa.gov>). Lab experiments suggest that the length of the pupal stage in these populations of *C. eriphyle* is approximately 14 days under typical field conditions (Higgins 2014) so we estimated temperatures experienced by pupae in the field as a moving average of the 14 days prior to an emergence/collection date. Then to estimate temperatures experienced by the adults, we used a moving average of the five days after the same emergence/collection date.

Statistical Analysis

All statistical analyses were performed in R (v. 2.15.3) (RCoreTeam 2013) using the nlme libraries (Pinheiro 2014). To look for seasonal patterns in ventral hindwing melanin, we used a linear mixed effects model with population, Julian date, and sex with year as a random intercept. To relate developmental temperature to ventral hindwing melanin, we used population, pupal mean temperature, pupal minimum temperature, and sex. Lab experiments have found that both pupal mean temperature (Hoffman 1978) and cold shocks (Shapiro 1976) influence melanism.

To determine the utility of developmental temperature as a seasonal cue, we correlated adult mean temperatures to pupal minimum and mean temperatures over the course of the season for both populations combined. We repeated each for adult maximum temperature to more closely reflect temperatures when adults are active.

Results

The temperature variation among days decreased while mean temperature increased (Fig 1) through the activity season. There was a significant decrease in mean wing melanin over the course of the season ($F_{n=595,df=1}=51.78$, $p<0.01$) regardless of population ($F_{n=595,df=1}=0.84$, $p=0.35$) or sex ($F_{n=595,df=1}=1.505$, $p=0.22$, Fig. 2). We related the mean and minimum

temperatures of the 14 days prior to collection, corresponding to the exposure period of pupae, to absorptivity for each individual. Lower pupal minimum temperatures were associated with greater wing melanin ($F_{n=595,df=1} = 39.78$, $p < 0.001$, Fig. 3) regardless of sex ($F_{n=595,df=1} = 2.19$, $p = 0.11$). Higher pupal mean temperatures were associated with increased melanin ($F_{n=595,df=1} = 50.26$, $p < 0.001$) but this reflects the increase in trait variability later in the season. A model of absorptivity as a function of pupal mean temperature, without pupal minimum, shows a significant negative relationship reflected in Figure 3. There was also a significant interaction between pupal minimum temperature and population, producing a steeper slope for the Gunnison than the Olathe population ($F_{n=595,df=1} = 19.73$, $p < 0.01$, Fig3).

Recorded temperatures during pupal development in these locations were strongly and significantly correlated with adult temperatures. Specifically, mean and minimum pupal temperature and mean adult temperature show a significant relationship ($R^2_{\text{pupal mean}} = 0.87$, $F_{,df=1} = 6192.08$, $p < 0.001$; $R^2_{\text{pupal minimum}} = 0.82$, $F_{,df=1} = 4409.52$, $p < 0.01$) with Olathe being significantly warmer (pupal mean: $F_{,df=1} = 116.67$, $p < 0.001$; pupal minimum: $F_{,df=1} = 22.00$, $p < 0.001$). Adult maximum temperature is significantly, but more weakly, correlated to both pupal mean ($R^2_{\text{pupal mean}} = 0.71$, $F_{,df=1} = 2350.53$, $p = 0.01$) and pupal minimum temperature ($R^2_{\text{pupal minimum}} = 0.65$, $F_{,df=1} = 1722.13$, $p < 0.01$). The high degree of correlation indicates that developmental environment is a predictor of adult experience.

Discussion

Phenotypic plasticity in response to seasonal environmental cues ensures the appropriate phenotype is produced in a given season. Seasonal trait variation is typically regulated by either highly reliable cues, such as photoperiod, or by a combination of cues, such as photoperiod and temperature or photoperiod and precipitation (Weismann 1875; Shapiro 1976). For organisms

with a short growing season, photoperiod may be of little use and temperature may be a more reliable cue (Hoffman 1978). However, temperature is variable and variability can limit the evolution of adaptive plasticity in insects and other taxa (Kingsolver & Huey 1998). This raises the question of the reliability of temperature as a seasonal cue, particularly in the induction of thermoregulatory traits wherein developmental temperature directly affects adult fitness by limiting activity time or decreasing survival (Kingsolver 1996; Ellers & Boggs 2004; Buckley & Kingsolver 2012).

