Physiological ecology of stress in a terrestrial ectothermic vertebrate (garter snakes, Thamnophis spp.)

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Physiological ecology of stress in a terrestrial ectothermic vertebrate
(garter snakes, Thamnophis spp.)

by

Kaitlyn Grace Holden

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Ecology and Evolutionary Biology

Program of Study Committee:
Anne M. Bronikowski, Major Professor
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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa

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ABSTRACT

Physiology mediates how organisms interact with and are constrained by their environment. Understanding the variation in physiological responses to normal and extreme environmental conditions, particularly thermal variation, is critical for predicting how organisms will respond to environmental change. As global temperatures rise, species are experiencing increased incidence of stressful climatic conditions at the local scale, including increased temperatures, increased variance in environmental conditions on seasonal and annual bases, and higher incidence of extreme weather events. Changes in the environment can result in alterations at every level of biological organization, from genetics to physiology to demography, and may have consequences for adaptation to stress and related phenotypes. Moreover, such variation in physiological responses to stress – within and among individuals, populations and species – may influence how populations will adapt, acclimate, or face extinction in rapidly shifting climatic conditions. Climatic variability is particularly significant for ectotherms, as thermal profiles and water availability are direct determinants of their performance and are often intimately linked to life history, making them vulnerable to environmental change.

Using two species of widespread North American ectotherms (garter snakes Thamnophis elegans and Thamnophis marcius), I examined the broad influence of temperature on aspects of physiology in lab studies, and then investigated climatic influences on physiology in the wild. In the first study, I tested for thermal effects on a number of physiological measures: metabolic rate ($\dot{V}_{O_2}$), stress physiology (corticosterone levels, and ratio of heterophils to lymphocytes in circulation), and energy availability (glucose and insulin levels) before, during, and after a
simulated brumation in the checkered garter snake (*Thamnophis marcianus*). My results demonstrate not only a temperature dependence across physiological axes, but seasonal variation in thermal responsiveness. In the second study, I found that measures of both whole-animal metabolic rate (\(V_{O_2}\)) and cellular oxygen consumption rate (OCR) increased with increasing temperature but at different rates. Additionally, within any given temperature the measures of metabolic physiology were not correlated within individuals. These findings show that, cellular metabolism does not directly dictate whole-animal oxygen consumption, yet both have substantial phenotypic plasticity across temperature. In the final study, natural populations of garter snakes (*Thamnophis elegans*) that are characterized by divergent life-history strategies displayed significant annual variation in measures of baseline and stress-induced plasma corticosterone and blood glucose concentrations, and in their reactivity (the magnitude of change between baseline and stress-induced measures), in an ecotype-dependent manner across a 10-year study. Moreover, the effects of precipitation and temperature also manifested in an ecotype-specific manner. This suggests that physiological regulation of stress via glucocorticoids and glucose may not be the most important mechanism by which garter snakes in this system deal with large-scale environmental perturbations such as drought. Instead, ecotypic differences are likely driving physiological responses to stress, and climate indirectly influences these ecotypes through their distinct life histories.
CHAPTER 1
INTRODUCTION

Since Levins’ seminal work on evolution in changing environments – and how the predictability in ecological heterogeneity impacts the evolution of phenotypes – understanding the evolutionary and ecological causes in the variation in organismal responses to stress (both acute and chronic) has been a fundamental theme in evolutionary biology (Levins 1968). How individuals, and by extension populations, respond to stress in their environment is an active area of research with implications for both conservation (Baker et al. 2013; Dantzer et al. 2014; Wikelski and Cooke 2006) and evolutionary processes (Crespi et al. 2013; Patterson et al. 2014).

Physiology mediates how organisms interact with and are constrained by their environment. Organisms are buffered against the potential negative impacts of adverse or “stressful” conditions through activation of a variety of mechanisms which collectively make up the stress response. A primary characteristic of the vertebrate stress response is the upregulation of the hypothalamic-pituitary-adrenal/interrenal (HPA/HPI) axis (Greenberg and Wingfield 1987). In response to a real or perceived stressor (e.g., changes in thermal conditions, predation events, food deprivation), glucocorticoid steroid hormones are released into circulation (i.e., cortisol [in mammals and fish] or corticosterone [in rats and reptiles, including birds]; “CORT” hereafter) providing a biomarker of the activation of the HPA/HPI axis (Romero and Wikelski 2001). It is well understood that the effects of glucocorticoids, specifically CORT, are context dependent and thus can have different effects under varying conditions. However, CORT primarily acts to mediate daily and seasonal metabolic processes such as the acquisition, storage,
and utilization of energy stores (Johnstone et al. 2012; Landys et al. 2006; Sapolsky et al. 2000). In concert with catecholamines, such as epinephrine and norepinephrine, stress-induced concentrations of CORT can also activate downstream pathways that mobilize or prevent the uptake of additional biomarkers, such as glucose (“GLUC” hereafter) by initiating processes of glycogenolysis and gluconeogenesis (reviewed in Sapolsky et al. 2000). Under homeostatic conditions, insulin reduces the production, and promotes the uptake, of glucose into tissues (Strack et al. 1995). Thus, insulin and CORT serve as antagonistic, long-term regulators of energy acquisition and storage. However, glucocorticoids can also stimulate insulin secretion. Thus insulin may participate in organismal responses to stress in a glucose-independent manner, such as has been similarly reported in mammals (Baumgard and Rhoads 2013). Accordingly, in response to stress, circulating levels of CORT, GLUC and INS generally covary (e.g., Chapter 2; Gangloff et al. 2016; Gangloff et al. 2017) and provide biomarkers of both the activation of the HPA/HPI axis (CORT) and its downstream effects on energy mobilization (GLUC and INS) (Romero and Wikelski 2001). Additionally, glucocorticoids can act to redistribute leukocytes, with increased release of heterophils into circulation and movement of lymphocytes into peripheral tissues (Dhabhar et al. 1996; Dhabhar et al. 1994; Vleck et al. 2000). This redistribution of immune cells is thought to be a preparatory shift to mitigate negative effects of stressful encounters (Goessling et al. 2015; Sapolsky et al. 2000). Stress-induced elevations of physiological biomarkers can shift the physiological state of an individual as a means of adaptively maintaining homeostasis (Greenberg and Wingfield 1987; Romero et al. 2009), increasing/enhancing physiological function (e.g., increased respiratory rate and cardiovascular tone) and behavior (e.g., increased awareness and cognition, enhanced analgesia) while temporarily diminishing/suppressing feeding, digestion, growth, reproduction and immunity
(McEwen and Wingfield 2003; Palacios et al. 2012; Sapolsky et al. 2000). Shunting resources from these latter processes in the short-term can increase an individual’s chances of survival; thus, the magnitude of increase in circulating biomarkers, such as glucocorticoids, glucose, and insulin, are indicators of the reactivity or sensitivity of an individual to a given stressor (Romero 2004). However, prolonged and/or extreme elevations in biomarkers of the stress response can reduce an individual’s fitness, induce pathology, or lead to population level decline (Romero and Wikelski 2001; Sapolsky et al. 2000).

Variation in stress responses can have consequences for evolutionary adaptation to stress and related phenotypes, and may be shaped by the environment. Moreover, such variation – within and among individuals, populations and species – may influence how species will acclimate, adapt, or go extinct in currently changing environments. Global climate change has profoundly affected terrestrial organisms across diverse landscapes (Parmesan 2006; Walther et al. 2002). With the rapid changes in climate, species are experiencing increased incidence of stressful climatic conditions, including not only increased temperatures and increased variance in environmental conditions both on seasonal and annual bases, but also higher incidence of extreme events (Stott 2016). Exposure to prolonged periods of environmental disturbance or highly variable environments are likely to affect organisms either directly, such as through effects on growth, reproduction and survival, or indirectly, through habitat alteration or access to resources (Parmesan 2006; Walther et al. 2002). Depending on the behavioral and physiological abilities of an individual to buffer against environmental perturbations, local conditions may result in altered community dynamics, geographic range, phenology, or declines in population persistence (Bay et al. 2018; Janzen et al. 2018; Urban et al. 2014). The effects of thermal stress in particular are important as global temperatures continue to warm (IPCC 2014). Temperature,
mediated through physiology, affects how organisms interact with and are constrained by their environment (Angilletta 2009; Huey 1982; Huey et al. 2012). Measures of energetics, such as metabolic rate, are highly temperature dependent and are governed through temperatures effect on rates of biochemical reactions (Gillooly et al. 2001). The effects of temperatures, both above and below a species’ optimal range, on biochemical and metabolic processes can lead to disruptions of homeostasis and declines in performance (Angilletta 2009; Huey and Kingsolver 1989). Thermal performance curves (TPC) can be used to describe the temperature dependence of physiological performance. As global climate warms, it becomes increasingly important to quantify the thermal reaction norms of metabolic and hormonal function to gain a more mechanistic understanding of how populations will respond to environmental stressors (Buckley et al. 2012).

Climatic variability is particularly significant for reptiles, as thermal profiles and water availability are direct determinants of their performance (Angilletta 2001; Huey 1982) and are often intimately linked to life history (Janzen 1994), making them particularly vulnerable to environmental change (Deutsch et al. 2008; Huey et al. 2012). At extreme temperatures (both above and below a species optimal range) individuals are unable to maintain homeostasis and performance declines (Angilletta 2009; Huey and Kingsolver 1989). This is particularly prominent in ectotherms that have limited ability to metabolically control their body temperature, and thus their body temperature is fundamentally associated with the thermal conditions of their environment (Angilletta et al. 2002; Avery 1982; Peterson 1987).

Garter snakes (Thamnophis spp.) are relevant models in the areas of ecology, evolution, behavior and physiology (Castoe et al. 2011). They have a broad geographic range across much of North America (Rossman et al. 1996), and thus have adapted to a vast array of environmental
conditions, food sources, and predation pressures (e.g., Arnold 1981a; Burghardt and Schwartz 1999; Kephart and Arnold 1982). Additionally, garter snakes are relatively long-lived, exhibit indeterminate growth (i.e. size), and, within species, there are examples of divergences in life-history strategies (Bronikowski and Arnold 1999). These characteristics make garter snakes ideal study subjects for studying the broad importance of thermal tolerance and how physiology can react to reduce thermal stress. I used established laboratory colonies of two species of garter snakes (western terrestrial garter snakes: *Thamnophis elegans*, and checkered garter snakes: *Thamnophis marcianus*) to test the effects of ecologically relevant ranges of temperatures, both above and below the species optimum, on aspects of their physiology. Additionally, to investigate climatic influences on physiology in the wild, I used natural populations of western terrestrial garter snakes (*Thamnophis elegans*) in the Eagle Lake basin of Lassen County, California, US, whose ecology and behavior have been studied for more than 40 years (Arnold 1977; 1981b). Here, two distinct ecotypes have evolved along the pace-of-life continuum that differ in their life-history strategies, resource availability and predation pressure, and numerous physiological measures (e.g., Bronikowski and Arnold 1999; Reding et al. 2016; Sparkman et al. 2009). Populations of a fast-living ecotype live around the lakeshore and exhibit fast growth, high annual reproduction and low annual survivorship; in contrast, populations of a slow-living ecotype reside in nearby montane meadows and exhibit slow growth, low annual reproduction, but high annual survival (Bronikowski and Arnold 1999; Sparkman et al. 2013). The selective forces driving these ecotypic differences include local climate, thus providing a unique opportunity for a comparative study of the ability of divergent populations to respond to differing climates. These populations in this long-term study system provide an ideal natural laboratory to test how physiological plasticity within and among individuals, as well as among populations,
responds to environmental change and predicts local population extinction. Persistent drought conditions have led to major changes in the hydrology of the study area, resulting in many local population extinctions within the garter snake study system. Yet other populations in the system are thriving despite close geographic proximity (25 km²). This study system provides a unique opportunity to test the premise that physiology can determine a species’ ability to survive a changing environment.

**Dissertation Organization**

**Chapter 2 Physiological regulation of energy balance beyond simple temperature dependence in an ectotherm during winter dormancy**

This chapter characterizes the physiological response to prolonged cold exposure, and its effects on the maintenance of long-term energy balance, in the checkered garter snake (*Thamnophis marcianus*). We tested the effects of environmentally-relevant temperatures for overwintering preparation and emergence on measures of physiology by characterizing how these processes integrate and thereby facilitate seasonal brumation. We found temperature-specific effects on measures of oxygen consumption, such that \( \dot{V}_{\text{O}_2} \) monotonically decreased with decreasing temperature, remained depressed during brumation, and increased with increasing temperature following brumation. Additionally, post-brumation measures of oxygen consumption were depressed relative to pre-brumation measures at the same temperature, suggesting a decrease in the thermal sensitivity of \( \dot{V}_{\text{O}_2} \) following extended periods of cold exposure. In contrast, measures of corticosterone, insulin, and heterophil-lymphocyte ratios exhibited the opposite pattern of metabolic rate and responded similarly to pre- and post-brumation temperatures (increasing with decreasing temperature), but with differing thermal
response curves among measures. Concentrations of plasma glucose did not change under acute cold exposure pre-brumation, but declined during the sustained cold exposure of brumation and rose with increasing temperature. Our results suggest coordination among physiological axes to mitigate deleterious effects of extended cold exposure as experienced during winter dormancy. The integrated physiological state presented here demonstrates not only the existence of temperature dependence across physiological axes, but seasonal variation in thermal responsiveness.

Chapter 3 Temperature dependence of metabolic fuel selection and oxygen consumption in cells and whole organisms

This chapter examines how temperature dependence of whole-organism metabolic rates and cellular rates of oxygen consumption covary in the western terrestrial garter snake (*Thamnophis elegans*) during acute exposure to a range of environmentally relevant temperatures. Characterizing the temperature-dependence of rates of oxygen consumption across levels of biological organization gives insight into the range of active temperatures that influence whole-organism performance. We found that metabolic rate at the level of the whole animal, as well as cellular rates of basal and ATP-linked respiration, increased with temperature. However, maximal rates of cellular respiration were temperature-insensitive. Although whole-organism and cellular measures of oxygen consumption rate demonstrated temperature dependence, they did not exhibit intra-individual covariation – they both increased with temperature but did so differently. Additionally, we examined, but found no evidence that temperature influenced the dominant fuel source (i.e., carbohydrates, lipids, proteins) oxidized to support metabolism. The difference in the way these measures of respiration respond to temperature suggests that the
increase in \( V_{O_2} \), is not simply driven by a shift in cellular oxygen consumption rates, but instead are governed by other processes at a larger scale.

Chapter 4 A decade of stress physiology reveals life-history specific responses to habitat change in garter snakes (Thamnophis elegans)

This chapter incorporates a decade of physiological data to quantify the effects of climate on a natural system that has been characterized by extended and repeated cycles of severe drought. Using divergent-life history ecotypes of western terrestrial garter snakes (Thamnophis elegans) we tested for annual variation in stress physiology and whether that variation was consistent with the expectations of life-history strategies. Additionally, we examined whether patterns of intra- or inter-annual variation in physiology were predicted by aspects of local climate. We found significant annual variation in baseline and stress-induced measures of plasma corticosterone, baseline blood glucose, as well as in the reactivity of both corticosterone and glucose (i.e., the magnitude of change between baseline and stress-induced measures, \( \Delta \text{CORT} \) and \( \Delta \text{glucose} \)) within both ecotypes. Importantly, yearly variation in aspects of physiology (baseline CORT, baseline glucose and \( \Delta \text{glucose} \)) were explained, to some extent, by climate, but only in the context of ecotype. Additionally, we found within-year differences between ecotypes. Specifically, M-slow snakes exhibited higher levels of both baseline and stress-induced CORT, while L-fast snakes exhibited higher levels of glucose for both baseline and stress-induced measures. Overall, our results show that there is significant inter- and intra-annual variation in this system. However, this variation is not explained, at least directly, by variation in temperature and precipitation. This suggests that physiological regulation, via glucocorticoids or glucose, is likely not the mechanistic means by which garter snakes in this system deal with large-scale environmental perturbations such as drought. Instead, ecotypic differences are likely driving
physiological responses, and climate may indirectly influence these snakes through the
differences in habitat utilized by each ecotype.

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CHAPTER 2

PHYSIOLOGICAL REGULATION OF ENERGY BALANCE BEYOND SIMPLE TEMPERATURE DEPENDENCE IN AN ECTOTHERM DURING WINTER DORMANCY

Kaitlyn G. Holden, Eric J. Gangloff, Anne M. Bronikowski

Abstract

Characterizing the physiological response to prolonged cold exposure is essential for understanding the maintenance of long-term energy balance. As part of their natural life cycle, temperate ectotherms are often exposed to seasonal variation in temperatures, including extended periods of cold well outside of their activity range. Relatively little is known about variation in physiological responses as animals enter and exit brumation during sustained cold temperatures. We tested the influence of temperature on physiology before, during, and after a simulated brumation in the checkered garter snake (*Thamnophis marcianus*), a widespread ectothermic vertebrate. We tested for thermal effects on metabolic rate (\(\dot{V}_{O_2}\)), measures of physiology and energy availability. Our results demonstrate a temperature dependence of measures of plasma corticosterone, glucose, and insulin, as well as immune cell heterophil: lymphocyte ratios. Though physiological measures all responded to temperature, they did so with different thermal response curves. This was evident both among variables and within variables depending on whether animals were going into or coming out of brumation. Additionally, \(\dot{V}_{O_2}\) responded to temperature such that pre- and post-brumation thermal response curves differed in a manner suggesting reduced thermal sensitivity of metabolic rate following a period of extended cold...
exposure. The integrated physiological state presented here demonstrates not only a temperature
dependence across physiological axes, but seasonal variation in thermal responsiveness.