In the case of ventral hind wing melanin in *C. eriphyle*, as the season progresses the mean absorptivity decreases and we can explain this with pupal temperature. This is true in both of the measured populations despite differences in elevation, duration of flight season, and relative mean temperature. The air temperatures in the summer, are 5°-10°C warmer in Olathe compared to Gunnison (Figure 1). Olathe, CO is largely farm land with irrigated alfalfa fields, early springs and warmer temperatures. As a result, the flight season is longer, starting in late April to early May and continuing until early October with several generations contained within. By contrast, Gunnison, CO is one of the coldest cities in the United States. Because of the topography of the area, Gunnison experiences a climatic inversion in which cold air is trapped in the Gunnison valley making it colder than the peaks of surrounding mountains for the better part of the winter (King 2007). The flight season of *C. eriphyle* is much shorter here, typically with two flights, starting in mid-June and continuing until early September. While pupal temperature seems to have a similar effect on ventral hindwing melanin in the populations of *C. eriphyle* in which we sampled, adults from the Gunnison population appear darker. We know that there are fixed elevation differences in melanin as well as plasticity in melanin (Ellers & Boggs 2002,

Hoffman 1978). We present data that is representative of early-season and mid-season morphs for each population.

Here we also observed an increase in ventral hindwing variability with season in both populations. What might be driving this? Perhaps habitat changes over the course of the season. In the spring, following the snow melt, the vegetation is just exiting dormancy. It is sparse and short. As the season progresses, temperatures increase, and irrigation systems are employed. The vegetation grows tall rapidly and creates a host of microhabitats. Thus, early in the season, the pupae are highly synchronized and the probability that all pupae are experiencing similar conditions is high. Later in the season, the generations are spread out temporally and within site heterogeneity of microhabitat, and thus variation in the thermal environment, that pupae may experience increases. It is unclear from this data if going through diapause contributes to the lack of variation early in the season; however, previous work done on *C. eriphyle* implicate pupal temperature, when larval temperatures were held constant, in the plasticity in ventral hind wing melanin (Hoffman 1978; Higgins 2014).

There have been two studies that explored how pupal temperatures in controlled environments relate to the Gunnison population (Hoffman 1978) or both the Gunnison and the Olathe populations of *C. eriphyle* (Higgins 2014). Specifically, they both show that constant pupal temperatures can drive the plasticity of ventral hindwing melanin such that lower temperatures produce darker, more melanic adult phenotypes. The main effect was the same in these studies, Higgins (2014) also compared ramping treatments to constant temperatures and found that *C. eriphyle* that were exposed to pupal temperatures ramping between 21°- 29°C, with an average of 25°C induced more melanin than pupal temperature held at a constant 25°C. This suggests that exposure to high or low temperatures may, in fact, be driving melanin deposition.

Work done in other species has shown that exposure to cold temperatures or a cold-shock can lead to an increase in melanin (Weismann 1875; Shapiro 1976). It appears that pupal temperature can account for much of the seasonal variability in ventral hind-wing melanin.

By integrating temperature over the pupal development period, ventral hindwing melanin is well matched for the environment experienced over the adult life span. In the field, the diurnal temperature fluctuation and day-to-day variability create a thermal environment that is far from constant. Here we set a fixed window for pupal development of 14 days based on laboratory data (Higgins 2014), even though pupal temperature can effect pupal duration. When we look at the mean temperature for pupal experience, the variability is almost completely lost and we are left with a gradual increase in temperature. The mean pupal temperature highly correlates to the mean adult temperature taken over a five day period, (Fig 4) at both sites. If we related pupal temperature to the temperature on the day of collection, rather than an average of adult experience, there is no significant relationship ($p=0.199$). Mean temperatures encompass the nighttime low temperatures which may contribute to the deposition of wing melanin during pupal development, and the daytime highs, which relate to the temperature experienced during flight time for adults.

We had the opportunity with *C. eriphyle* to ask how the reliability of the cues affect adaptive patterns of seasonal trait variation. Adaptive plasticity, particularly irreversible developmental plasticity, can be limited by the lag time between the developmental cue and the environment experienced by the adult (discussed in DeWitt, Sih & Wilson 1998). By using reliable environmental cues, in the special case of seasonal polyphenism- reliable seasonal cues, the cost of this lag time can be ameliorated. In his work, Hoffman suggested that by using pupal temperature as a cue *C. eriphyle* allow for “last minute adjustments” to phenotype for dealing

with rapidly changing thermal environments (1978). However, given previous estimates of seasonal predictability relating daily maximum temperatures averaged over the first and third week in July saw little correlation ($<7\%$), we did not expect that pupal temperature would correlate to adult temperature (Kingsolver & Huey 1998). In our case, we use moving averages relating temperatures two weeks ago to temperatures five days from now and saw a high degree of correlation, and thus a limited cost of developmental lag time between developmental environment and adult environment. Thermally driven patterns of seasonal variation in ventral hind wing melanin currently appear to be adaptive in these two populations of *C. eriphyle*.

Cues that have historically been good indicators of seasonality, such as photoperiod and temperature, may become less reliable with climate change. Within season variability and novel environmental conditions are predicted to increase world-wide over the next 100 years (Williams, Jackson & Kutzbach 2007; Battisti & Naylor 2009). The topography of mountain ecosystems make them particularly vulnerable and prone to changes in temperature and precipitation (Thompson 2000; Diaz, Grosjean & Graumlich 2003). With *C. eriphyle*, we have an example of a species that uses developmental temperature to determine adult phenotype and that relationship is currently adaptive. As climate change continues to impose increased summer temperatures, droughts, early snowmelt, and overall increased temporal seasonal variation it is unclear if this currently reliable cue will remain a good indicator of adult experience across the elevation gradient for this species. For other organisms that use a single, or even multiple cues to produce a seasonally appropriate phenotype, the increase in novel climatic conditions may still leave them poorly equipped for their environment. This will likely depend on the lag time between the induction of the phenotype and the environment experienced by the resultant phenotype. By understanding more about the mechanism of seasonal trait variation in this and

other species, we can begin to make more accurate predictions of activity, fitness, and ultimately population persistence under different climatic conditions.

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Figure Legends

Figure 1: Daily mean temperatures over the course of the flight season for both Gunnison (solid line) and Olathe (dashed line). Day to day variability decreases over the flight season. Gunnison remains approximately 10°C cooler than Olathe throughout the season. Colors represent each of the four years in which we collected.

Figure 2: Mean absorptivity \pm sd at 650nm for both Gunnison (circle) and Olathe (triangle) plotted first A) by collection date (Julian day) and then by B) pupal mean temperature. Colors represent the year in which each sample was collected. There is a decrease in mean absorptivity both as a function of day and pupal temperature.

Figure 3: The relationship between pupal temperature and adult temperature for each population Gunnison (circle) and Olathe (triangle). Colors represent each of the four years in which we collected. Adult temperatures were calculated as a moving average of 5 day flight mean plotted as a function of moving average of 14 day pupal mean from late spring through late summer.