**Introduction**

Many temperate organisms experience predictable annual cycles of temperatures, which
includes a season below their activity threshold (Williams et al. 2015). Lower temperatures
associated with this “winter” season often preclude normal function for ectotherms, as
temperatures outside of an organism’s optimal range reduce physiological and behavioral
performance (Huey and Stevenson 1979). Therefore, ectotherms often stay inactive or dormant
for extended periods of the year when temperatures are too cold for normal activity (Stevenson
1985; Ultsch 1989). This period of dormancy (“brumation” hereafter) is described by lowered
metabolism, heart rate, and energy demands in ectotherms (Voituron et al. 2000; Williams et al.
2015). In this way, many animals mitigate low temperatures and negligible food availability that
may otherwise be lethal (Capraro et al. 2019; Fanter et al. 2020; Ultsch 1989). In temperate non-
avian reptiles (hereafter “reptiles”), as in other taxa, extended periods of brumation prompt a
complex adaptive physiological phenotype and not simply a cold-induced torpor (Gregory 1982).
Thus, an understanding of the physiological processes associated with seasonal body cooling,
brumation, re-warming, and reperfusion are critical for understanding how animals cope with
extended periods of cold, in general, and variation in the temperature profiles and duration of
winter specifically. How animals generally cope with extended periods of cold is especially
relevant, as historical temperature profiles and duration of the winter season are severely
interrupted by changing climates (IPCC 2007; Williams et al. 2015).
Coordinated physiological systems are expected to respond to temperature in order to facilitate an animal entering, enduring, and emerging from brumation. In this view, the animal exhibits an integrated physiological state that includes alterations in hormones, energy reserves, blood cell counts, and respiratory capacities. We hypothesized that ectothermic vertebrates experiencing brumation will exhibit a coordination of: (i) metabolism, (ii) the hypothalamic-pituitary-adrenal/interrenal axis (HPA/HPI axis), (iii) regulation of glucose as a primary energy metabolite, (iv) insulin dynamics, and (v) circulating immune cells. Temperature governs metabolism by affecting rates of biochemical reactions; extended periods of cold temperatures are characterized by metabolic depression (Gregory 1982; Hailey and Loveridge 1997; Patterson and Davies 1978). This seasonal metabolic depression can be considered an inverse acclimation, where an animal not only conforms to the decreased environmental temperature but actively suppresses metabolic processes (Tsuji 1988), which serves to conserve energy (Patterson and Davies 1978). Increasing circulating levels of plasma glucocorticoids, such as corticosterone in reptiles (“CORT” hereafter), in response to non-optimal temperatures is likely an important driver (Greenberg and Wingfield 1987; Romero et al. 2009). Both temperature and glucocorticoid production directly affect metabolism (Bradshaw 2003; DuRant et al. 2008; Preest and Cree 2008). Glucocorticoid production, resulting from changes in the regulation of the HPA axis, mediates daily and seasonal metabolic processes such as acquisition, storage, and utilization of energy stores (Johnstone et al. 2012; Landys et al. 2006; Romero 2002; Romero and Butler 2007; Sapolsky et al. 2000). In temperate ectotherms, which endure extended periods of cold exposure (i.e., seasonal period of dormancy), CORT levels are elevated relative to baseline concentrations and oppose depressed metabolic rates under cold exposure (Brischoux et al. 2016; Dupoué et al. 2013). Circulating levels of plasma glucose positively covary with CORT at warm
temperature (Gangloff et al. 2016), but temperate ectotherms also exhibit substantially reduced levels of glucose during winter dormancy when metabolic rates are depressed (Haggag et al. 1966a). Under homeostatic conditions, insulin reduces the production, and promotes the uptake, of glucose into tissues (Strack et al. 1995). Thus, insulin and CORT serve as antagonistic, long-term regulators of energy acquisition and storage. However, glucocorticoids can also stimulate insulin secretion. At temperatures above a species’ thermal optimum, insulin and glucose simultaneously increase alongside rising concentrations of CORT (Gangloff et al. 2016). Thus insulin may participate in organismal responses to extreme temperatures in a glucose-independent manner, such as has been similarly reported in mammals (Baumgard and Rhoads 2013). Finally, glucocorticoids can act to redistribute leukocytes, with increased release of heterophils into circulation and movement of lymphocytes into peripheral tissues (Dhabhar et al. 1996; Dhabhar et al. 1994; Vleck et al. 2000). This redistribution of immune cells is thought to be a preparatory shift to mitigate negative effects of stressful encounters (Goessling et al. 2015; Sapolsky et al. 2000).

To characterize how physiological processes integrate and thereby facilitate seasonal brumation in long-lived ectotherms, we quantified the thermal response curves of physiological markers over a 6-month cooling, brumation, and warming experiment. An animal’s physiological state was represented by the integrated physiological phenotype comprised of whole-organism oxygen consumption ($\dot{V}_{O2}$); levels of circulating CORT, glucose, and insulin; and heterophil:lymphocyte ratios (H:L) in whole blood. We conducted this experiment in a research colony of the widespread temperate checkered garter snake (*Thamnophis marcianus*). If these axes of physiological function coordinate brumation, we expect the thermal response curves to covary. Specifically, we predict that:
1. \( \dot{V}_{O_2} \) will decrease with cooling, remain low, and increase with warming,

2. Circulating CORT will rise with cooling, remain elevated during brumation, and fall again with warming,

3. Circulating glucose will increase with cooling in coordination with CORT, decrease during brumation as metabolism slows, and increase with warming,

4. Insulin, if acting to solely regulate glucose, will remain static or even exhibit increased circulating levels during cooling, decrease during brumation when glucose levels are low, and remain low as temperatures increase during emergence from brumation and glucose is mobilized. Alternatively, if insulin functions outside of its role in glucose regulation, as seen in response to heat exposure, insulin levels will increase in response to cold temperatures and decrease as temperatures warm post-brumation, and

5. H:L ratios will increase with cooling, remain static during brumation when metabolism is low, and decrease with warming.

Finally, we tested whether thermal response curves entering and exiting brumation were mirror images – whether the slopes of increase or decrease varied pre- and post-brumation as a means of elucidating the temperature-dependence of each physiological parameter across a simulated winter season. Symmetrical thermal response curves, pre- and post-brumation, indicate simple temperature-dependence of these physiological parameters, whereas, asymmetrical responses indicate that systems are responding in a plastic manner to their current or anticipated conditions.
Materials and Methods

Study System

We studied the effects of cold on physiological state in the checkered garter snake (Thamnophis marcianus) transitioning into, during, and emerging from winter dormancy. The study subjects were the third generation (F3) offspring of wild progenitors from the southwest United States. In their natural habitat, checkered garter snakes brumate for several months of the year (typically Nov – Jan; Rossman et al. 1996). Study subjects were offspring born in a three-week period in June-July, 2013 from adults paired at the Ophidian Research Colony at the University of Texas, Tyler (see Holden et al. 2019 for details of laboratory husbandry of the parents of these F3 study subjects). Siblings from 22 families were raised in a common-garden environment at Iowa State University from age two weeks until this experiment (age two years). Animals were housed individually with 12:12 light:dark cycle, water available ad libitum, and thawed pinky mice offered weekly. Animal enclosures were situated on heating elements in a 20°C room such that they had a thermal range of 20–32°C in their home cages. Husbandry approximated photoperiod and temperature regimes commonly experienced throughout their range during the active season (Rossman et al. 1996). This common-garden rearing benefits the careful study of physiological processes, as we were able to control temperature, lighting, and feeding, as well as disallow infectious disease.

Experimental Procedure

Our experiment was conducted over 22 weeks from December 2015 through May 2016. Each physiological metric included a maximum of 63 individuals split between males and females (limited blood volume precluded measuring every variable for every animal at every
temperature). Animals were last fed two weeks prior to the start of this experiment so that they were fasted with cleared digestive tracts. Supplemental heating elements were turned off one week prior to the start of the experiment at which point the environmental chamber was used to control temperature. Animals were measured (SVL in mm) at the start of the experiment and were weighed (mass in grams) twice pre-brumation (at 20°C and 5°C) and twice post-brumation (at 5°C and 20°C). Animals were maintained for six days at each temperature, pre-brumation, in the following order: 20°C, 15°C, 10°C, 5°C for a total of 24 days. Animals were then maintained at 5°C for 12 weeks of brumation. After 12 weeks, temperature was stepped up to 5°C, 10°C, then 15°C with animals spending six days at each temperature, consistent with the pre-brumation protocol (see Figure 1 for experimental timeline). After the final measurements at 15°C, animals were warmed to room temperature, and supplied with supplemental heat and two weeks of feeding. We then turned off supplemental heat for one week and conducted the 6-day experimental protocol on the animals at 20°C. This approach mirrors the pre-brumation procedure at 20°C and allowed us to compare whether initial feedings post-brumation returned measures of physiology to a pre-brumation state.

Each 6-day temperature exposure followed the protocol: acclimate for days 1 and 2 in home cages; draw small blood sample (50 µL from caudal vein using a heparin-rinsed syringe) on day 3 for measurement of hormones, metabolites, and leukocytes; measure whole-organism rates of oxygen consumption on days 4-6. As increases in glucocorticoids, above baseline, occur in garter snakes after 10 minutes of handling (Palacios et al. 2012) all blood samples were obtained within 10 minutes of handling. Handling time (3.4 ± 2.7 minutes) did not affect CORT ($F_{1, 436} = 0.34, P = 0.5609$) and thus was not included in additional analyses. Blood smears, for
leukocyte counts, were prepared from a drop of freshly drawn blood and the remaining whole blood was centrifuged, plasma aliquoted, flash-frozen, and stored at -80°C until analysis.

**Oxygen Consumption Rate**

We quantified $\dot{V}_{O_2}$ using a stop-flow respirometry system as a proxy for metabolic rate—a measure of steady state maintenance in post-absorptive individuals at rest (Bennett and Dawson 1976). At each experimental temperature, metabolic rate was measured on 54-63 animals over days 4-6 (nine batches, three batches per day). Each respirometry run included 6-7 animals and a blank (empty chamber to measure baseline values). Animals were acclimated overnight in experimental chambers (500 mL glass jars custom-fitted with in- and out-flow tubing). A flow rate of 400 mL/min was maintained by an MFS-2 mass flow generator and cycled through the eight chambers with an RM-8 flow multiplexer (Sable Systems International, Las Vegas, NV, USA). Animals were held, undisturbed, for 61-minute or 93-minute intervals (pre- and post-brumation, respectively) before reestablishing air flow and measuring oxygen content. Post-brumation hold time was increased to more precisely measure metabolic rates given the low levels of gas exchange exhibited by these animals following extended exposure to cold temperatures. Results from trial animals, run under both hold intervals (post-brumation) showed no bias in measurements of $\dot{V}_{O_2}$ for either length of time ($t_s = -0.462$, $P = 0.676$). Air outflow was run through a Drierite drying column, scrubbed of carbon dioxide with soda lime, and analyzed for proportion of oxygen with Sable Systems CA-10 analyzer. Volume of oxygen consumed was corrected for variation in barometric pressure, then converted to rates (mL/hour) of oxygen consumed ($\dot{V}_{O_2}$) using Sable System’s ExpeData software (Sable Systems; N = 482 individual measurements).
**Corticosterone**

We measured CORT using a double-antibody radioimmunoassay (ImmuChem Double Antibody Corticosterone I-125 RIA kit, MP Biomedicals, Irvine, CA, USA). The assay was conducted following previously described protocols adapted for use with garter snakes (Palacios et al. 2012). All samples ($N = 465$) were run at 1:80 dilution in duplicate with a pooled sample run with each batch to assess intra-assay (average coefficient of variation (CV) of 3.13% within a given assay) and inter-assay variability (CV of 10.3% across assays). Sample duplicates with a CV > 10% were rerun as were any samples outside of the standard curve.

**Glucose**

The concentration of plasma glucose was measured using a colorimetric assay (Autokit Glucose C2, Wako Chemicals, Richmond, VA, USA) following manufacturer’s protocols with slight modification to sample (4 µL) and reagent (150 µL) volumes. All samples ($N = 330$) were run in triplicate on 16 plates. A pooled sample was run on each plate to assess intra-plate variability (average within plate CV of 5.0%) and inter-plate variability (CV of 7.6% across plates).

**Insulin**

Circulating levels of plasma insulin were measured using a radioimmunoassay (catalog no. SRI-13K, EMD Millipore, St. Louis, MO, USA) following manufacturer’s protocols, except that sample and reagent volumes were reduced by one-half. To validate this kit for use with garter snakes we tested the parallelism of kit-provided controls with a serially diluted sample
(pooled sample from multiple individuals) from our species of interest (no difference in slopes; $F_{3, 6} = 77.1, P = 0.58$). Although caution is warranted for assessing absolute concentrations from a heterologous assay, this method is valid for interpreting patterns of relative change. Due to low plasma volume, samples were pooled by sex within each temperature. All pooled samples ($N = 120$, representing 705 individual samples) were run in duplicate and the quality control pooled sample was run with each batch to assess intra-assay (average within batch CV of 3.8%) and inter-assay variability (CV of 4.8% across batches).

**Differential White Blood Cell Counts**

Blood smears were prepared from a drop of freshly drawn blood. Smears were fixed in methanol and stained with Wright-Giemsa stain. Relative abundances of leukocytes (i.e., lymphocytes, heterophils, monocytes, and basophils) were estimated by classifying the first 100 leukocytes encountered with a compound microscope under oil submersion with the $100\times$ objective (i.e., a total of $1000\times$ magnification; described in Sparkman et al. 2014). The number of heterophils and lymphocytes was used to calculate an H:L ratio for each individual ($N = 462$).

**Statistical Methods**

For analysis of our dependent physiological variables ($\dot{V}_{O_2}$, CORT, glucose, insulin, and H:L), we conducted repeated-measures mixed-effects linear models with the *lme4* package (Bates et al. 2015) in the programming language R (R Development Core Team 2018). Fixed effects were assessed using type III sums of squares using corrected denominator degrees of freedom (Kenward and Roger 1997). Figures were made with the ggplot2 package (Wickham 2009) showing back-transformed least-squares means. To meet assumptions of normality of
model residuals, we log$_{10}$-transformed $\dot{V}_{O2}$ and measures of plasma CORT, insulin, and H:L ratios, and square-root-transformed measures of plasma glucose. Temperature was modeled as a categorical factor, allowing for examination of post hoc linear contrasts. Explanatory variables included the fixed effects of sex, brumation (pre or post period of dormancy), temperature, and their interactions, as well as a covariate of mass (with the exception of our models of insulin, as samples represent pooled individuals). Individual nested within family was modeled as a repeated-measures random effect, with up to eight repeated observations for each individual, thus our models took the following form where $\mu$ represents the grand mean and $\varepsilon$ is the error term:

$$Y \sim \mu + \text{Mass} + \text{Sex} + \text{Brumation} + \text{Temperature} + \text{Sex} \times \text{Brumation} + \text{Sex} \times \text{Temperature} + \text{Brumation} \times \text{Temperature} + 1|\text{Family/Individual} + \varepsilon$$

To test whether physiological measures were responding similarly to temperature before and after brumation, we examined the homogeneity between slopes (i.e., whether or not thermal response curves were symmetrical pre- and post-brumation) and differences in pre-/post-brumation intercepts (as slopes may be similar but differ in elevation). Models took the same form as stated above with the exception that temperature was modeled as a continuous variable. The interaction of pre-/post-brumation and temperature allowed us to test for differences in slope. To test for differences in intercepts, models were run without the interaction term.

Additionally, we calculated the temperature coefficient ($Q_{10}$) for pre- and post-brumation measures of each dependent variable. $Q_{10}$ is a measure of the rate of change of a biological system as a consequence of changing the temperature by 10°C (Clarke 2017). $Q_{10}$ is a unitless quantity that expresses the temperature dependence of biological processes. The $Q_{10}$ is calculated
simply as the ratio of biological measures at two temperatures differing by approximately 10°C using the following equation.

\[ Q_{10} = \left( \frac{R_2}{R_1} \right)^{\frac{10}{T_2-T_1}} \]

Results

Physiological Measures

As expected, oxygen consumption increased with increasing body mass (Table 1). Oxygen consumption decreased as temperature decreased and increased with increasing temperature following brumation \((P < 0.0001; \text{Fig. 2A})\). All measures of blood-borne physiological markers were affected by temperature (all \(P<0.0001\), Table 1). CORT increased with decreasing temperature and remained elevated over the 12-week brumation; CORT rose at 10°C after brumation before decreasing back towards pre-brumation baseline (Fig. 2B). Glucose did not change under short-term exposure to cold temperatures pre-brumation; however, after prolonged exposure to 5°C, circulating levels of glucose were substantially decreased. The depressed glucose levels were maintained through 10°C and began to increase thereafter, though did not increase to pre-brumation levels by the end of the experiment (Fig. 2C). Insulin did not change pre-brumation until animals were exposed to 5°C; as temperatures increased post-brumation, circulating levels of insulin decreased such that by the time animals had acclimated to 20°C there was no difference between pre- and post-brumation baseline measures (Fig. 2D). Heterophil: lymphocyte ratios were affected by temperature such that animals increased their H:L as temperatures decreased. As animals were stepped-up in temperature emerging from
brumation, H:L decreased such that there was no distinguishable difference between measures at 20°C pre- and post-brumation (Fig. 2E).