Figure 1

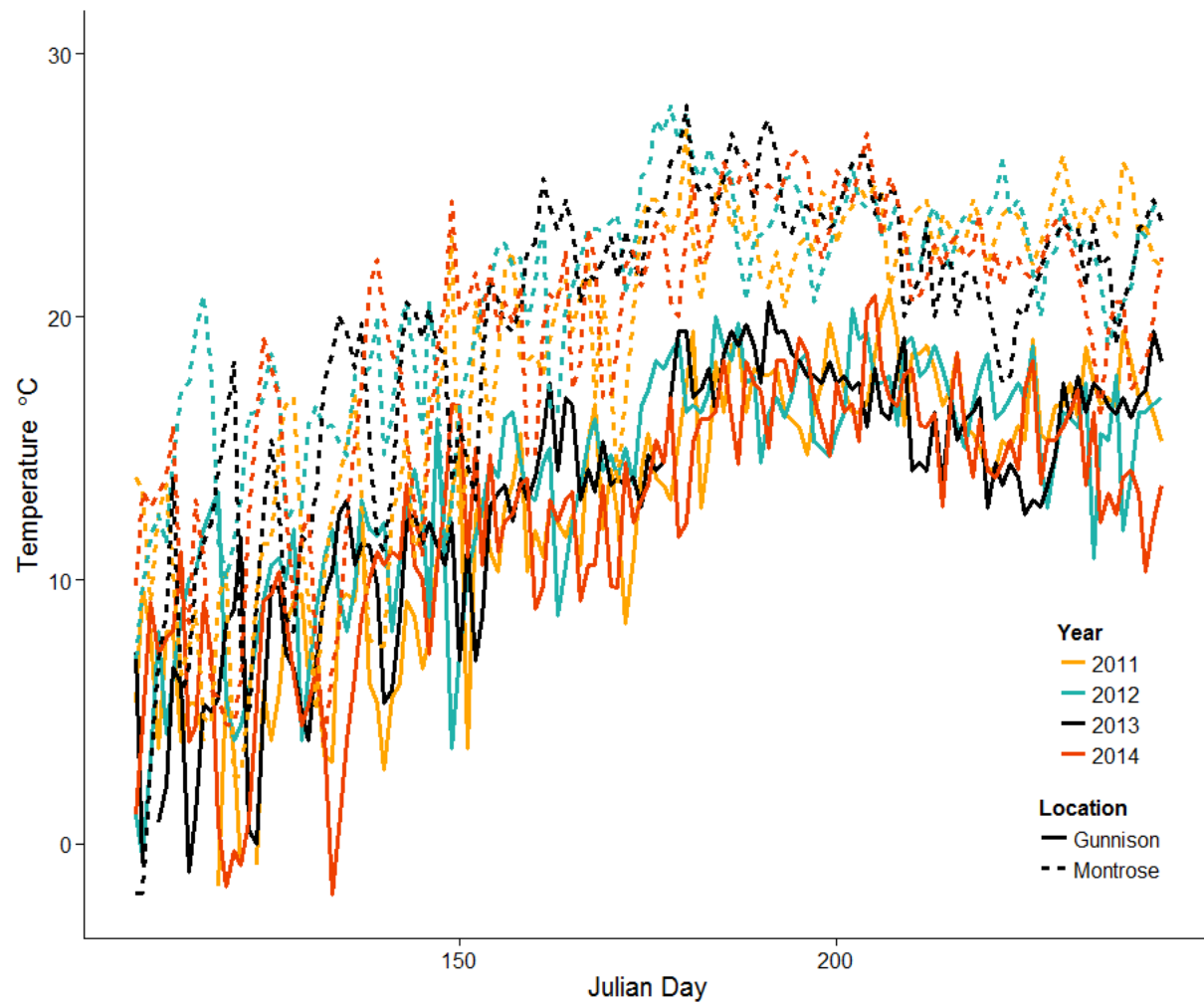


Figure 2