_Heterogeneity of Slopes_

Measures of \( \dot{V}_\text{O}_2 \), CORT, and glucose were differentially affected by temperature before and after the period of winter dormancy. The rate of change across experimental temperatures (evidenced by model slopes) and their intercepts pre-/post-brumation demonstrate asymmetrical temperature dependence (all \( P < 0.0001 \); Table 2 and Fig. 3A, 3B and 3C respectively). The thermal response curves for circulating levels of insulin and H:L did not differ in their rates of change across temperatures before and after brumation (\( P = 0.987 \) and 0.907, respectively), but did exhibit different intercepts (both \( P < 0.0001 \); Fig. 3D and 3E, respectively). Similarly, \( Q_{10} \) values of \( \dot{V}_\text{O}_2 \), CORT, and glucose demonstrate a difference in temperature dependence pre-versus post-brumation, while measures of H:L and insulin suggest a similar response to changes in temperature whether entering or exiting brumation (Table 3).

_Discussion_

We tested the effects of environmentally-relevant temperatures for overwintering preparation and emergence on measures of physiology by characterizing how these processes integrate and thereby facilitate seasonal brumation. Our results suggest a temperature-dependence of all physiological variables measured, but with differing thermal response curves both among physiological measures and depending if animals were entering or exiting brumation. Measures of metabolic rate responded monotonically to temperature but in an opposite pattern than measures of blood physiology, with \( \dot{V}_\text{O}_2 \) decreasing with decreasing
temperature, and increasing with increasing temperature, pre- and post-brumation respectively. Additionally, post-brumation measures of oxygen consumption were depressed relative to pre-brumation measures at the same temperature, suggesting a decrease in the thermal sensitivity of $\dot{V}_{O_2}$ following extended periods of cold exposure. In contrast, measures of CORT, insulin, and H:L exhibited the opposite pattern of metabolic rate. Concentrations of plasma glucose did not change under acute cold exposure pre-brumation, but declined during sustained cold exposure and rose with increasing temperature.

We found temperature-specific effects on measures of oxygen consumption, such that $\dot{V}_{O_2}$ monotonically decreased with decreasing temperature, remained depressed over the 12-week period of brumation, and increased with increasing temperature following brumation. This finding supports our prediction of the thermal-dependence of metabolic rate under cold exposure. Additionally, this depressed metabolism generally agrees with literature of other ectotherms that experience extended dormancy, and has been posited as a means of reserving energy stores (Gregory 1982; Guppy and Withers 1999; Hailey and Loveridge 1997; Patterson and Davies 1978). However, it’s notable, that we did not find a temperature insensitive range as has been shown in the temperate common garter snake (*Thamnophis sirtalis*) under exposure to cool temperatures (Aleksiuk 1971; 1976). This insensitive range has been suggested as means of compensating for the depressive effects of low temperatures on metabolism in ectotherms from cooler climates (Aleksiuk 1971). Though $\dot{V}_{O_2}$ responded in an expected temperature-dependent manner, the slopes of the temperature response curves, between pre- and post-brumation, were heterogeneous (Fig. 3A; Table 2). The thermal response of $\dot{V}_{O_2}$ as temperatures warmed was much shallower than when animals were stepped down in temperature. This was true even of our post-brumation measurement at 20°C collected after animals were acclimated, fed and fasted for
three weeks. This pattern was also characterized by a change in the temperature coefficient (Q_{10}), with a larger pre-brumation Q_{10} than post-brumation (Table 3), suggesting a decrease in the sensitivity of metabolic rate to temperature following a period of extended cold exposure. This apparent decoupling, or decrease in sensitivity, of $\dot{V}_{O_2}$ from temperature could be due minimal fuel stores following extended dormancy. Emerging from dormancy involves reviving organismal function at multiple levels and organisms with depleted energy reserves may not be able to meet the metabolic demands (de Souza et al. 2004; Zani et al. 2012). Individuals may respond to a limited ability, or even inability, to mobilize metabolic fuels such as glucose post-brumation (likely due to reduced glycogen stores in the liver) by plastically shaping the temperature-dependence of their metabolic rates to align with their current energetic state.

In contrast to measures of whole-organism metabolic rate, CORT, insulin, and H:L responded similarly to pre- and post-brumation temperatures (increasing with decreasing temperature). However, thermal response curves differed among measures (Fig. 2B, 2D, and 2E, respectively). Circulating glucose levels responded to long-term cold exposure (i.e., brumation) but had no response to acute cold exposure.

*Corticosterone*

CORT increased with decreasing temperature, remained elevated during brumation, and decreased as temperatures warmed (Fig. 2B). These findings suggest that CORT is upregulated in response to temperatures outside of the species’ optimal range. Interestingly, however, CORT did not decrease monotonically coming out of brumation. Elevated CORT levels during or following chronic cold exposure also occur in other snake species (Brischoux et al. 2016; Lutterschmidt and Mason 2009) as well as in reptiles under acute exposure to suboptimal
temperatures (Dupoué et al. 2013; Telemeco and Addis 2014). However, CORT rose between 5°C and 10°C post-brumation and decreased thereafter to levels similar to pre-brumation baseline. This peak in CORT at 10°C is interesting for two reasons: first, it suggests that the levels sustained throughout brumation were elevated but not maximal. Elevated CORT levels may buffer an individual from negative effects of long-term metabolic depression. However, chronic stress-induced changes in physiological state (i.e., prolonged and/or extreme elevations in glucocorticoids) can reduce an individual’s fitness and induce pathology (Romero 2004; Sapolsky et al. 2000). Sustaining CORT levels below maximal concentrations may balance the benefits and costs of glucocorticoid upregulation and allow for levels to increase in response to other potential perturbations. Second, mobilization of CORT at 10°C may “jumpstart” metabolic function coming from dormancy. Increases in plasma CORT occur in other ectotherms following dormancy and have been suggested to be linked to resource mobilization at the onset of emergence as a way to stimulate organismal function (Brischoux et al. 2016; Lutterschmidt and Mason 2009). The heterogeneous response to temperature before and after a period of winter dormancy, with CORT increasing with decreasing temperatures, is also characterized by Q_{10} values less than one (indicating a decrease) following brumation (Table 3). This pattern suggests an increase in the sensitivity of CORT to temperature following a period of cold exposure and may indicate an ability of the HPA axis to mobilize additional CORT to prepare an individual to emerge from their state of physiological dormancy.

**Glucose**

Levels of plasma glucose did not respond to acute cold exposure (Fig 2C). Glucose levels were low during the 12-week brumation and increased in response to post-brumation warming.
However, fed-fasted post-brumation measures at 20°C revealed that levels of plasma glucose had not returned to pre-brumation concentrations. Analyses of slope heterogeneity and $Q_{10}$ further elucidated a disparity between glucose dynamics under acute and long-term exposure to cold temperatures. Pre- and post-brumation slopes were non-homologous: $Q_{10}$ calculated across pre-brumation measures was approximately 1 suggesting thermal independence (Table 3), while a post-brumation $Q_{10}$ of 1.29 demonstrates increased sensitivity of glucose to rising temperature. Thus, acute exposure to suboptimal cold temperatures in checkered garter snakes regulates glucose pathways to promote the maintenance of energy metabolism. Glucocorticoids, such as CORT, are elevated in response to environmental perturbations and promote the mobilization of glucose, stimulate gluconeogenesis in the liver, decrease glucose uptake by peripheral tissues, and increase insulin resistance (i.e., decrease insulin sensitivity of tissues). These effects work in concert to either maintain plasma glucose levels or promote hyperglycemia (Kuo et al. 2015; Sapolsky et al. 2000). However, extended periods of cold exposure (as in winter dormancy) seems to decouple this regulation and glucose levels drop considerably. This hypoglycemic response during brumation agrees with findings in various ectotherms that endure prolonged cold exposure (e.g., Abdel-Raheem et al. 1989; de Souza et al. 2004; Haggag et al. 1966a; Khalil and Yanni 1959; Padgaonkar and Das 2000), but contrasts with ectotherms that are capable of freeze tolerance (Costanzo et al. 1993; Grenot et al. 2000; Storey and Storey 1996; Voituron et al. 2000). Additionally, the post-brumation increase in plasma glucose is in agreement with the upregulation of $\dot{V}_O_2$. However, similar to post-brumation measures of $\dot{V}_O_2$, circulating glucose concentrations at 20°C did not fully compensate for the extended period of metabolic depression and thus were lower than their pre-brumation equivalent. Though not measured here, reduced liver glycogen stores accompany hypoglycemia under the physiological demands of
overwintering in other ectotherms (Barwick and Bryant 1966; de Souza et al. 2004; Dessauer 1955; Haggag et al. 1966b; Patterson and Davies 1978; Zani et al. 2012). This suggests that depleted energy stores from an extended dormancy may preclude a rapid return to homeostasis. The overall dynamic of glucose – exhibiting maintenance under acute cold exposure and suppression under longer-term cold exposure – suggests that its regulation is not solely temperature dependent, but instead occurs across the winter season.

**Insulin**

Levels of plasma insulin generally varied inversely with temperature. Increased insulin under cold stress is seemingly a biological paradox; insulin is primarily considered a strict regulator of circulating glucose concentrations that works antagonistically with CORT. Under homeostasis, insulin modulates the glucose-relevant effects of glucocorticoids by reducing glucose production and promoting uptake into tissues (Dallman et al. 1995; Strack et al. 1995). However, levels of plasma insulin rose in the snakes independent of glucose, as reflected in differing thermal response curves (Fig. 2D). This finding agrees with work on fishes, where reducing temperature increased levels of circulating insulin in both fed and fasted animals (Gabillard et al. 2003; Navarro et al. 2002). Additionally, insulin increases under heat exposure, independent of increase in glucose, in a congeneric snake (Gangloff et al. 2016). This hyperinsulinemia when exposed to temperatures outside of the thermal optimum, despite reduced nutrient intake, suggests insulin has a temperature-dependent responsiveness beyond its role in glucose regulation. Specifically, the role of hyperinsulinemia may include activating and upregulating heat shock proteins, at least under extreme conditions above the thermal optimum (reviewed in Baumgard and Rhoads 2013; Li et al. 2006). Temperature dependence of insulin
and its role in energy balance, particularly under conditions of cold exposure, needs further consideration, however. Regardless, levels of plasma insulin were tightly coupled to thermal conditions in these snakes (Tables 2, 3; Fig. 3).

**Heterophil: Lymphocyte Ratios**

Like levels of plasma insulin, H:L ratios covaried inversely with temperature. Even so, H:L did not remain static during brumation but instead rose. These findings agree with other studies showing that H:L ratios correlate with environmental stressors (i.e., H:L increases with increasing magnitude of a given stressor: reviewed by (Davis et al. 2008)) and with circulating levels of glucocorticoids (Gangloff et al. 2017; Goessling et al. 2015). Interaction between the endocrine and immune systems is important for determining responsiveness of organisms to environmental perturbations, as glucocorticoids can act to redistribute leukocytes (Dhabhar et al. 1996; Dhabhar et al. 1994; Vleck et al. 2000). Changes in the profile of circulating leukocytes likely indicates an emergency life-history strategy, where increased circulating phagocytes (e.g., heterophils) fight immediate infection and decreased circulating lymphocytes preserve resources by preventing upregulation of the adaptive immune system (Goessling et al. 2015; Wingfield and Kitaysky 2002). Temperature-induced variation in H:L also could have survival implications, as immune responsiveness and overall fitness may be compromised if the temperature at which the individual’s immune system is most effective and the temperature at which a pathogen is most effective are mismatched (Pounds et al. 2006; Rohr and Raffel 2010).
Though a majority of studies focus on how extreme heat exposure constrains performance, we provide an integrated look at how ecologically relevant cold temperatures elicit physiological responses. Our results suggest coordination among physiological axes to mitigate deleterious effects of extended cold exposure as experienced during winter dormancy. The integrated physiological state presented here demonstrates not only the existence of temperature dependence across physiological axes, but seasonal variation in thermal responsiveness. As global climatic patterns continue to change, it becomes increasingly important to quantify the thermal reaction norms of metabolic and physiological function not only under high temperature conditions, but also during periods of brumation and inactivity. Some have suggested that increasing temperatures may actually be beneficial for some temperate ectotherms – warmer temperatures could allow for longer active periods resulting in increased growth, reproduction, and species distribution (Chamaille-Jammes et al. 2006; Stahlschmidt et al. 2015; Weatherhead et al. 2012) – however, the effects of milder winters are not as often considered (but see Brischoux et al. 2016; Williams et al. 2015; Zani 2008). Under milder winter conditions when the temperature is too cold for animals to be active, but not cold enough to reach dormancy, we would expect this to cause a state of chronic physiological stress (Brischoux et al. 2016; Voituron et al. 2000) and thus impede an individual’s ability to suppress metabolic and physiological function to the extent necessary to maintain energy balance.

Acknowledgements

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Authors’ contributions

KGH, EJG and AMB designed the experiment; KGH and EJG conducted the experiment; KGH completed physiological assays; KGH performed data analysis and drafted manuscript. All authors contributed to the interpretation of results and final manuscript.

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Table 1. Repeated-measures mixed linear model analysis of metabolic rate and physiology in captive-raised checkered garter snakes (*Thamnophis marcianus*). Metabolic rate is the log10 of the rate of oxygen consumption ($\dot{V}_{O2}$). Physiological measures of plasma corticosterone and insulin are log10-transformed and circulating glucose measures square-root transformed. Heterophil: lymphocyte ratios from whole blood are log10-transformed. Values are $F_{dfn,dfd}$. Significant effects (P<0.05) are in bold.

<table>
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<tr>
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<th>$\dot{V}_{O2}$</th>
<th>CORT</th>
<th>Glucose</th>
<th>Insulin</th>
<th>H:L</th>
</tr>
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<td></td>
<td></td>
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<td>0.2021</td>
<td>--</td>
<td>0.7974</td>
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<td>0.01</td>
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Table 2. Analysis of covariance for heterogeneity of pre-brumation and post-brumation slopes of metabolic rate and physiology in captive-raised checked garter snakes (Thamnophis marcianus). Metabolic rate is the log_{10} of the rate of oxygen consumption ($\dot{V}_{O_2}$). Physiological measures of plasma corticosterone and insulin are log_{10}-transformed and circulating glucose measures square-root transformed. Heterophil: lymphocyte ratios from whole blood are log_{10}-transformed. Values are $F_{df_n, df_d}$. Significant effects (P<0.05) are in bold. Linear model estimates of pre- and post-brumation slopes and intercepts are from models of raw data and include ± SE.

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<tr>
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<th>$\dot{V}_{O_2}$</th>
<th>CORT</th>
<th>Glucose</th>
<th>Insulin</th>
<th>H:L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Slope</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>$F$ (d.f., d.f.)</td>
<td>18.41 1, 431.1</td>
<td>22.63 1, 414.4</td>
<td>16.96 1, 268.1</td>
<td>0.00 1, 113</td>
<td>0.01 1, 412.9</td>
</tr>
<tr>
<td>$P_{r}&gt;F$</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.987</td>
<td>0.907</td>
</tr>
<tr>
<td><strong>Intercept</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F$ (d.f., d.f.)</td>
<td>15.26 1, 475.8</td>
<td>39.65 1, 458.9</td>
<td>344.61 1, 293.9</td>
<td>42.77 1, 116</td>
<td>44.25 1, 427.3</td>
</tr>
<tr>
<td>$P_{r}&gt;F$</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Pre-brumation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.05 ± 0.00</td>
<td>-5.18 ± 0.44</td>
<td>0.05 ± 0.09</td>
<td>-0.00 ± 0.00</td>
<td>-0.18 ± 0.02</td>
</tr>
<tr>
<td>Intercept</td>
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<td>133.78 ± 17.21</td>
<td>32.56 ± 3.18</td>
<td>0.13 ± 0.01</td>
<td>2.38 ± 0.63</td>
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<tr>
<td><strong>Post-brumation</strong></td>
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<tr>
<td>Slope</td>
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<td>-6.83 ± 1.63</td>
<td>0.54 ± 0.08</td>
<td>-0.01 ± 0.00</td>
<td>-0.31 ± 0.04</td>
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<tr>
<td>Intercept</td>
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<td>387.48 ± 75.10</td>
<td>16.28 ± 2.69</td>
<td>0.16 ± 0.01</td>
<td>4.57 ± 0.98</td>
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Table 3. Temperature coefficient ($Q_{10}$) calculated from back-transformed LSM model estimates (repeated-measures mixed linear models)

<table>
<thead>
<tr>
<th></th>
<th>$Q_{10}$ Pre-brumation</th>
<th>$Q_{10}$ Post-brumation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\dot{V}_{O_2}$</td>
<td>3.658</td>
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<tr>
<td>CORT</td>
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<td>Glucose</td>
<td>1.019</td>
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<tr>
<td>H:L</td>
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<td>0.065</td>
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<tr>
<td>Insulin</td>
<td>0.530</td>
<td>0.468</td>
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Figure 1. Experimental Timeline.
Figure 2. Effects of overwintering conditions on metabolism and physiology in captive-raised checkered garter snakes (*Thamnophis marcianus*). Physiological measures of energy regulation and stress response: (A) metabolic rate via rates of oxygen consumption (*N* = 482 data points), (B) Corticosterone (*N* = 465), (C) Glucose (*N* = 330), (D) Insulin (*N* = 120), and (E) Heterophil-Lymphocyte ratio (*N* = 446). All variables are plotted as back-transformed least squares means ± SE from analyses of metabolic and physiological function (see Table 1).
Figure 3. Heterogeneity of thermal response curves pre- and post-brumation in captive-raised checkered garter snakes (Thamnophis marcianus). Plots are the least squares means estimates ± SE from mixed effects linear models of metabolic rate and physiological function pre-brumation (circles) and post-brumation (triangles) for measures of (A) metabolic rate via rates of oxygen consumption (N = 482 data points), (B) Corticosterone (N = 465), (C) Glucose (N = 330), (D) Insulin (N = 120), and (E) Heterophil: Lymphocyte ratio (N = 462). Regression lines represent the effects of temperature on pre-brumation (solid lines) and post-brumation (dashed lines) measures of physiology.
CHAPTER 3

TEMPERATURE DEPENDENCE OF METABOLIC FUEL SELECTION AND
OXYGEN CONSUMPTION IN CELLS AND WHOLE ORGANISMS

Kaitlyn G. Holden, Ashley R. Hedrick, Eric J. Gangloff, Steven J. Hall,
Anne M. Bronikowski

Abstract

Temperature, mediated through physiology, affects nearly every aspect of how organisms interact with and are constrained by their environment. Measures of organismal energetics, such as metabolic rate, are highly temperature dependent and are governed through temperature effects on rates of biochemical reactions. However, how this temperature-dependence varies across scales of biological organization is not well characterized. We examined the temperature dependence of measures of whole-animal oxygen consumption ($\dot{V}_{O_2}$) and cellular metabolic rate (OCR) in the western terrestrial garter snake (Thamnophis elegans), a terrestrial ectotherm inhabiting a highly thermally variable environment. Characterizing the temperature-dependence of rates of oxygen consumption across levels of biological organization gives insight into the range of active temperatures that influence whole-organism performance. Additionally, we examined the effects of acute exposure to a range of temperatures on fuel selection in fasted animals – whether increasing temperature induces shifts in the primary macronutrient oxidized to support metabolism. Our results demonstrate a temperature dependence of metabolic rates across levels of biological organization. We found that metabolic rate at the level of the whole animal, as well as cellular rates of basal and ATP-linked respiration, increased with temperature.
However, maximal rates of cellular respiration were temperature insensitive. Thus, as
temperatures increased, basal cellular respiration approached maximal levels and the cellular
reserve capacity decreased. Although whole-organism and cellular measures of oxygen
consumption rate are both temperature dependent, they do not exhibit intraindividual covariation
– that is they both increase with temperature but do so differently. This suggests that, in our
study, cellular metabolism does not directly govern whole animal oxygen consumption.
Alternatively, measures of fuel consumption (i.e., RER and natural abundance of δ13C) did not
vary with acute exposure to extreme temperatures (in a fasted state). This consistency suggests
the maintenance of a single fuel source being oxidized to support metabolism across a broad
range of metabolic demands.

Introduction

Temperature broadly affects an organism’s performance and function (Angilletta 2009;
Huey and Stevenson 1979). The effects of temperatures both above and below the optimal range
on biochemical and metabolic processes can lead to disruptions of homeostasis and declines in
can be used to describe the temperature dependence of physiological performance. The
relationship generally defines a response where performance rises with increasing temperature
and rapidly drops off once the organism’s thermal optimum has been surpassed (Huey and
Stevenson 1979; Sinclair et al. 2016). Typically, TPCs quantify whole-organism performance
across a range of body temperatures (within the range of an organism’s critical thermal
minimum and maximum) as a means of identifying how organisms interact with and are
constrained by their environment (Williams et al. 2016). This is particularly prominent in
ectotherms that have limited ability to metabolically control their body temperature and thus their body temperature is fundamentally associated with the thermal conditions of their environment (Angilletta et al. 2002; Avery 1982; Peterson 1987). Consequently, there has been extensive investigation of the thermal sensitivity of different aspects of physiological function in ectotherms. Though the effects of extreme temperatures on performance, and ultimately fitness, often focus on the level of the whole-organism, impairment at the cellular level could provide the mechanistic link driving the breakdown of higher levels of organization (reviewed in Angilletta 2009; Gangloff and Telemeco 2018). Alternatively, higher-order systems (such as organ systems) may be more sensitive to thermal extremes and thus show declines in function at temperatures lower than observed at the level of the cell, setting thermal tolerances at the level of the whole organism (Pörtner 2002). Identifying the level of organization most susceptible to temperature extremes and characterizing the relationships between levels of organization can provide insight into how temperature affects whole-organism function.

The thermal dependence of metabolism in ectotherms has been widely studied (e.g., (Angilletta 2009; Grigg 1978; Pörtner et al. 2006), including in our study system of western terrestrial garter snakes (Thamnophis elegans; (Bronikowski and Vleck 2010; Gangloff et al. 2016; Gangloff et al. 2015). Temperature-driven increases in metabolism can have profound effects on all levels of biological organization (Brown et al. 2004; Gillooly et al. 2001). Eukaryotic cells primarily rely on energy substrate (adenosine triphosphate, ATP) produced in mitochondria to sustain cellular and organismal function (reviewed in Hood et al. 2018; Solaini et al. 2010). Consequently, the ability of cells to respond to challenges in maintaining energy flux, such as meeting energetic demands at cold temperatures or increased metabolic demands imposed by high temperatures, depends on the bioenergetic capacity of mitochondria (Galli and
Richards 2012; Ivanina et al. 2012; Portner et al. 1999). The primary goal of our study is to quantify the relationship between measures of whole-animal and cellular metabolism to understand both the temperature dependence of each metric and whether rates of cellular respiration may drive whole organism respiration (Salin et al. 2015; Salin et al. 2016b; Salin et al. 2019). This knowledge is important to questions across scales such as: how genomic and biochemical variation scale up, and how individuals can respond to novel environments.

A second question for this study is whether increased temperatures, specifically, induce a shift in the primary resource oxidized to support metabolism. Examining the temperature dependence of metabolism can provide quantitative measures of energy expenditure and give insight into energy budgets (Dillon et al. 2010; Kearney 2012; Williams et al. 2012). Particularly for ectotherm vertebrates, surviving fasting or dormancy relies on stored energy reserves. Thus, animals may experience increased mortality if the variability in their thermal conditions accelerates the pace of energy usage (Williams et al. 2012). Fasting animals pass through sequential phases of macronutrient oxidation from carbohydrates, to lipids, then proteins (Caloin 2004; Castellini and Rea 1992). Whole-organism respirometry has been used to differentiate fuel usage through its variables: respiratory gas exchange ratio (RER, the proportion of CO₂ produced to O₂ consumed), and respiratory quotient (RQ). Under steady-state conditions RER is equivalent to RQ and provides an indication of the dominant fuel being catabolized to support metabolism (Kleiber 1961). However, RER cannot differentiate between protein and mixed fuel catabolism, thus stable isotopes approaches are becoming more common. Specifically, measures of the stable carbon isotope content in breath (reported as δ¹³C) can be used for fuel selection assays because respired CO₂ is a direction byproduct of metabolism (Hatch et al. 2002; reviewed in: McCue and Welch 2015; Voigt et al. 2008; Welch et al. 2016). The δ¹³C values from the exhaled breath of
an animal represent the $\delta^{13}C$ values of the metabolic fuel being oxidized and thus, resource use (McCue and Welch 2015). Following the expectation of sequential fuel usage, differences in $\delta^{13}C$ values among macromolecules derived from a single food source should exhibit a pattern whereby carbohydrate oxidation yields a higher $\delta^{13}C$, protein and mixed fuel oxidation display intermediate $\delta^{13}C$, and lipid metabolism has the lowest $\delta^{13}C$ (reviewed in Bowling et al. 2008; McCue and Welch 2015; Welch et al. 2016). However, lower than expected values, given the known range for macronutrient catabolism, have been found in several studies of non-avian reptiles (e.g., da Silva et al. 2013; Farmer and Carrier 2000; Grigg 1978; Hall 1924; Henriksen et al. 2015). Here again, fuel selection provides insights into how individuals metabolically respond to novel or stressful environments.

To address our two goals, we first quantified how measures of oxygen consumption at the level of the whole-animal ($\dot{V}_{O2}$) relate to the level of the cell (OCR). Second, we tested the effects of acute exposure to high temperatures on fuel selection in fasted ectotherms. Snakes have the ability to regulate resource usage over extended periods of time, sparing their structurally critical endogenous protein stores and relying on lipid oxidation when resources are limited (McCue 2007). Moreover, measures of digestion energetics (e.g., specific dynamic action, gut motility, nutrient assimilation) have been shown to be influenced by increased temperatures (McConnachie and Alexander 2004; McCue 2006; Plasman et al. 2019; Secor 2009). However, the effects of temperature on fuel usage in fasted ectotherms is not well documented. Using two metrics of fuel usage (i.e., RER and $\delta^{13}C$ content of breath) we examined the agreement between our methodologies as well as the temperature-dependence of fuel selection.
Materials and Methods

Study System

We studied the relationship between whole animal metabolic rate and cellular rates of oxygen consumption in the western terrestrial garter snake (*Thamnophis elegans*). The study subjects (*N* = 11) were all same-age females of similar size (mean mass ± SD: 69.98 ± 12.28g), born in captivity in August 2010 from wild caught gravid females (see (Gangloff et al. 2015) for full details). All snakes were raised in a common garden: housed in 150-liter (40 gallon) glass aquaria each filled with 3-5 cm of pet bedding with water available *ad libitum*, fed weekly thawed mice, maintained on a 12:12 light: dark cycle with a thermal gradient ranging from 25-34°C across their enclosure in 28C rooms (preferred body temperature, see Gangloff et al. 2020) and brumated annually at 4°C from December 15 – April 15. Animals were maintained in accordance with the Iowa State University Institutional Animal Care and Use Committee protocol 3-2-5125-J.

Experimental Procedure

Our experiment was conducted over 28 days beginning 11 October 2018 (as four identical protocols at four different temperatures). Snakes were divided into two groups (of 5 and 6 snakes) that were measured on two consecutive days. Each group was further divided into batches (with each batch consisting of 1-2 snakes) for measures of whole organism respirometry. Each group had the following sequence: Day1 Feed, Days2-6 fast, Day7 measure OCR, day 8 measure $\dot{V}_{O2}$, see Figure 1 for experimental timeline. Throughout the experiment, group was maintained but batch within group was randomized. Each batch was subjected to a 2.75-hour treatment at each of four ambient temperatures (24, 28, 32, 36°C) encompassing the activity
range of this species (Arnold et al. 1995; Huey et al. 1989; Peterson et al. 1993), thus there were
11 snakes x 4 temperatures for N = 44 combinations of whole organism and cellular metabolism
(Fig. 1). Animals were fed then fasted for 1 week prior to each measure of whole-body metabolic
rate to ensure that animals were in the same state of post-absorption and to remove the influence
of digestion (i.e., specific dynamic action) so that our measures were reflective of maintenance
metabolism (Álvarez and Nicieza 2005; Stevenson et al. 1985). Animals were maintained at
constant 28°C between measurement temperatures. Prior to data collection, animals were
acclimated to experimental conditions by moving them into the respirometry chambers (946 mL
glass jars custom-fitted with in- and out-flow tubing) and held overnight before whole-animal
rates of oxygen consumption (\( \dot{V}_{O_2} \)) were collected.

**Whole-Animal Oxygen Consumption Rate**

Rates of oxygen consumption (\( \dot{V}_{O_2} \)) and carbon dioxide production (\( \dot{V}_{CO_2} \)) were measured
using stop flow respirometry. As a proxy for metabolic rate, \( \dot{V}_{O_2} \) is a measure of steady state
maintenance in post-absorptive animals at rest during normal periods of activity (Bennett and
Dawson 1976). A flow rate of 500 mL/min was maintained by an MFS-2 mass flow generator
and cycled through three chambers over the course of 150 minutes. One chamber served as a
blank and was sampled for three 15-minute intervals at the start of each run, and an additional
15-minute interval at the end of each run to establish baseline values during the experiment. The
other two chambers, containing experimental animals, were each sampled twice in alternating
15-minute intervals (N = 88 measures: 11 animals \( \times \) 4 temperatures \( \times \) 2 measures each of \( O_2 \) and
\( CO_2 \)). Air outflow was scrubbed of water with Drierite desiccant and subsequently measured for
oxygen and carbon dioxide content with FC-10 and CA-10 analyzers, respectively (Sable
Systems). We corrected measures of oxygen and carbon dioxide for barometric pressure and integrated the change in instantaneous gas concentrations over the period for which the chamber was sealed to calculate $\dot{V}_{O2}$ and $\dot{V}_{CO2}$ (mL/hour) using ExpeData software (Sable systems; (Lighton 2008)). At each temperature, animals within their glass measurement chambers were placed into experimental incubators, 15 minutes before the start of each run; this, combined with the initial intervals measuring the blank (45-minutes), provided 60 minutes for snakes to acclimate to experimental conditions before the measurements.

**Cellular Oxygen Consumption Rate**

To test for effects of temperature on cellular respiration and its relationship to whole-animal metabolism, we measured the oxygen consumption rate (OCR) of isolated peripheral blood mononuclear cells (PBMCs; i.e., white blood cells) with a Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, California, USA). For full assay details see Gangloff et al (2020). Whole blood was drawn four times for each snake; i.e., once for each experimental temperature. 400 µl whole blood (equating to < 1% of each animal’s total body mass) was collected from the caudal vein in heparin rinsed syringes. PBMCs were isolated via centrifugation, resuspended in 500 µl Seahorse SF Base Medium (Agilent Technologies), and supplemented with glucose (10 mM), pyruvate (1 mM), and glutamine (2 mM). Cells were plated on 24-well plates with densities of $1 \times 10^6$ cells per well in triplicate or quadruplicate (per each sample; $N=168$) in a total volume of 525 µL and incubated at 28°C until assayed. Two to three wells on each plate were medium-only blanks to correct for background variation. Samples from the two groups of animals were run on consecutive days with batches randomized within day between two plates to accommodate all samples and replicates. We followed manufacturer’s
protocol of the Cell Mito Stress Test (Agilent Technologies) with modifications (Gangloff et al. 2020) for snake PBMCs to measure: basal respiration, rates of ATP-linked respiration, and maximal cellular respiration. The Seahorse XFe24 directly measures the OCR of cells for each aspect of cellular metabolic rate following the sequential injection of compounds (oligomycin, carbonyl cyanide m-chlorophenyl hydrazone [CCCP], and Rotenone) known to target specific regions of the electron transport chain and alter OCR. Following measures of basal oxygen consumption, compounds were injected in sequence to first inhibit ATP-synthase (oligomycin 1 µM) as a means of elucidating the proportion of basal respiration driving ATP production – a measure of the ATP produced that contributes to meeting energetic needs of the cell. Second, the addition of CCCP (1.5 µM), a H⁺ ionophore, acts to dissipate the proton gradient and thus uncouples electron transport from ATP synthesis and allows the transport of protons into the cell without participation of ATP synthase (Ghoul et al. 1989). This uncoupling simulates a scenario of maximum energy demand and induces maximal rates of respiration within the cell. Finally, inhibition of complex I of the electron transport chain was accomplished via the addition of Rotenone (0.5 µM).

**Fuel Selection**

We measured two metrics of fuel selection to test the temperature-dependence of macronutrient usage as a means of supporting metabolism, the respiratory gas exchange ratio (RER) and the δ¹³C composition of CO₂ in exhaled breath samples. We calculated RER from measures of whole animal oxygen consumption and carbon dioxide production (\( \dot{V}_{CO2} / \dot{V}_{O2} \); equivalent to the respiratory quotient, RQ, under steady-state conditions). When metabolism is primarily supported by lipid oxidation the RER is approximately 0.7 while pure carbohydrate
oxidation is represented by an RER of 1.0. Protein oxidation, as well as estimates of mixed fuel oxidation, yield an intermediate RER around 0.83 (Kleiber 1961). To measure the carbon isotope values in breath samples from our experimental animals, metabolic chambers were custom fitted with 5.4 mm butyl rubber septa. We used 15 mL syringes to sample excurrent air from each metabolic chamber at the end of each respirometry run (i.e., animals had been exposed to experimental temperature for 2.75 hours and were hermetically sealed for 15 minutes before sample collection; N = 44) and dispensed the sample into evacuated glass Exetainers (12 ml, LabCo Limited, Buckinghamshire, UK). These samples were analyzed for the naturally occurring stable carbon isotopes ($^{12}$C and $^{13}$C) of CO$_2$. These are traditionally expressed in $\delta$ notation relative to an international standard (Vienna Pee Dee Belemnite) with units of parts per thousand (‰):

$$\delta = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000$$

Here, R is the molar ratio of heavy to light isotope of the sample or standard. Air samples were analyzed for $\delta^{13}$C of CO$_2$ using a tunable diode laser (Campbell Scientific TGA200A; Logan, UT, USA) following the method described in Hall et al. (2017). Precision of standards analyzed concurrently with these samples (1σ) measured 0.3‰. To calculate the $\delta^{13}$C of CO$_2$ from animal breath, we used a two-source mixing model to account for dilution from background CO$_2$ in ambient air:

$$\delta^{13}C_{\text{breath}} = \frac{\delta^{13}C_{\text{sample}} [CO_2]_{\text{sample}} - \delta^{13}C_{\text{ambient}} [CO_2]_{\text{ambient}}}{[CO_2]_{\text{sample}} - [CO_2]_{\text{ambient}}}$$
where sample represents the mixture of breath-derived and ambient CO₂, ambient represents the background CO₂ in the room, and \([\text{CO}_2]\) denotes the mole fraction of CO₂ in the respective sample.

**Statistical Methods**

For analysis of our dependent variables of whole-animal metabolic rate (\(\dot{V}_{O_2}\)), respiratory exchange ratio, measures of cellular oxygen consumption rate (basal, ATP-linked, and maximal respiration), and values of breath \(\delta^{13}\text{C}\) we conducted repeated-measures mixed-effects linear models with the \textit{lme4} package (Bates and others 2015) in the programming language R (R Core Team 2018). Fixed effects were assessed using type III sums of squares using corrected denominator degrees of freedom (Kenward and Roger 1997). Figures were made with the \textit{ggplot2} package (Wickham 2009) showing back-transformed least-squares means for measures of whole-organism and cellular oxygen consumption rate, and RER and raw data for breath \(\delta^{13}\text{C}\).