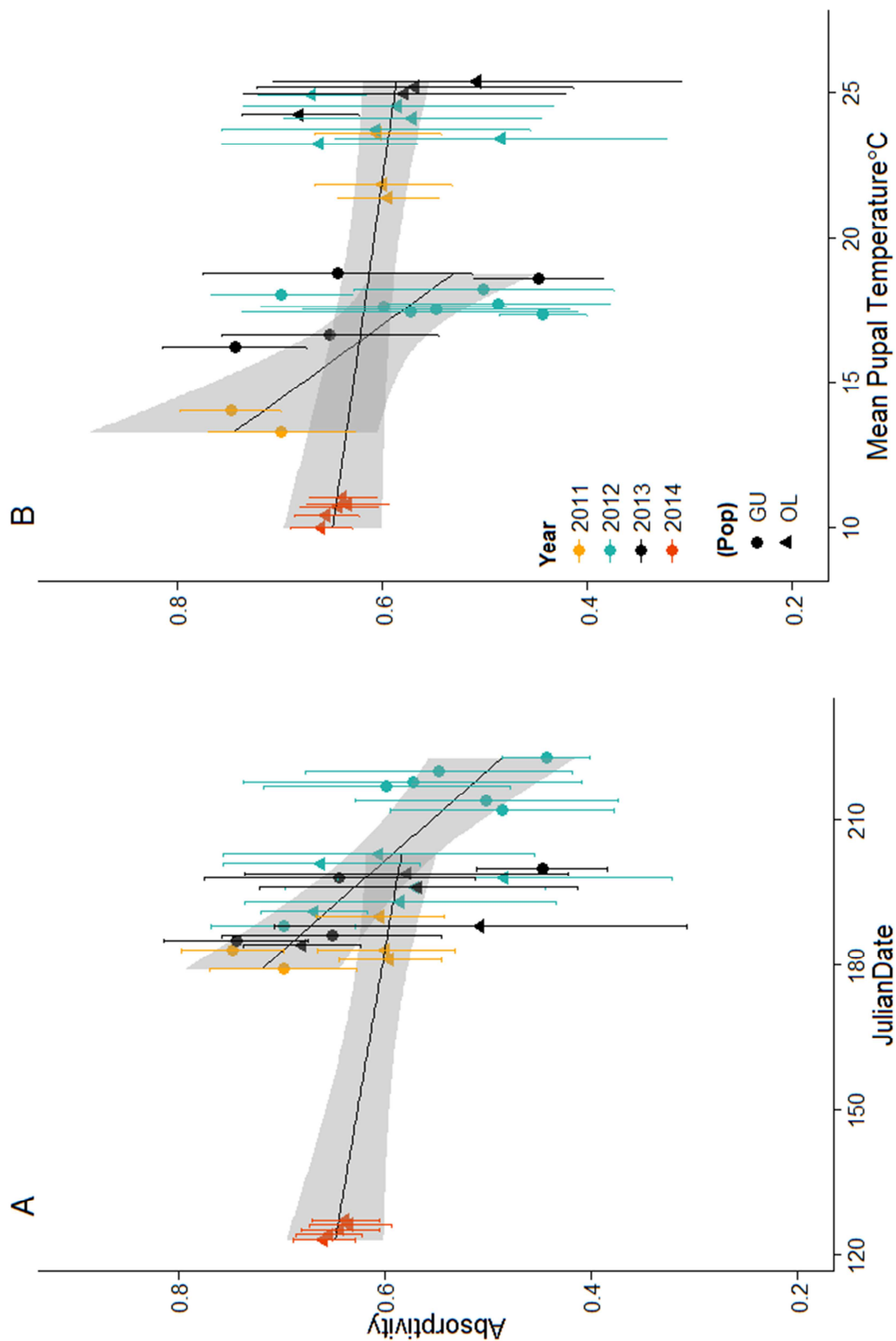
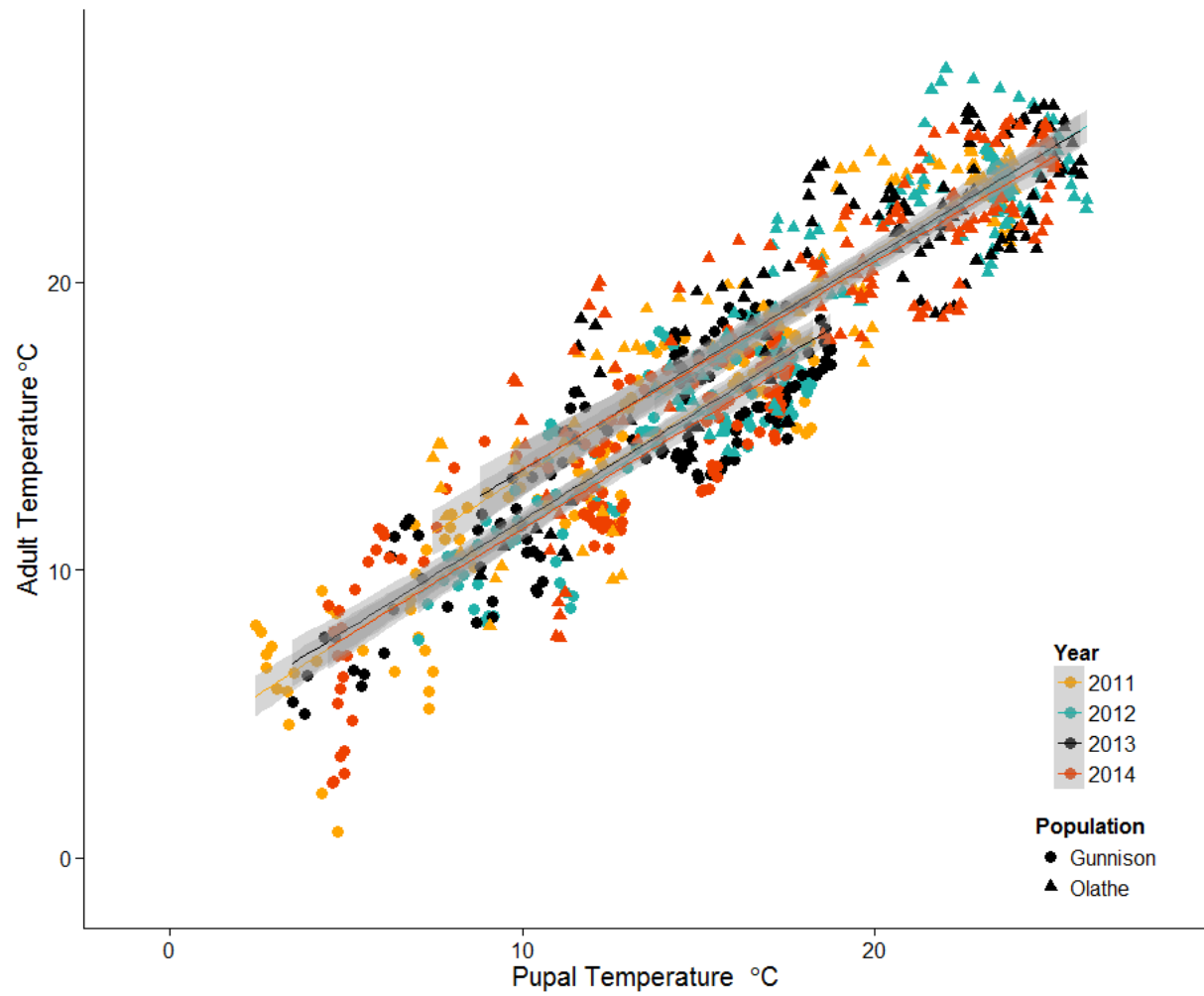