Measures of \(\dot{V}_{O_2}\) and cellular OCR were log\(_{10}\)-transformed, and measures of RER were cubed to meet model assumptions of normality. Outliers were identified using twice the interquartile method, and for cellular measures, wells where there was an injection failure were identified, and removed (\(N = 5\) for VO₂, 9 for CO₂, 56 for Basal, 63 for ATP-linked, and 58 for Maximal). Temperature was modeled as a categorical factor, allowing for examination of post hoc linear contrasts. Models included temperature as an explanatory fixed effect as well as a covariate of body mass and individual and individual nested within temperature as random effects. Our models took the following form where \(\mu\) represents the grand mean and \(\varepsilon\) is the error term:

\[
Y \sim \mu + \text{Mass} + \text{Temperature} + 1|\text{Individual} + 1| \text{Individual}(\text{Temperature}) + \varepsilon
\]
To determine the relationship between whole-animal measures of oxygen consumption ($\dot{V}_{O_2}$) and cellular level oxygen consumption (basal OCR) we first calculated mass-specific metabolic rate for measures of whole-animal respirometry by dividing $\dot{V}_{O_2}$ (mL/min) by the body mass (g) of the individual. The transformed measures of oxygen consumption were scaled, within each temperature, to a mean of zero and unit variance and we used a linear mixed effects model with $\dot{V}_{O_2}$ as the dependent variable, basal OCR as the independent variable, and individual as a random effect. Similarly, we used a linear mixed effects model to determine the relationship between scaled measures of fuel selection, where RER was the dependent variable, $\delta^{13}C$ the independent variable, and individual as a random effect.

**Results**

*Oxygen Consumption Rates*

Rates of whole-animal oxygen consumption increased with temperature ($P < 0.0001$; Table 1, Fig. 2A). There was no significant effect of mass on $\dot{V}_{O_2}$ ($P = 0.4109$) as mass was similar across the 11 subjects, but we retained mass as a covariate in our model because of the known relationship between mass and metabolic rate. Measures of basal and ATP-linked cellular oxygen consumption increased with temperature (both $P < 0.0001$; Table 1, Fig. 2B and 2C respectively). Maximal rates of cellular oxygen consumption were not affected by temperature ($P = 0.8458$, Table 1, Fig. 2D). Additionally, we found no significant relationship between measures of whole-animal $\dot{V}_{O_2}$ and cellular (basal) oxygen consumption other than both measures are temperature dependent ($F_{1,37.36} = 0.6138$, $P = 0.4383$, Fig. 3). Similarly, we found no direct relationship between measures of metabolic rate at the level of the whole animal and either
cellular ATP-linked respiration ($F_{1,39.502} = 0.0298, P = 0.8637$) or maximal respiration ($F_{1,36.991} = 0.3619, P = 0.5511$).

**Fuel Selection**

Breath $\delta^{13}C$ and RER, within any given experimental temperature, ranged from $-19.32\%$ to $-16.32\%$. and $0.31 – 0.65$, respectively. Although neither RER nor $d^{13}C$ responded directly to temperature, within individuals, they were negatively correlated across each temperature, Table 2, Figure 4.

**Discussion**

In this study we tested how temperature dependence of whole-organism metabolic rates and cellular rates of oxygen consumption covary in the western terrestrial garter snake during acute exposure to a range of environmentally relevant temperatures. As expected, our results show a strong temperature dependence of whole-animal metabolic rate (Bronikowski and Vleck 2010; Gangloff et al. 2020). As well, cellular measures of basal and ATP-linked respiration increased with temperature, whereas maximal cellular OCR did not. However, though both whole-organism and basal cellular measures of oxygen consumption increased with temperature the thermal reaction norms differed – there was no significant relationship between the two measures, i.e., the slopes representing the rate of increase in oxygen consumption at the scale of the whole-animal and cell differed. In contrast, we found no effect of temperature on measures of maximal cellular respiration or in measures of fuel selection. Though there was no indication that the type of fuel used to support metabolism changed with temperature, measures of RER and $\delta^{13}C_{CO2}$ covaried within individuals.
We found that, similar to metabolic rates at the scale of the whole organism, basal and ATP-linked measures of cellular oxygen consumption also increased without plateau with increasing temperature. When considering ATP-linked respiration, the injection of oligomycin, an ATP synthase inhibitor, decreased the overall rate of oxygen consumption. This decrease in respiration from basal measures is representative of the portion of basal respiration that was being used to drive ATP production and thus sustain cellular function. Our results suggest that increasing temperatures increase the proportion of basal respiration (and thus the amount of ATP produced by the mitochondria) that contributes to meeting the energetic needs of the cell. The addition of CCCP uncoupled electron transport from ATP synthesis, simulating a scenario of maximal energy demand and promoting maximum rates of respiration within the cells. Given this, it is not surprising that maximal rates of oxygen consumption did not change with changing thermal exposure. Cells often function at a baseline level that is below their total bioenergetic capacity, such that under conditions of increased energetic demand they can upregulate their rates of respiration. If the energy requirement supersedes that of the reserve capacity (i.e., energy demand increases beyond the cellular maximum), the cell and its associated tissue risk not only death but system failure on a larger scale (reviewed in Desler et al. 2012). Additionally, we did not find evidence that whole-animal rates of respiration were driven exclusively by rates of cellular respiration. Though whole-animal measures of $\dot{V}_{O2}$ and cellular measures of basal OCR both responded in a temperature dependent manner, their thermal response curves differed (Fig. 2). The difference in the way in which these measures of respiration respond to temperature suggests that the increase in $\dot{V}_{O2}$, particularly at $36\degree C$, is not simply driven by a shift in cellular oxygen consumption rates, but instead are governed by other processes at a larger scale. Furthermore, here we measured metrics of cellular respiration in immune cells isolated from
whole blood. Research has shown that there can be significant variation in mitochondrial properties and performance (e.g., oxidation, phosphorylation, reactive oxygen species production) across tissue types (Brown et al. 2012; Hulbert et al. 2006; Salin et al. 2016a) which in turn has consequences for metabolic rate at the level of the whole-organism. Thus, the relative metabolic contribution of a tissue or organ system may inform animal performance and change as a function of an animal’s current metabolic state (Salin et al. 2015; Salin et al. 2016b).

**Fuel Selection**

In contrast to the temperature dependence of (non-maximal) measures of oxygen consumption rate, we found no evidence that temperature influenced the dominant fuel source (i.e., carbohydrates, lipids, proteins) oxidized to support metabolism. Though RER values were lower than expected given the known range of values for macronutrient catabolism, we expect that animals were primarily oxidizing lipids to support metabolism throughout the experiment. Previously this phenomenon of low RER has been explained in reptiles as a loss of respiratory CO$_2$ as urinary bicarbonate (Grigg 1978). However, more recently it has been suggested that patterns of low RER in some ectothermic vertebrates may be due to modulation of arterial P$_{CO_2}$, particularly when subjected to high temperatures (reviewed in da Silva et al. 2013). It has been fairly well established that in ectotherms, arterial pH decreases with increasing temperature (da Silva et al. 2013; Glass et al. 1985; Jackson 1978). This decreased pH is primarily driven by increased CO$_2$ retention in blood plasma as bicarbonate. Depending on the efficiency of a species, or individual, acid-base balance can be adjusted via changes to ventilation such that increased ventilation removes more CO$_2$ from the body, increasing blood pH and increasing RER. It is possible that our study species was unable to compensate for the increased arterial
\( P_{\text{CO}_2} \) via ventilation alone over the duration of their heat exposure. Alternatively, there may be a complex interplay between multiple mechanisms involved in acid-base balance that may prevent a rapid removal of \( \text{CO}_2 \) from the system (reviewed in Burggren and Bautista 2019). Additionally, animals consumed frozen/thawed mice – which are high in protein and lipids, and low in carbohydrates (McCue et al. 2005; Secor and Nagy 1994). Other studies investigating energetics in snakes have found a general pattern of exogenous amino acid oxidation following ingestion of a meal, shifting to oxidation of exogenous lipids, and, as the snakes became post-absorptive, a reliance on endogenous lipid metabolism (McCue 2007; McCue et al. 2015; McCue and Pollock 2008). As dietary carbohydrates are not a major component of a carnivore’s diet, there is little intermediate or mixed fuel oxidation. As a secondary metric to try and elucidate substrate usage in our study system under varying thermal conditions, we measured \( \delta^{13}\text{C} \) of \( \text{CO}_2 \), as \( \text{CO}_2 \) is a direct product of an animal’s metabolism (Hatch et al. 2002; McCue and Welch 2015; Voigt et al. 2008; Welch et al. 2016). Though we did not know the bulk \( \delta^{13}\text{C} \) value of the meals fed the animals during this study (or of their previous diet, contributing to endogenous lipid formation), in combination with RER estimates and the known relationships between the characteristic \( \delta^{13}\text{C} \) values of each metabolite we can make inferences about fuel usage. The carbon isotope ratio of lipids is naturally depleted in \( \delta^{13}\text{C} \) (with some estimates suggesting a difference as great as 5–15‰) relative to either carbohydrates or proteins (Welch et al. 2016). Similar to the expectation of fuel usage under measures of RER, \( \delta^{13}\text{C} \) values should therefore decrease as oxidation of carbohydrates shifts to lipid oxidation, and then increase again when the primary metabolic fuel source consists of either proteins or mixed fuels (Welch et al. 2016). Animals that are in a state of fasting have been shown to shift toward lower \( \delta^{13}\text{C} \) values, indicative of increased reliance on lipid vs. non-lipid substrates (Lee et al. 2017; Perkins and Speakman 2001; Voigt et al. 2008).
Additionally, a study examining metabolite transitions in rats reported a decrease in δ\textsubscript{13}C of CO\textsubscript{2} corresponding to a reduction in carbohydrate oxidation and shift toward lipid oxidation. This was followed by a gradual increase in δ\textsubscript{13}C of CO\textsubscript{2} corresponding to increased reliance on protein metabolism as animals depleted their lipid stores (reviewed in Welch et al. 2016). The values reported for fasting lipid metabolism in this study align with the δ\textsubscript{13}C values we found in fasting garter snakes. Though we found no temperature dependence of fuel use, there was a negative correlation between intra-individual measures of δ\textsubscript{13}C and RER such that, regardless of temperature, higher RER was associated with lower δ\textsubscript{13}C. This trend could reflect a shift in the partitioning of inorganic carbon among tissues, such as bicarbonate retention of δ\textsubscript{13}C in circulation (Mook et al. 1974; Panteleev et al. 1999; Tabiri et al. 2002) or incorporation into proteinaceous tissues (Thompson et al. 2000; Williams et al. 2007) causing a disproportionate loss of lighter δ\textsubscript{12}C in exhaled CO\textsubscript{2}. Alternatively, δ\textsubscript{13}C signatures could be a direct reflection of selective mobilization of stored lipids, such that shifts in fuel reserves from more recent dietary lipids to “old” lipids, or due to tissue-specific fractionation, are manifested in δ\textsubscript{13}C values of breath samples.

Conclusion

Taken together these results contribute to our understanding of the thermal dependence of measures of metabolism. In addition to traditional measures of oxygen consumption at the level of the whole organism via respirometry, we use a novel approach to examine the effects of thermal extremes on key metabolic parameters of cellular respiration. This combination of methodologies allows for the direct comparison of metabolic rate across levels of biological organization in a living animal in a repeated measures context. Additionally, this study provides
insight into the relationship between two metrics assessing fuel usage. Our results suggest that though oxidative metabolism and energetic expenditure are increasing with temperature we do not see a corresponding shift in macronutrient usage away from metabolism of a single metabolic substrate under acute exposure to extreme temperatures in this species.

Acknowledgements

We thank D. Delaney for assistance in customizing metabolic chambers; Animals were maintained at Iowa State University following IACUC protocol #3-2-5125-J under Dr. Anne Bronikowski. This research was supported in part by a grant from the National Science Foundation (IOS-1558071).

Authors’ Contributions

KGH and ARH conducted the experiment; KGH and EJG collected data; KGH performed data analysis and drafted manuscript; SJH provided guidance in stable isotope data collection, analysis and interpretation. AMB provided guidance in data analysis and interpretation. All authors contributed to experimental design, interpretation of results and manuscript revisions.

References


Hall FG. 1924. The respiratory exchange in turtles. The Journal of metabolic research. 6:393-401.


Table 1. Repeated-measures mixed linear model analysis of whole organism and aspects of cellular oxygen consumption rates in the garter snake *Thamnophis elegans*. All measures were log_{10}-transformed for analysis. Values are $F_{\text{dfn, dfd}}$. Significant effects ($P<0.05$) are in bold.

<table>
<thead>
<tr>
<th></th>
<th>$\dot{V}_{O2}$</th>
<th>Basal OCR</th>
<th>ATP-linked OCR</th>
<th>Maximal OCR</th>
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<tbody>
<tr>
<td><strong>Mass</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>$F$ (d.f., d.f.)</td>
<td>0.64 1, 8.98</td>
<td>0.112 1, 8.11</td>
<td>0.11 1, 8.69</td>
<td>0.01 1, 8.85</td>
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<td>$P_r &gt; F$</td>
<td>0.4428</td>
<td>0.7464</td>
<td>0.7472</td>
<td>0.9078</td>
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<tr>
<td><strong>Temperature</strong></td>
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<td></td>
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</tr>
<tr>
<td>$F$ (d.f., d.f.)</td>
<td>24.31 3, 29.96</td>
<td>13.75 3, 30.43</td>
<td>15.11 3, 29.18</td>
<td>0.30 3, 29.72</td>
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<tr>
<td>$P_r &gt; F$</td>
<td><strong>&lt; 0.0001</strong></td>
<td><strong>&lt; 0.0001</strong></td>
<td><strong>&lt; 0.0001</strong></td>
<td>0.8281</td>
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</table>

Table 2. Repeated-measures mixed linear model analysis of measures of metabolic fuel selection in the garter snake *Thamnophis elegans*. Measures of respiratory gas exchange ratio (RER) from whole-animal respirometry were $X^3$ transformed. Measures of carbon stable isotopes from breath samples ($\delta^{13}C_{CO2}$) were not transformed. Values are $F_{\text{dfn, dfd}}$.

<table>
<thead>
<tr>
<th></th>
<th>RER</th>
<th>$\delta^{13}C_{CO2}$</th>
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<tr>
<td><strong>Mass</strong></td>
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<tr>
<td>$F$ (d.f., d.f.)</td>
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<td>0.76 1, 8.94</td>
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<tr>
<td>$P_r &gt; F$</td>
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<td>0.4057</td>
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<tr>
<td><strong>Temperature</strong></td>
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<tr>
<td>$F$ (d.f., d.f.)</td>
<td>0.48 3, 26.81</td>
<td>0.24 3, 29.25</td>
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<tr>
<td>$P_r &gt; F$</td>
<td>0.7023</td>
<td>0.8633</td>
</tr>
</tbody>
</table>
**Fig 1.** Experimental timeline depicting the sequence of events under one temperature treatment. This design was repeated four times for each of our experimental temperatures (24, 28, 32, 36°C).
Fig 2. Thermal reaction norms for (A) Whole organism level oxygen consumption ($\dot{V}_{\text{O}_2}$; N = 83) in ml/hour (B) Cellular level basal oxygen consumption in pmoles/hour (N = 110), (C) Cellular ATP production rate in pmoles/hour (N = 107), and (D) Cellular maximal respiration rate in pmoles/hour (N = 105) measured at 4 temperatures. Data are represented as least squares means estimates from repeated-measures linear models. Error bars represent +/- SE.
Fig 3. Relationship of whole-animal metabolic rate ($\dot{V}_O_2$) and basal measures of cellular oxygen consumption (OCR) in *Thamnophis elegans* at ambient temperatures from 24 to 36°C ($F_{1,37.36} = 0.6138, P = 0.4383, R^2 = 0.009$). $\dot{V}_O_2$ and Basal OCR were standardized to a mean of 1 and unit variance to assess within temperature covariance between measures of fuel selection.
Fig 4. Effect of temperature on (A) Respiratory exchange ratio (N = 74) and (B) $\delta^{13}$C content of excurrent breath (N = 43) for *Thamnophis elegans*. Data are plotted as the least squares mean +/- SE from repeated-measures mixed linear model analysis.
CHAPTER 4
A DECADE OF STRESS PHYSIOLOGY REVEALS LIFE-HISTORY SPECIFIC RESPONSES TO HABITAT CHANGE IN GARTER SNAKES (THAMNOPHIS ELEGANS)

Kaitlyn G. Holden, Amanda Sparkman, Eric J. Gangloff, David A.W. Miller, Anne M. Bronikowski

Abstract

Changing environmental conditions can manifest at every level of biological organization in wild animals. The increasing frequency of record high temperatures and more variable precipitation may impact population persistence via effects on individual physiology and fitness. Extreme environmental events may override expectations of resiliency based on traditional theory, specifically life-history strategies that prioritize survival over current reproduction should respond more to stress. We tested the hypotheses that stress physiology – baseline and reactivity of plasma corticosterone and glucose – vary across years and with directionalities consistent with life-history theory. We further test for an effect of local climate. Over a decade that included a record three-year drought, we found significant ecotype-specific annual variation in stress physiology. The fast pace-of-life (POL) ecotype of garter snakes had greater variability both in baseline and stress-induced measures of corticosterone and in stress-induced measures of glucose. Furthermore, fast POL animals had greater stress-induced measures of glucose (and, in one-year, greater reactivity), whereas slow POL animals had greater stress-induced measures of...
glucocorticoids (and, in three years, reactivity). This variation was not explained directly by local climate, and instead may be mediated by climate-induced changes to snake habitats. We discuss our findings in the contexts of life-history theory and population persistence.

**Introduction**

Over the last two decades, there has been a resurgence in studies examining life-history trade-offs focused on the connections between life history and physiology (e.g., reviewed in: Crespi et al. 2013; Promislow and Harvey 1990; Stearns 1992; Zera and Harshman 2001). Life-history theory posits resource-based trade-offs among traits such as growth and maintenance on one hand, and reproduction and survival on the other (Stearns 1992). These trade-offs are hypothesized to constrain the covariation of life-history traits along a slow-to-fast continuum (Ricklefs and Wikelski 2002; see also Pace-of-Life syndromes Réale et al. 2010). In this framework, populations of the same species, that experience different habitat conditions, are expected to diverge in life histories, and concomitantly, in metabolic and energetic traits that support life-history traits (Réale et al. 2010). Slow “pace-of-life” (POL) populations are characterized by individuals that grow slow, mature late, reproduce over a longer timeframe, and live longer - maximizing fitness by prioritizing self-maintenance and survival. Alternatively, fast POL populations have individuals that grow quickly, mature early, and prioritize current reproduction, often with shorter lifespans (Promislow and Harvey 1990). Having similarities with the ideas of “r and K selection” (Pianka 1970), which hypothesize natural selection as shaping rapid versus slow generation times across species, POL ideas are connected to populations diverging over this continuum due to environmental pressures and subsequent resource allocation strategies.
Physiological hypotheses that result from POL theory include predictions about immune function and metabolism, i.e., more reliance on and investment in acquired immune function (e.g., Lee 2006), and slower metabolic rates (e.g., Auer et al. 2018) in slow POL individuals. Less clear is how stress physiology should associate with POL, in part because of the vast number of physiological markers that could be characterized as mediators of a stress response. Predictions include elevated baseline and stress-induced measures of glucocorticoids in longer-lived, slow POL populations which should invest more into maintenance and survival (Hau et al. 2010; see also Ricklefs and Wikelski 2002; Wingfield et al. 1998), as well as exhibit less variation within measures. A further prediction is that baseline measures of glucose reflect resource availability and increase as part of the stress response, thus populations in more stable environments (characterized by consistent food and water) should have higher circulating baseline and stress-induced measures of glucose.

POL and life-history theory are imagined over ecological timescales and within normal climate variability. However, contemporary climate change has profoundly caused extinctions to terrestrial species across diverse landscapes (Parmesan 2006; Walther et al. 2002) due to an increase in stressful climatic conditions. Changes in climate include elevated temperatures, increased variance in environmental conditions on seasonal and annual bases, and higher incidence of extreme events such as droughts (Stott 2016). Exposure to prolonged periods of environmental disturbance or highly variable environments impact individual growth, reproduction, and survival, either directly or indirectly, through habitat changes and access to resources (Parmesan 2006; Walther et al. 2002). Climatic variability is particularly significant for reptiles; thermal profiles and water availability directly determine reptile performance (Chapters 2 and 3, Angilletta 2001; Huey 1982), making reptiles particularly vulnerable to environmental
change (Deutsch et al. 2008; Huey et al. 2012). Depending on the behavioral and physiological abilities of an individual to buffer against rising temperatures and more frequent drought conditions, changing environmental conditions can alter community dynamics, geographic range, phenology, and rates of population extinction (Bay et al. 2018; Janzen et al. 2018; Urban et al. 2014), and may render normal stress physiology and plasticity ineffective.

How the environment affects organismal growth, reproduction, and survival is largely mediated through physiology (Crespi et al. 2013). The endocrine system, primarily glucocorticoid hormones such as corticosterone (“CORT” hereafter), mediate daily and seasonal metabolic processes such as the acquisition, storage, and utilization of energy stores, such as glucose (“GLUC” hereafter) (Johnstone et al. 2012; Landys et al. 2006; Sapolsky et al. 2000). Under adverse or “stressful” conditions (e.g., changes in thermal conditions, food deprivation), activity of the hypothalamic-pituitary-interrenal (HPI) axis is upregulated (Greenberg and Wingfield 1987; Sapolsky et al. 2000), increasing circulating levels of plasma glucocorticoids (Romero and Wikelski 2001). In concert with catecholamines, such as epinephrine and norepinephrine, CORT can activate downstream pathways that mobilize and prevent the uptake of GLUC and stimulate production of GLUC via glycogenolysis and gluconeogenesis (reviewed in Sapolsky et al. 2000). Thus, in response to stress, circulating levels of CORT and GLUC generally covary (e.g., Gangloff et al. 2016; Gangloff et al. 2017; Neuman-Lee et al. 2020). Under extreme stress, or in pervasively harsh environments, physiological parameters that support homeostasis may deviate from their functional range, resulting in allostatic overload or “wear and tear” as predicted by the allostatic load hypothesis (McEwen and Wingfield 2003; McEwen and Wingfield 2010) or reactive scope (Romero et al. 2009) models respectively. Exceeding the “adaptive scope” – the range of tolerance for stress – can shift the physiological
state of an individual as a means of adaptively maintaining homeostasis (Greenberg and Wingfield 1987; Romero et al. 2009). This happens by increasing and enhancing physiological function (e.g., increased respiratory rate and cardiovascular tone) and behavior (e.g., increased awareness and cognition, enhanced analgesia), while diminishing/suppressing feeding, digestion, growth, reproduction and immunity (Holden et al. 2019; McEwen and Wingfield 2003; Palacios et al. 2012; Sapolsky et al. 2000). Shunting resources from these latter processes in the short-term can increase an individual’s chances of survival, but if sustained, can reduce an individual’s fitness, induce pathology, or lead to population-level decline (McEwen and Wingfield 2010; Romero and Wikelski 2001; Sapolsky et al. 2000). Thus, the magnitude of increase in circulating stress biomarkers, such as CORT and GLUC, reveals the reactivity or sensitivity of an individual to a given stress (see Chapter 2).

Though physiological responses to environmental perturbations have been examined in a variety of ectotherms, few studies have addressed long-term trends in physiological responses to environmental fluctuations. Variation in stress responses can be partitioned at different levels of analysis, each of which may have consequences for evolutionary adaptation to stress and related phenotypes, and may be shaped by the environment. Moreover, such variation – within and among individuals, populations, and species – may influence how populations will adapt, acclimate, or perish in currently changing landscapes. Here, we test hypotheses on the magnitude and directionality of variation in the stress physiology markers of CORT and GLUC in terrestrial ectotherms with two divergent life-history ecotypes, across a decade in an area with dramatic climate and habitat change. We test for associations with each life-history strategy (fast and slow), and whether stress physiology mirrors thermal and hydrologic climate patterns. Our study focuses on populations of western terrestrial garter snakes (*Thamnophis elegans*) in the Eagle
Lake Basin of Lassen County, California, USA, which have been the subject of over 40 years of research on ecology, behavior, and evolution (e.g., Arnold 1977; 1981), and that have experienced several droughts over this period. Thus, this long-term study afforded us a unique opportunity to address important questions (a la Reinke et al. 2019). We collected data over the last decade to test the prediction that baseline and stress-induced changes in circulating CORT and GLUC vary annually and in a manner consistent with each ecotype’s specific POL expectation and that this variation is covaries with temperature and precipitation. Specifically, snakes inhabiting more stochastic environments should have relatively high baseline and stress-induced CORT as a means of promoting self-maintenance and immediate survival, and variable or depressed baseline GLUC levels due to inconsistent resource availability. In contrast, fast-living animals that favor current reproduction and live in less variable environments should exhibit lower circulating levels of both baseline and stress-induced CORT and higher, less variable circulating levels of GLUC.

**Materials and Methods**

**Study System**

Our study system consists of lower elevation populations situated along the rocky shoreline of Eagle Lake, and higher elevation snakes inhabiting the surrounding mountain meadows. Meadow habitats have cooler air temperatures, lower prey and water availability, and lower predation rates relative to lakeshore sites (Bronikowski and Arnold 1999; Kephart 1982; Kephart and Arnold 1982; Sparkman et al. 2013). Lakeshore and meadow snakes have contrasting life-history ecotypes, which can be characterized along the POL continuum. Lakeshore snakes exhibit: faster growth, earlier maturation, larger adult body sizes, higher
reproductive effort per bout, and lower annual survival than meadow snakes (summarized in Schwartz et al. 2015) (see also Bronikowski and Arnold 1999; Reding et al. 2016; Sparkman et al. 2009) (“L-fast” and “M-slow” hereafter). Even so, substantial heterogeneity exists among populations of the same ecotype, particularly in physiological traits (e.g., metabolism: Gangloff et al. 2020; immune function: Sparkman and Palacios 2009), and often in the context of climate variation (e.g., Combrink et al. 2020; Miller et al. 2011). For this study we focused on three replicate L-fast (L2, L4, L7) and four replicate M-slow (M1, M2, M3, M5) populations (Figure S1).

During the focal decade (2010-2019), California experienced a multi-year severe drought (2013-2015) characterized by decreased winter snowfall and snowpack, decreased spring precipitation, and record high temperatures (Griffin and Anchukaitis 2014; Mao et al. 2015; Vose et al. 2014). The 2013-2015 drought was preceded by another drought period (2007-2009) characterized by similarly low amounts of annual precipitation. But the extreme drought conditions of the more recent event altered the habitat structure and impacted prey availability such that multiple snake populations declined or were extirpated during our focal decade. Indeed, survival rates in M-slow populations are sensitive to variation in hydrology through impacts on survival rates, likely through variation in prey availability (Miller et al. 2011; Miller et al. 2014).

**Field Work**

Garter snakes in this region of California have an annual cycle of consisting of: emerging from hibernacula in late spring (ca. mid-May); mating and foraging over the summer months of June and July; decreasing activity in August and September; and returning to hibernacula in the fall for overwintering. Over a 10-year period (2010 – 2019, excluding 2011), we hand-captured
free-ranging garter snakes in the open or under rocks in L-fast and M-slow populations from late-May through early July. Immediately upon capture we collected a blood sample from the caudal vein with a heparin rinsed syringe. We used a drop of fresh whole blood to prepare blood smears (Sparkman et al. 2014) and to measure GLUC (see below). Remaining blood was kept on ice until centrifugation where we separated packed red blood cells from plasma. We flash-froze and kept samples in liquid nitrogen, until shipment to Iowa State University on dry ice where they were stored at -80°C until time of assays.

We used plasma samples to assess baseline levels of circulating plasma corticosterone (CORT; sample sizes summarized in Table 1). Because circulating glucocorticoids increase after 10 minutes of handling (Palacios et al. 2012), we used this time as a cutoff for inclusion as a baseline measure. To assess the reactivity of CORT and GLUC, we conducted an additional bleed at 3 hours post-capture, following established restraint protocols (snakes were held in individual cloth bags for the duration; Gangloff et al. 2017; Moore and Mason 2001; Romero and Wikelski 2001). Based on the CORT stress reactivity response curves reported in Palacios et al. (2012), peak stress-response in this species occurs at 3 hours post-capture. To further corroborate this finding, in 2013 we conducted a 9 time-point assessment of stress-induced CORT and GLUC, ranging from baseline to 3 days (Supplemental Figure 2a and 2b). For most animals, therefore, we have three measures of interest for circulating CORT and GLUC (Table 1): baseline (CORT $N = 2,307$; GLUC $N = 2,279$), stress-induced maximum (CORT $N = 1,850$; GLUC $N = 1,635$), and within-individual change ($\Delta$CORT $N = 1,603$; $\Delta$GLUC $N = 1,241$). After blood collection and separation, we sexed, weighed (g), and measured from snout-to-vent (SVL, mm) all snakes; females were palpated to determine gravidity and number of embryos. We excluded immature snakes in the current study (less than 300mm SVL or low body weight
(L-fast < 20g; M-slow < 15g)). After processing, we released all snakes at their point of capture. Fieldwork was conducted under permits administered by the California Department of Fish and Game, and all procedures were approved by the Iowa State University Institutional Animal Care and Use Committee (protocol 3-2-5125-J).

**Corticosterone and Glucose Measurement**

We measured CORT concentration using a double-antibody radioimmunoassay (ImmuChem Double Antibody Corticosterone I-125 RIA kit, MP Biomedicals, Irvine, CA, USA). We conducted the assay following previously described protocols adapted for use with garter snakes (Palacios et al. 2012). We measured all baseline plasma samples at either a 1:40 dilution ($N = 1803$) or a 1:80 dilution ($N = 504$) to fit within the bounds of the standard curve. We measured all stress-induced samples at 1:160 dilution ($N = 1850$). We ran a subset of samples ($N = 20$) at a dilution factor of 1:40, 1:80, and 1:160 to test for an effect of dilution. We found good agreement across dilutions (average coefficient of variation across dilutions [CV] 20.5%), with better agreement between 40-fold and 80-fold dilutions (CV = 14.4%). 160-fold dilutions were uniformly lower than 40-fold and 80-fold, thus the stress-induced samples represent a conservative estimate of CORT concentration. All runs included from 1 to 3 pooled samples (inter-assay CV 13.8%). We re-ran sample duplicates with an intra-assay CV > 10% and any samples outside the standard curve (average intra-assay CV for all final samples = 4.15%).

We measured the concentration of GLUC at the time of blood collection from a drop of fresh whole blood using a Freestyle Lite Glucometer (Abbott Diabetes Care, Alameda, CA). The lower limit of this glucometer is 20 mg/dL; lower concentrations return a value of “low”. We assigned a value of 19 mg/dL to “low” measures ($N = 427$ of 3914 baseline and stress-induced GLUC
samples). We also assigned a random number between 3 and 19 to these individuals (3 mg/dL being the lowest concentration we have recorded using a laboratory glucose ELISA [Autokit Glucose C2, Wako Chemicals, Richmond, VA, USA]). Results of analyses using both approaches to setting a numerical value to “low” were not qualitatively different, so we equated “low” to 19 mg/dL.

**Climate Variables**

To assess the impact of climate on stress physiology, we obtained climate data from the National Oceanic and Atmospheric Administration (NOAA) National Climate Data Center (NCDC) for the region (California division 3). We already know that prey and water availability during the summer depend upon preceding winter snowpack and spring rains (e.g., Miller et al. 2011). June through October is relatively dry, whereas November through April is relatively wet; May is highly variable (Figure 1a). We generated three climate estimates from the NOAA database related to precipitation, drought severity, and temperature. For our estimate of relevant precipitation, we summed monthly precipitation (“Precip”; cm) for November through April preceding the field season. For an assessment of overall drought condition, we averaged the Palmer Hydrological Drought Index (PHDI) across the same November–April as Precip. PHDI is a monthly index that reflects the severity of a wet or dry spell by accounting for soil moisture, ground water, and temperature (Jacobi et al. 2013; Palmer 1965). Average monthly minimum, maximum, and mean temperature (MinTemp, MaxTemp, MeanTemp; °C) were computed for May–July, which corresponds to the peak activity months for Eagle Lake garter snakes and the months across which we collected samples (see Fig. 1b). MeanTemp was highly correlated with both MinTemp ($R^2 = 0.93$, $P < 0.0001$) and MaxTemp ($R^2 = 0.96$, $P < 0.0001$) and models of the
effects of each on measures of CORT and GLUC provided the same qualitative result, so we consider only MeanTemp here. In models where we tested for an effect of climate, we assessed effects of the current year’s climate (e.g. for the 2012 field season, we used the preceding Nov, 2011 – April, 2012 hydrology variables) and the previous year’s climate (e.g. preceding Nov, 2010 – April, 2011 hydrology in the above example) on measures of CORT and GLUC to address the possibility of a time-lag in stress from climatic conditions (Fig. 1b).

**Statistical Methods**

For analyses of stress physiology, we considered baseline concentration, stress-induced concentration, and within-individual reactivity (the difference between baseline and stress-induced measures) for CORT and GLUC. We had several related but separate questions to address, thus we have three separate analyses. All analyses were conducted in SAS (v9.4, SAS Institute, Cary, NC) using Proc MIXED for repeated-measures mixed-effect linear models. We estimated denominator degrees of freedom for model F-tests using the Kenward-Roger Degrees of Freedom Approximation (Kenward and Roger 1997). Graphs were made in the programming language R (R Development Core Team 2018) with package ggplot2 (Wickham 2009). CORT and GLUC were log_{10}-transformed, while measures of reactivity (ΔCORT and ΔGLUC) were square-root transformed to meet assumptions of normality of model residuals (Sokal and Rohlf 2012). All analyses included the explanatory fixed effects of Sex (mature snakes of 3 levels: male (M), female (F), or gravid female (G)); Day (-of-year) to account for any variation across the sampling season (ordinal day-of-year where our earliest sampling date May 17 = 138, and last date July 12 = 193); and body size (zSVL), which we Z-transformed to mean of 0 with unit variance because size varied between ecotypes, and among sexes within ecotypes (Range L-fast:
M = 305-655 mm, F = 315-724 mm, G = 456-780 mm; Range M-slow: M = 307-591 mm, F = 316-627 mm, G = 351-619 mm). This size standardization allows us to assess the variation among relatively small and relatively large animals within each ecotype-by-sex group, not confounded with ecotype or sex. All models also included the repeated measures random effect of individual nested in within-year recaptures (four levels: 1, 2, 3, 4) and among-year recaptures (four levels: 1, 2, 3, 4) to account for the non-independence of repeated samples collected from the same individual over seasons and years.