Figure 3



CHAPTER SIX: DISCUSSION OF FINDINGS AND FUTURE DIRECTIONS

Here I used Rocky Mountain *Colias* butterflies to look at adaptation to climate and climate change. Specifically, I quantified: (1) lower and upper thermal limits on flight activity; (2) upper thermal limits on survival of eggs and adults; (3) and historic changes in, and seasonal patterns of, ventral hindwing melanin of adults. First, I quantified the thermal limits on flight activity to evaluate the components of local adaptation of morphology and thermal sensitivity in two species of *Colias*. Then, I looked at high temperature tolerances in both a sessile life stage and a mobile one to look for geographic variation between populations in upper thermal limits across life stages. Because morphological traits are heritable and determine activity and ultimately fitness, I measured museum specimens to look for directional selection in *Colias meadii* traits in response to recent climate warming in this region during the past 60 years. Finally, because pupal temperature also contributes to adult phenotype, most notably in the seasonal variability in *Colias eriphyle*, I used field-caught individuals from early and late season collections to ask if temperatures during the pupal stage were a good predictor of temperatures during the adult flight season.

When activity is crucial to fitness, evolved differences in lower thermal limits are not uncommon (Sunday, Bates & Dulvy 2011) but thermoregulatory behavior tends to lead to conserved thermal limits (Buckley, Ehrenberger & Angilletta 2015). Because *Colias* species have a conserved thermal optima and morphological adaptations across elevations, I looked for differences in the lower and upper limits on activity in adults of two *Colias* species. I found

evidence that species along an elevation gradient in the Rockies exhibit differences in low temperature thermal sensitivity of flight initiation. This finding suggests that knowing the thermal optima may not be sufficient for modeling temperature dependent activity (Buckley & Kingsolver 2012). This approach is useful, especially given the context dependent nature of measuring thermal limits in the lab, but it may under-predict activity at cool temperatures for high elevations.