*The effect of annual variation on stress physiology*

To assess variation across year, our models contained the effects listed above. As well, we included “Year”, modeled as a categorical variable to allow for post hoc linear contrasts (nine levels: 2010-2019, no observations in 2011), and the fixed effects of “Time” (two levels: baseline and 3hr stress); “Ecotype” (two levels: L-fast versus M-slow), and “Population-nested-within-ecotype” (L-fast, three levels: L2, L4, L7; M-slow, four levels: M1, M2, M3, M5). Thus, our models took the following form where Y is either CORT, GLUC, ΔCORT, or ΔGLUC, and μ represents the grand mean and ε is the error term:

\[
Y \sim \mu + zSVL + \text{Day} + \text{Sex} + \text{Time} + \text{Year} + \text{Ecotype} + (\text{Sex} \times \text{Time}) + (\text{Year} \times \text{Time}) + (\text{Ecotype} \times \text{Time}) + (\text{Year} \times \text{Ecotype}) + (\text{Year} \times \text{Ecotype} \times \text{Time}) + \text{Population(Ecotype)} + |\text{ID(within year(among year))}| + \varepsilon
\]

where “Time” and its interactions were excluded in the analyses of (ΔCORT and ΔGLUC).
The effect of climate variation on stress physiology

To assess whether variation in our stress physiology variables was driven by variation in local climate, we used repeated mixed-effects models similar to the above model to analyze baseline CORT and GLUC and within-individual (ΔCORT and ΔGLUC). We removed Time and its interactions, and we replaced Year with the climate variable of interest (Precip, PHDI, or MeanTemp) and included Year as a random effect.

\[ Y \sim \mu + zSVL + \text{Day} + \text{Climate} + \text{Ecotype} + \text{Sex} + (\text{Climate} \times \text{Ecotype}) + \text{Population} + \epsilon \]

The effect of annual and climate variation on populations.

The above models are focused on ecotype, and individual populations are nested with ecotype. However, because we witnessed extirpation of populations of both ecotypes extirpated during the 40 years of study, we also ran all models above substituting “Population” in place of “Ecotype” and removing the nesting of Population. This approach allowed a test of whether certain populations are more resilient to climate-induced stress than others (irrespective of their specific life-history phenotype).

Results

Correlations among variables.

Baseline measures of CORT and GLUC were negatively correlated \((R^2 = -0.65, P = 0.01)\), stress-induced measures of CORT were positively correlated with ΔCORT \((R^2 = 0.83, P < 0.0001)\), and baseline measures of GLUC were positively correlated with stress-induced
measures of GLUC ($R^2 = 0.78$, $P < 0.0001$). No other physiological variables (baseline, stress-induced, or $\Delta$ CORT and GLUC) were significantly correlated. For climate variables (Precip, PHDI, MeanTemp) only Precip and PHDI were significantly correlated ($R^2 = 0.91$ $P < 0.0001$). Accordingly, results for Precip and PHDI were in agreement; for simplicity we report results for Precip only.

The effect of annual variation on stress physiology.

Both baseline and stress-induced CORT and GLUC varied across years by ecotype (year-by-ecotype-by-time interaction; Table 2). For seven of nine years, M-slow snakes had higher levels of baseline CORT than L-fast snakes (Figure 2a). In three of seven years, L-fast snakes had higher levels of baseline GLUC than M-slow snakes (Figure 3a). These results were mirrored in stress-response (3hr) CORT and GLUC (Table 2). For four of eight years, M-slow snakes had higher levels of stress-induced CORT than L-fast snakes (Figure 2b and S3a). And in five of six years, L-fast snakes had higher levels of stress-induced GLUC than M-slow snakes (Figure 3b and S3b). The maximum value of stress-induced CORT was 867 ng/mL in an M-slow snake; and for stress-induced GLUC was 212 mg/dL in an L-fast snake. Additionally, we found that L-fast snakes were more variable in their baseline measures of CORT (CV 28.9%), as well as stress-induced measures of GLUC (12.8%), when compared to M-slow snakes across years (CV baseline CORT: 17.5%; stress-induced GLUC: 4.8%), while M-slow snakes were slightly more variable in their baseline measures of GLUC (CV 9.3%) compared to L-fast snakes (CV 6.7%) across years.
**Effects of covariates on physiology**

Measures of baseline CORT increased with increasing body size (zSVL), while baseline GLUC, stress-induced GLUC, and ΔGLUC decreased with increasing body size. Baseline CORT decreased across sampling period (May 17 – July 12), whereas baseline GLUC increased with advancing day, with a corresponding decrease in ΔGLUC. Males (M) had higher baseline CORT than females (F), with gravid females (G) being intermediate and not different than Males or Females (rank order: M > G > F, M ≠ F). Females had significantly higher values of baseline GLUC than males and gravid females (rank order: F > (M, G)).

**The effect of climate variation on stress physiology.**

Using the previous year’s (i.e., 1-year lag) climate variables did not yield models with greater explanatory power. Models assessing the relationship between current year’s climate and physiology detected meaningful interactions of ecotype and climate, including both Precipitation (Cumulative November through April) and Temperature (Mean May through July) on baseline CORT and GLUC, and ΔCORT and GLUC (Table 3). For L-fast snakes, baseline CORT increased with increasing Precip (Fig. 2C) with a corresponding decrease in ΔCORT (Fig. 2D); M-slow snakes remained the same (Fig. 2C). Conversely, baseline GLUC increased in M-slow snakes with increasing temperature; L-fast snakes remained the same (Fig. 3C). For ΔGLUC, M-slow snakes had increased reactivity (i.e., larger ΔGLUC) with increasing Precip; L-fast snakes were not affected (Fig. 3D).
The effect of annual and climate variation on populations.

Our models which substituted Population for Ecotype (and removed Population nested within Ecotype) were qualitatively similar to our models focused on ecotype differences. This justifies an ecotype approach in our analyses and these results are not considered further. Nonetheless, we retained population nested within ecotype as a fixed effect in all models as it explained a significant amount of variation in physiology, which demonstrates variation among similar-ecotype populations.

Discussion

In this study, we tested whether replicate populations of a terrestrial ectotherm (garter snakes), with divergent life-history ecotypes, along a slow-to-fast pace-of-life (POL) continuum, exhibited annual variation in biomarkers of stress physiology, and whether such variation was predicted by precipitation, drought, and temperature climate variables. Specifically, we tested the prediction that snakes inhabiting more stochastic environments (M-slow; e.g., Bronikowski and Arnold 1999) should have relatively high baseline and stress-induced CORT levels as a means of promoting self-maintenance and immediate survival. As well, M-slow populations should display variable or lower baseline GLUC levels due to inconsistent resource availability relative to L-fast populations. In contrast, we predicted L-fast populations to have lower circulating levels of both baseline and stress-induced CORT and high, less variable circulating levels of GLUC. We found significant annual variation in baseline and restraint-stress induced CORT and GLUC that corresponded to ecotype-specific differences in many years in the direction predicted by POL theory. We detected limited support for the hypothesis that our measures of stress physiology were significantly impacted by climate. We discuss our findings in the context of
POL trade-offs and population sensitivity to climate, per se, versus indirect manifestations of climate on resource availability, and explore the ramifications for population persistence.

**Pace of life sensitivity to climate change**

Life-history theory is based on the premise of trade-offs. Life-history theory posits that organisms must make energetic decisions and allocate resources between survival and reproduction in response to variation in their environment. Understanding physiological responses to environmental stress can offer insight into life-history strategies and energetic allocation of resources. Highly variable environments are predicted to drive availability of resources and ultimately life-history evolution. Through variation in reproductive success, stochastic environments are expected to select for slow POL (Tuljapurkar et al. 2009). However, relatively few empirical studies have examined the effects of habitat variation on intraspecific life-history variation. Cayuela et al. (2019) examined variation in habitat dynamics in populations of the yellow-bellied toad (*Bombina variegata*) across multiple years and found that populations inhabiting more variable environments had higher rates of survival and longer lifespans compared to populations in more stable environments. Another study, comparing the life-history strategies of black-browed albatross (*Thalassarche melanophrys*), found that populations with breeding grounds characterized by increased climatic variability had lower reproductive success but longer lifespans compared to populations breeding in more stable environments (Nevoux et al. 2010). Similarly, a semi-natural study examining the effects of climate variability on survival in the European common lizard (*Zootoca vivipara*) found that populations experiencing more predictable precipitation had higher rates of survival than those populations exposed to unpredictable and more variable precipitation (Maso et al. 2020).
Additionally, Colchero et al. (2019) found that, with increasing environmental variation, the probability of population extinction is greatly reduced in species that exhibit survival-fecundity trade-offs, compared to species that exhibit either positive covariation between survival and fecundity or no covariation.

Physiology is purported to be the mechanistic link mediating the relationship between survival and reproductive trade-offs (Promislow and Harvey 1990; Ricklefs 2000). The endocrine system and energy stores are broadly implicated in mediating such trade-offs (reviewed in Crespi et al. 2013), and with facilitating the interaction between organisms and their environment (Cohen et al. 2012). Slow-paced populations or species – whose energetic demands maximize fitness by prioritizing self-maintenance over rapid growth and annual fecundity – should exhibit higher levels of stress-induced glucocorticoids relative to fast-paced organisms that favor current reproduction over self-maintenance (Ricklefs and Wikelski 2002). Thus, slow-living populations should exhibit greater physiological plasticity, and in turn be better buffered against local extinction. Patterson et al. (2014) found that, in wild mountain white-crowned sparrows (Zonotrichia leucophrys oriantha), high rates of survival were correlated with relatively high circulating baseline and stress-induced CORT levels, while increased reproductive success corresponded with relatively high baseline measures but low stress-induced measures. However, the relationships between measures of physiology, stress, and fitness have been mixed across studies (Bonier et al. 2009; Breuner et al. 2008).

In our system, environmental stochasticity among populations has produced divergent ecotypes aligned along the slow-fast POL continuum that support the role of glucocorticoids in mediating life-history strategies. Specifically, the slow-living ecotype (M-slow) exhibits reduced among-year variability in biomarkers of physiological status, relative to fast-living (L-fast)
populations, suggesting the former have the capacity to accommodate a wider range of extrinsic conditions (i.e., a broader allostatic or reactive scope; reviewed in Crespi et al. 2013). M-slow populations also exhibit higher baseline and stress-induced CORT levels relative to their L-fast counterparts. This finding agrees with documented patterns of ecotypic differentiation in this system (Palacios et al. 2012): in years where ecotype differences were evident, M-slow animals displayed higher circulating levels of CORT. However, another study of these same populations of garter snakes found no evidence of POL differentiation in measures of physiology (CORT, GLUC, insulin, lactate, or metabolic rate) under conditions of heat exposure (Gangloff et al. 2016). Although measures of CORT rose with increasing temperature, animals from each ecotype responded similarly and CORT levels remained below maximum levels attained by wild-caught animals under handling stress, suggesting these animals had the capacity to increase their stress response if exposed to additional perturbations. A similar response to heat exposure was found in the Children’s python (Antaresia childreni), where circulating CORT was elevated, relative to baseline in response to heat exposure, but did not reach maximum levels. This was evidenced by an even greater increase in CORT when pythons were exposed to cold (Dupoué et al. 2013). Additionally, circulating CORT levels were negatively correlated with temperature in response to an extended heatwave in wild Z. vivipara (Dupoué et al. 2018), suggesting these lizards were downregulating activity levels to limit exposure to the extreme heat. Animals thus may respond differently to heat exposure than to other environmental perturbations, behaviorally mitigating negative effects of thermal stress. Alternatively, in years where ecotypes differed, L-fast populations exhibited higher baseline and stress-induced measures of GLUC than M-slow populations. This is likely due to more stable resources, as L-fast populations are characterized by having access to continuous food and water, while resources for M-slow populations are more
variable and depend on annual precipitation (Bronikowski and Arnold 1999). Additionally, L-fast populations overall were more variable in their baseline and stress-induced measures of CORT and stress-induced measures of GLUC. This pattern of increased GLUC in L-fast population may also give context to the greater variability in circulating levels of CORT. If modulation of glucocorticoids via the HPI axis functions to maintain homeostasis through regulation of energy substrates (i.e., glucose), we would expect more variability in circulating levels of CORT to maintain available GLUC within a narrow range.

*Climate, stress-physiology, and population persistence*

To understand impacts of stress physiology on population dynamics in the face of changing climate, longitudinal studies of physiology and demography are needed to identify the fitness effects of physiological variation. While many acknowledge the importance of long-term physiological studies on natural populations to understand how changing climates and habitats affect survival (Reinke et al. 2019), they remain rare. Long-term studies typically focus on population range shifts and decline or extinction (Berry et al. 2013; Blumstein et al. 2006; Bucciarelli et al. 2020; Moritz et al. 2008; Smith et al. 2013; Whitfield et al. 2007) and changing phenology (Berteux et al. 2004; Burgess et al. 2018; Charmantier et al. 2008; Janzen et al. 2018) and morphology (Bucciarelli et al. 2020). Far fewer have looked at behavioral (Dantzer et al. 2012; McGaugh et al. 2010; Pirotta et al. 2018) or physiological (Combrink et al. 2020; Ouyang et al. 2011; Patterson et al. 2014; Sonnweber et al. 2018) plasticity. While these long-term studies provide valuable insight into how populations are changing and adapting across space and time, the generalizability across systems remains unclear.
Although long-term physiological field studies are rare, even more so are those directly considering the effects of local climate variation on trait variation. The impacts of climate change on population persistence will depend on its members’ capacity to buffer against changing environments, through acclimation, adaptation, or dispersal (Deutsch et al. 2008). Phenological shifts and behavioral plasticity are common mechanisms by which organisms mitigate the negative effects of changing environments (Bradshaw and Holzapfel 2006; Huey et al. 2003; Walther et al. 2002), however, organisms also cope with variation in their environment through physiological plasticity that is largely mediated by the endocrine system (McEwen and Wingfield 2003; McEwen and Wingfield 2010; Romero et al. 2009). Glucocorticoids function as primary mediators of energy metabolism, acting to both mobilize energy stores and maintain longer-term homeostasis (Johnstone et al. 2012; Wingfield 2013; Wingfield and Kitaysky 2002). Thus, glucocorticoids often fluctuate in parallel with other biomarkers of energy expenditure such as glucose and act as indicators of the reactivity or sensitivity of an individual to a given stressor (chapter 2). In response to a perceived stressor, blood GLUC levels are typically elevated to provide the metabolic substrate necessary to support a stress response (Sapolsky et al. 2000). Endocrine-mediated stress responses are likely fundamentally important for population-level responses to climate change. Accordingly, variation in aspects of local weather – such as temperature and precipitation – have been associated with increased CORT and GLUC levels in a variety of taxa in the short-term (see meta-analysis in de Bruijn and Romero 2018), suggesting that environmental stimuli related to climate change act as stressors. Stressors, in turn, depending on the pervasiveness, magnitude or frequency, can affect population fitness (Wingfield et al. 1998). However, although glucocorticoids, and associated metabolites are modulated in response to stressful events, most studies examine the effects of acute stress exposure. Studies of long-
term exposure to climate-related stress (i.e., extreme or pervasive weather events), find considerably more variation (de Bruijn and Romero 2018). Our results are concordant with this perspective; annual variation in CORT and GLUC was not linked to simple measures of drought, precipitation, or temperature. Moreover, weather variables were important only in the context of contrasting ecotypes – with ecotype-specific results (Figs 2c, 3c, 3d).

This area of California has experienced several multi-year droughts since the study of these populations of garter snakes began (1987–1989, 2001–2003, 2007–2009, 2013–2015), with 2013–2015 having the driest and hottest years on record (Bales et al. 2018; Griffin and Anchukaitis 2014; Mann and Gleick 2015). Extreme drought has been linked to reductions in body condition across populations of California newts (Taricha torosa; Bucciarelli et al. 2020) and rapid species decline in anurans across much of the Sierra Nevada mountains (Drost and Fellers 1996; Fellers and Drost 1993). Additionally, Prugh et al. (2018) quantified responses of sympatric plants, arthropods, reptiles, and mammals to the most recent drought in the Carrizo plain (central valley of California), finding that more than 25% experienced appreciable declines in abundance. Given the dramatic climatic changes throughout California, we were additionally interested in evidence of acclimation to drought, at the population level, between extreme climatic events. We collected baseline CORT measures from 2006–2010 (Palacios et al. 2012) for the same populations (2010 rerun with current samples). Unlike in the present study, the effects of climate on baseline CORT from 2006–2009 did not interact with ecotype. Together these findings of small or nonexistent direct effects of climate on CORT and GLUC suggest that the indirect effects of climate on habitats are more important in mediating stress physiology. Aspects of local climate have also been used to assess effects of climate change on population viability. Extreme deviations in local climate have been linked to changes in the elevational
range of many species. Ranges have shifted and, in some cases contracted, for multiple species of birds (Tingley et al. 2012), reptiles (Barrows 2018), and small mammals (Moritz et al. 2008; Rowe et al. 2015). Miller et al. (2018) found that many amphibian communities are especially sensitive to fluctuations in precipitation immediately prior to, and during, the breeding season. They further noted that winter conditions in montane habitats predicted population persistence and distribution. Species distribution models, incorporating metrics of climate and habitat, have also been used alongside observed distributions in a large-scale re-sampling effort of the “Cascade transects”, including transects within Lassen National Forest (for details see Moritz et al. 2008), to assess range dynamics for several small mammal species. These efforts found limited support for either climate or habitat being a sole predictor in range shifts for many species. However, for species in which measures of local climate, habitat structure, and range shifts were tightly coupled, high elevation (2000–3200 m) population range shifts were best predicted by climate directly, while low (< 1500 m) and mid (1500–2500 m) elevation population range shifts were best predicted by the combined influence of climate and habitat alterations (Santos et al. 2017; Smith et al. 2013). The diversity of organismal responses to various climatic measures highlights the importance of utilizing multiple predictors when assessing organismal responses to climate change. However, by only assessing effects of local climate, such as temperature and precipitation, studies of ectotherms may miss potentially more important drivers of physiology such as indirect effects of climate on food availability, access to refugia, and predator dynamics.

Physiological plasticity in response to climate variation in this system may reflect adaptation to habitats characterized by seasonal variation and repeated exposure to extreme climatic events (i.e., droughts) rather than a stress response per se. Alteration of landscapes by
climate change has affected many species across the state of California. Notably, during droughts in the 1980s and 2000s, meadow habitats experienced pronounced local extirpation. For snakes inhabiting meadows, droughts limit availability of food and water resources (Bronikowski and Arnold 1999; see also Kephart and Arnold 1982). The most recent drought, however, has been accompanied by record declines in lake levels; minimum lake levels during the 1980s and early 2000s droughts were between 1555.1 and 1555.6 m, whereas minimum lake levels in 2015 fell to 1551.4 m (per Lassen County Public Works), leaving exposed shoreline with no vegetation or retreat rocks. This exposed stretch between suitable habitat and the lake could render snakes at higher risk of predation as they traverse the shoreline to their food source. Altered resource availability, such as microhabitat structure and access to retreat sites (Huey et al. 1989), can leave individuals more vulnerable to their local climate, more movement-challenged (Kelley et al. 1997), or more exposed to predators and ultimately lead to decreases in population density (Berry et al. 2013; Whitfield et al. 2007). At present, no snakes reside in historically large L-fast populations (L1, L3, L5, and L6; Fig. S1) where vegetation and retreat sites are now absent, whereas demographically healthy L-fast populations have this appropriate habitat structure. It appears that real-estate, rather than food and water per se, limits L-fast snakes. Whether dispersal of snakes from meadow sites to lakeshore sites will result in recolonization is doubtful given that stream corridors remain dry (see Gangloff et al. 2020; Manier and Arnold 2005 for estimates of migration rates and gene flow).

**Conclusion**

Overall, our results reveal considerable inter- and intra-annual variation in snake physiology, yet little of this variation is explained by measures of local temperature and
precipitation. Still, populations disappeared during our focal decade, likely in response to habitat change resulting from climatic shifts. Whether physiology, demography, or genetic structure best predicts population persistence is therefore an ongoing pursuit or urgent import.

**Acknowledgements**

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**Authors’ Contributions**

KGH, AMS, EJG, DAWM, and AMB performed field work and collected samples; KGH performed lab work, analyzed data and wrote the manuscript; AMB provided guidance in data analysis and interpretation

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**Table 1.** Sample sizes for baseline, stress-induced, and reactivity (change between baseline and stress-induced; Δ) measures of plasma corticosterone (CORT) and blood glucose (GLUC) by ecotype and year. Numbers in parentheses represent the number of populations that contribute to the total number of samples for a given ecotype by year combination.

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Table 2. Repeated-measures mixed linear model analysis of physiology in wild-caught western terrestrial garter snakes (*Thamnophis elegans*). Physiological measures of baseline plasma corticosterone and blood glucose are log_{10}-transformed. Measures of physiological responsiveness (ΔCORT and ΔGLUC) are square-root transformed. Values are $F_{dfn, dfd}$. Significant effects ($P \leq 0.05$) are in bold.

<table>
<thead>
<tr>
<th></th>
<th>CORT</th>
<th>ΔCORT</th>
<th>Glucose</th>
<th>ΔGLUC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>zSVL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F$ (d.f., d.f.d)</td>
<td>26.41</td>
<td>0.63</td>
<td>65.77</td>
<td>29.72</td>
</tr>
<tr>
<td>$P_{r} &gt; F$</td>
<td>&lt; 0.0001</td>
<td>0.429</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Ordinal Day</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F$ (d.f., d.f.d)</td>
<td>3.08</td>
<td>0.65</td>
<td>11.29</td>
<td>48.85</td>
</tr>
<tr>
<td>$P_{r} &gt; F$</td>
<td>0.079</td>
<td>0.421</td>
<td>0.001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>$F$ (d.f., d.f.d)</td>
<td>5.53</td>
<td>6.36</td>
<td>29.92</td>
<td>13.63</td>
</tr>
<tr>
<td>$P_{r} &gt; F$</td>
<td>0.004</td>
<td>0.002</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>Time</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F$ (d.f., d.f.d)</td>
<td>1064.24</td>
<td>--</td>
<td>1877.82</td>
<td>--</td>
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<tr>
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<td>&lt; 0.0001</td>
<td>--</td>
<td>&lt; 0.0001</td>
<td>--</td>
</tr>
<tr>
<td><strong>Year</strong></td>
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<tr>
<td>$F$ (d.f., d.f.d)</td>
<td>14.93</td>
<td>9.86</td>
<td>4.01</td>
<td>4.22</td>
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<tr>
<td>$P_{r} &gt; F$</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Ecotype</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>$F$ (d.f., d.f.d)</td>
<td>18.80</td>
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<td>27.45</td>
<td>0.63</td>
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<td>$F$ (d.f., d.f.d)</td>
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<td>--</td>
<td>3.30</td>
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<tr>
<td>$P_{r} &gt; F$</td>
<td>0.004</td>
<td>--</td>
<td>0.037</td>
<td>--</td>
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<tr>
<td><strong>Ecotype × Time</strong></td>
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<td></td>
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<tr>
<td>$F$ (d.f., d.f.d)</td>
<td>8.55</td>
<td>--</td>
<td>0.75</td>
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<tr>
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<td>0.387</td>
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<tr>
<td><strong>Ecotype × Year</strong></td>
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<tr>
<td>$F$ (d.f., d.f.d)</td>
<td>1.62</td>
<td>1.63</td>
<td>2.24</td>
<td>1.97</td>
</tr>
<tr>
<td>$P_{r} &gt; F$</td>
<td>0.124</td>
<td>0.124</td>
<td>0.037</td>
<td>0.080</td>
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<tr>
<td><strong>Year × Time</strong></td>
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<tr>
<td>$F$ (d.f., d.f.d)</td>
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<td>--</td>
<td>2.99</td>
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<td>0.011</td>
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<tr>
<td><strong>Year × Ecotype × Time</strong></td>
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<tr>
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<td>6.17</td>
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<tr>
<td>$P_{r} &gt; F$</td>
<td>0.002</td>
<td>--</td>
<td>&lt; 0.0001</td>
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</tr>
<tr>
<td><strong>Population(ecotype)</strong></td>
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<td></td>
</tr>
<tr>
<td>$F$ (d.f., d.f.d)</td>
<td>11.81</td>
<td>3.94</td>
<td>4.36</td>
<td>17.76</td>
</tr>
<tr>
<td>$P_{r} &gt; F$</td>
<td>&lt; 0.0001</td>
<td>0.002</td>
<td>0.001</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>
Table 3. Repeated-measures mixed linear model analysis of physiology and the interaction with climate (precipitation and temperature) in wild-caught western terrestrial garter snakes (*Thamnophis elegans*). Here we report the effects of precipitation and temperature on physiology. Physiological measures of baseline and stress-induced plasma corticosterone and blood glucose are log_{10}-transformed. Measures of physiological responsiveness, or the change between baseline and stress-induced measures of physiology (ΔCORT and ΔGlucose) are square-root transformed. Values are $F_{d_{fn}, d_{df}}$. Significant effects (P < 0.05) are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Cumulative Precipitation</th>
<th>Mean Temperature</th>
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<tbody>
<tr>
<td></td>
<td>Baseline CORT</td>
<td>ΔCORT</td>
</tr>
<tr>
<td>zSVL</td>
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<td>$F$ (d.f., d.f.)</td>
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<td>0.70 1, 1577</td>
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<td>$F$ (d.f., d.f.)</td>
<td>3.97 1, 2270</td>
<td>0.87 1, 1577</td>
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<tr>
<td>$P_t &gt; F$</td>
<td>0.046</td>
<td>0.352</td>
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<tr>
<td>Sex</td>
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<td>$F$ (d.f., d.f.)</td>
<td>6.02 2, 2275</td>
<td>7.18 2, 1576</td>
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<td>0.001</td>
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<td></td>
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<td>22.06 1, 2271</td>
<td>1.35 1, 1577</td>
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<td>0.245</td>
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<td>0.89 1, 8,08</td>
<td>1.18 1, 5.47</td>
</tr>
<tr>
<td>$P_t &gt; F$</td>
<td>0.374</td>
<td>0.323</td>
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Table 3 Continued.

<table>
<thead>
<tr>
<th></th>
<th>Cumulative Precipitation</th>
<th>Mean Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline CORT</td>
<td>ΔCORT</td>
</tr>
<tr>
<td>Climate × Ecotype</td>
<td></td>
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</tr>
<tr>
<td>$F$ (d.f., d.f.)</td>
<td>5.49 1, 2275</td>
<td>4.35 1, 1577</td>
</tr>
<tr>
<td>$P_r &gt; F$</td>
<td>0.019</td>
<td>0.037</td>
</tr>
<tr>
<td>Population(ecotype)</td>
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<td></td>
</tr>
<tr>
<td>$F$ (d.f., d.f.)</td>
<td>10.4 5, 2250</td>
<td>3.93 5, 1575</td>
</tr>
<tr>
<td>$P_r &gt; F$</td>
<td>&lt; 0.0001</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Figure 1. Climate variables. (A) Mean monthly precipitation and temperature from 2005 – 2019 with corresponding annual snake activity cycle noted. (B) Mean climate variables for 15 years, including the five years preceding the focal decade, where Precip is the cumulative precipitation for the relevant wet season preceding the active period (previous November – April), Temp is the mean temperature during the active season (May – July) and PHDI is a mean value for relative dryness by snake year (for example mean snake year for 2012 constitutes monthly values from November 2011 – October 2012). Red dashed line indicates PHDI cutoff for extreme drought (< -3) (corresponding to 2007, 2008, 2009, 2014, 2015).
Figure 2. Annual baseline plasma CORT levels (A), stress-induced measures of CORT (B), and CORT response to annual cumulative precipitation (C) in free-ranging western terrestrial garter snakes (*Thamnophis elegans*) sampled in 9 years (2010-2019, excepting 2011). Data are back-transformed least squares means ± SE from statistical models in Table 3. Asterisks in panel (A) denote years in which there were significant differences in baseline corticosterone between ecotypes. Panel (C) depicts the significant effect of precipitation on circulating levels of baseline CORT in L-fast snakes. Panel (D) depicts the significant effect of cumulative precipitation on ΔCORT (reactivity or magnitude of change between baseline and stress-induced measures) in L-fast snakes. The effect of precipitation on ΔCORT for the M-slow ecotype is not significant.
**Figure 3.** Annual baseline blood GLUC levels (A), stress-induced measures of GLUC (B), mean temperature effects on baseline GLUC (C), and ∆GLUC response to annual cumulative precipitation (D) in free-ranging western terrestrial garter snakes (*Thamnophis elegans*). Baseline glucose was sampled across 7 consecutive years while responsiveness to a simulated stressor was measured in 2014-2019. Data are back-transformed least-squares means ± SE from statistical models in Table 3. Asterisks in panel (A) denote years in which there were significant within year differences in baseline glucose levels between ecotypes. Panel (C) depicts the significant effect of temperature on baseline glucose in M-slow snakes. Panel (D) depicts the significant effect of cumulative precipitation on ∆GLUC (reactivity or magnitude of change between baseline and stress-induced measures) in M-slow snakes. The effect of precipitation on ∆GLUC for the L-fast ecotype is not significant.
Supplemental Information

Figure S1. Locations of focal lakeshore (L-fast) and meadow (M-slow) populations in the Eagle Lake Basin, Lassen County, CA. Populations that are not viable at present (L1, L3, L5, L6, M4) are denoted with a circle-backslash symbol and population L4 that is in decline is denoted with a gray circle-backslash. Of note, M-slow population M4 was likely disbanded due to road construction that removed habitat structure.
**Figure S2.** Back-transformed least-squares means ± SE of plasma CORT concentration (A), and blood GLUC concentrations (B) across 3 days in captivity. Data are from wild-caught L-fast and M-slow ecotypes of *Thamnophis elegans* from Eagle Lake, CA. There was no significant effect of Ecotype (*P* = 0.45, *F* = 0.62, 1, 12.1) or Ecotype by Time (*P* = 0.77, *F* = 0.60, 8, 115) on CORT, and a marginally significant effect of Ecotype (*P* = 0.055, *F* = 4.5, 1, 12.2) on GLUC. For both ecotypes, time series measurements support using a 3hr post-capture bleed as representative of maximal stress-induced concentrations for both CORT and GLUC.
Figure S3. Annual stress-induced plasma CORT concentrations (A) and stress-induced whole blood GLUC concentrations (B) in free-ranging western terrestrial garter snakes (*Thamnophis elegans*) sampled over 9 years (2010-2019, excepting 2011) for CORT and over 6 years (2014-2019) for GLUC. Data are back-transformed least-squares means ± SE. Asterisks denotes years in which there was a significant difference between L-fast and M-slow ecotypes.
**Figure S4.** Annual reactivity of plasma CORT (ΔCORT) concentrations (A) and reactivity of whole blood GLUC (ΔGLUC) concentrations (B) in free-ranging western terrestrial garter snakes (*Thamnophis elegans*) sampled over 8 years (2010-2019, excepting 2011 and 2012) for ΔCORT and over 6 years (2014-2019) for ΔGLUC. For measures of the within individual reactivity, CORT was greater in M-slow animals in 1 of the 8 years sampled. For measures of GLUC L-fast reactivity was greater in one year. Data are back-transformed least-squares means ± SE. Asterisks denotes years in which there was a significant difference between L-fast and M-slow ecotypes.
CHAPTER 5
SUMMARY AND CONCLUSIONS

Collectively, the work presented here contributes to the growing body of literature on the pervasive effects of temperature on the physiological performance of biological systems. As global temperatures rise, species are experiencing increased incidence of stressful climatic conditions at the local scale. These changes are characterized not only by increased temperatures, but also increased variance in environmental conditions on seasonal and annual bases, and also higher incidence of extreme weather events. Thus, having a mechanistic understanding of how organisms respond to thermal variation, and extremes, gives insight into how organisms may interact with their environment to mitigate deleterious effects of changing environments.

Using two widespread ectotherms, in both lab-based and natural experiments, I focused on how the thermal environment affects an organism’s performance and predicts population level patterns. This included measuring physiological profiles of snakes across their range of ecologically relevant temperatures in the lab – providing an integrated look at how warm and cold (i.e., brumation) temperatures elicit physiological responses – as well as measuring the response of natural populations to annual variation in local climate (including the extreme events of multiple droughts years). For my first chapter I looked at how physiological processes integrate to support seasonal brumation by facilitating body cooling, sustained brumation, and subsequent warming in the checkered garter snake. This is particularly relevant when considering global climate change, as temperature profiles and duration of winter seasons are being disrupted. I found temperature dependence across all of the biomarkers measured: circulating levels of corticosterone, glucose, insulin and the ratio of heterophils to lymphocytes, as well as in measures of whole organism metabolic rate. Though measures of physiology all
responded to temperature, they did so with different thermal response curves – with heterogeneity both within and among variables, depending on whether animals were entering or exiting brumation. Together the results from this experiment suggest that physiological axes coordinate to mitigate deleterious effects of prolonged exposure to cold temperatures and that this coordination is plastic such that we see seasonal variation in thermal responsiveness. While my first chapter looked at the integration of physiological biomarkers at the level of the whole organism, I was also interested in temperature dependence across scales of biological organization. For my second chapter I measured the thermal dependence of whole organism and cellular level oxygen consumption, including temperatures above the preferred range, and nearing the critical maximum for the western terrestrial garter snake. In addition to traditional measures of metabolic rate at the level of the whole organism via respirometry, I used a novel approach to examine the effects of thermal variation on aspects of cellular respiration. I found that although metabolic rate at whole organism and cellular levels is temperature dependent, the two levels of biological organization do not exhibit intraindividual covariation – that is, they both increase with increasing temperature but do so with different thermal response curves. This suggests that, in this system, cellular metabolism does not directly govern whole animal rates of oxygen consumption. Additionally, I found that measures of fuel selection (i.e., respiratory exchange ratio and natural abundance of δ¹³C) did not vary across the range of temperatures included in this study. This consistency suggests the maintenance of a single fuel source being oxidized to support metabolism across a broad range of metabolic demands. For my final chapter, I examined whether patterns of intra- or inter-annual variation in measures of physiology were consistent with specific pace-of-life expectations in two divergent life-history ecotypes, and if that variation is predicted by local temperature and precipitation. I measured
baseline and stress-induced concentrations of corticosterone and glucose, and their reactivity (the magnitude of change between baseline and stress-induced measures), over a 10-year period that was characterized by a multi-year drought. I found significant annual variation in both baseline and stress-induced measures of corticosterone and glucose, as well as in their reactivity. However, importantly, I found that annual variation in physiology was not characterized by drought versus non-drought years and that temperature and precipitation explained little of the annual variation in corticosterone and glucose, and only in the context of ecotype. Additionally, I found within-year differences between ecotypes. Specifically, slow-living (M-slow) populations exhibited higher levels of baseline CORT for both baseline and stress-induced measures while L-fast snakes exhibited higher levels of glucose for both baseline and stress-induced measures. Overall, the results from this study show that there is significant inter- and intra-annual variation in this system. However, this variation is not explained, at least directly, by variation in temperature and precipitation. This suggests that physiological regulation of stress via glucocorticoids and glucose may not be the most important mechanism by which garter snakes in this system deal with large-scale environmental perturbations such as drought. Instead, ecotypic differences are likely driving physiological responses to stress, and climate indirectly influences these ecotypes through their distinct life histories.

Taken together these results show that in ectothermic garter snakes physiology is profoundly influenced by temperature. However, in the wild, local climate does not seem to directly predict physiological variation. This may mean that studies explicitly focusing on single aspects of climate (e.g., temperature or precipitation) may miss important biologically relevant variation that may either contribute to population persistence or, alternatively, leave populations more vulnerable to extinction.