Although there is documented geographic variation in lower thermal limits across geographic gradients, upper thermal limits tend to be more conserved (Sunday *et al.* 2014), especially in thermoregulating species (Buckley, Ehrenberger & Angilletta 2015). I expected little to no difference in either the body temperature for shade seeking behavior or the survival following high temperature heat shocks even though high elevation organisms see greater diurnal and seasonal temperature swings than lowland organisms (Ghalambor *et al.* 2006). To test this in *Colias*, I looked at the upper thermal limits in adults from the same two species. There was no observed difference between species in the body temperature for behavioral cooling adults from the alpine habitat. There was a difference in high temperature tolerance, contrary to my expectation. High elevation *C. meadii* have higher thermal tolerance than low elevation *C. eriphyle*. Taking both of my studies of thermal limits in concert, we can conclude that high-elevation *C. meadii* has a broader range of temperatures over which it can be active and survive compared to the lower-elevation *C. eriphyle*. This is interesting because behavioral inertia typically slows the evolution of physiological adaptations in thermoregulating organisms (Huey, Hertz & Sinervo 2003). This study highlights how effective thermoregulation and morphological adaptations may not be sufficient for some species to evolve local adaptation to climate across an elevation gradient.

Insects with complex life cycles vary in size, mobility, and thermal ecology across life stages. Consequently, sessile life stages, such as eggs or pupae, may evolve different thermal sensitivities or plasticity to temperature (Loeschcke *et al.* 1997; Kingsolver *et al.* 2011). Here I explored this idea in two ways. First, I used eggs from two population of *C. eriphyle* from two elevations. While many studies have compared geographic differences in thermal limits at a single life stage (reviewed in Sunday, Bates & Dulvy 2011), or thermal tolerances across life stages (reviewed in Bowler & Terblanche 2008)), few studies have explored geographic differences in thermal limits across life stages (but see Krebs & Loeschcke 1995; Hammond & Hofmann 2010; Potter, Davidowitz & Arthur Woods 2011). Because eggs are small and sessile, and because the ambient temperature is different between sites, I expected to see a population difference in the effects of high temperature; however, I found no difference in egg thermal tolerance between populations. Similar findings have been reported in lizards (Angilletta *et al.* 2013), but both studies lack a mechanistic explanation for conserved thermal limits in a sessile stage. This highlights that understanding more about the determinants of upper thermal limits across life stages and across geographic populations and species is important for understanding the biological responses to recent and future climate changes (Deutsch *et al.* 2008; Kingsolver, Diamond & Buckley 2013).

I also looked at the plasticity in adult thermoregulatory traits in response to changes in pupal temperatures in the field in two ways. First, I used museum specimens over the last 60 years for *C. meadii*, a univoltine species, and related ventral wing melanin to pupal temperature. Second, I used field samples of *C. eriphyle*, a multivoltine species, collected over the course of the flight season and related wing melanin to pupal temperature. In both cases, I observed a significant negative relationship to pupal temperature for ventral hind wing melanin such that

colder years or seasons produce darker individuals. This suggests phenotypic plasticity in response to developmental temperatures. However, it is not clear if this plasticity is adaptive.

Environmental variability can limit the evolution of adaptive plasticity in insects and other taxa (Kingsolver & Huey 1998). In *Colias*, morphological adaptations in ventral hind wing melanin, which increase the absorptivity of solar radiation, and thorax setal, length which retains heat, vary with elevation (Watt 1968). The predictability of the environment determines when morphological adaptations and plasticity in these thermoregulatory traits are adaptive. In the case of *C. eriphyle*, there is a strong correlation between pupal air temperatures and adult air temperatures over the course of the season suggesting that they are currently exhibiting adaptive plasticity. For *C. meadii*, the correlation between adult air temperatures and trait values is weaker over time and space. There are two important distinctions between these species; *C. eriphyle* is multivoltine and within habitat heterogeneity is high, *C. meadii* is univoltine and their habitat is more uniform with greater fluctuations in temperature, solar radiation and precipitation over the course of their flight season. Thermoregulatory traits are essential to activity and ultimately fitness. From other work done in this system, we know that hind wing melanin is, in part, controlled both plastically and genetically, making it a target for selection under climate change (Hoffman 1978; Ellers & Boggs 2002). With climate change over the last 60 years, we expected to see a decrease in ventral hind wing melanin in *C. meadii*. To test this, we sampled museum specimens of *C. meadii* from across the species range. Focusing our attention on the southern part of the range, we looked over time and saw that *C. meadii* appeared to be increasing in melanin over time. We then related this to flight temperature and saw that there is no significant relationship with melanin but a significant relationship with thorax fur, suggesting that phenotypes being produced do not necessarily match the environments being experienced. It was

only in relating ventral hind wing melanin to pupal temperatures, that we saw a significant relationship. These data suggest plasticity may help maintain high trait variation across the species range. This study highlights how critical the lag time between an environmental cue and the resultant phenotype is in determining how adaptive the phenotypic plasticity is (DeWitt, Sih & Wilson 1998).

I set out to understand how morphological, behavioral, and physiological mechanisms contribute to adaptation to climate and climate change. I used the *Colias* butterfly system as a model because they exhibit all three adaptations. I found that high elevation sites, with their short vegetation and variable temperatures, have butterflies with a broader range of thermal performance and a high degree of trait variability. Whereas low elevation sites, with their tall vegetation and heterogeneous microclimates, have butterflies with a narrower range of thermal performance and more predictable seasonal trait variation owed in part to multiple generations per year. Understanding the mechanisms of adaptation to local climate and climate change are essential to making accurate predictions of how organisms will respond to an unpredictable future. By comparing sister species that have a varying degrees of predictability in their environment over development and flight, I found that less predictability leads to broader thermal performance and greater trait variability. Thus, environmental variability can limit local adaptation to current climatic conditions, but it may help buffer high elevation species from some of the negative effects of ever increasing variability under climate change.

Future directions:

While this work addressed questions of local adaptation to climate and climate change, some questions remain. Based on my analysis of *C. m. meadii* over the past 60 years, it seems that pupal temperature may be partially controlling melanin deposition in the chrysalis. Because

we have not successfully reared *C. meadii* to pupation, this was not something we were able to test experimentally; however, this could be done in the field. Using the methods outlined in Hayes to mark eggs laid in the field (1981), eggs from a single female could be flagged, caged, and allowed to overwinter in their natal environment. Then, in early spring, the pupae could be retrieved and brought into the lab. To my mind, there are two questions: 1) Does temperature in fact determine melanin in alpine species and 2) how long is the critical window of temperature exposure that determines melanin deposition in the imaginal disc? To address this critical window, gene expression studies could be performed on the relevant region of the imaginal disc in sister individuals exposed to different temperatures. Hoffman alluded to the idea that *Colias* are making “last minute” adjustments to their phenotypes (1978). How last minute determines the length of the lag time between the developmental environment and adult experience which can determine the quality of the developmental cue (DeWitt, Sih & Wilson 1998).

Environmental variability and season length are important factors in the local adaptation in these species. My work on the differences in upper thermal limits for different populations raises important questions about habitat heterogeneity, population connectivity, and geographic differences in between life stage thermal tolerances. For *C. eriphyle*, I suggest microhabitat variability as a possible explanation for both the conserved upper thermal limits in eggs and the increased trait variability in adults. Measuring the microhabitat variability through the season at these sites would be the first step in validating this hypothesis. Because I observed no significant differences in behavior, patterns of variation in hindwing melanin, or upper thermal limits in eggs or adults between the two populations of *C. eriphyle* measuring the gene flow between these populations may help explain why. Finally, while I observed no geographic difference in cross-stage thermal tolerances in *C. eriphyle*, I think that looking across a greater geographic

disparity may yield different results. I believe that clinal populations of *Colias* or other holometabolis insects may exhibit interesting cross-stage differences in thermal tolerance. Furthermore, I believe that understanding these geographic patterns may be important in predicting population growth under climate change.

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