ABSTRACT

Title of Dissertation: THERMAL PHYSIOLOGY IN A WIDESPREAD LUNGLESS SALAMANDER
Alexander Joseph Novarro, Doctor of Philosophy, 2018

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Understanding species responses to climate change has become a top priority for conservation biologists. Unfortunately, current models often treat species as a single entity, ignoring population-level variation. This approach may result in major oversights when managing widespread species, which generally exhibit physiological variation across their geographic range. The eastern red-backed salamander (Plethodon cinereus) is the most widely distributed Plethodon species, extending farther north than any other lungless salamander. This species’ geographic distribution raises two major questions: How does P. cinereus thrive across a wide range of temperatures, and does it possess thermal adaptations that will buffer the ecological consequences of climate change? To explore these questions, I first examined the effects of elevated temperature on metabolic hormone release rates and physiological performance (i.e., ingestion rate and mass gain) across a latitudinal population gradient. I found that physiological traits and populations differ in their
thermal flexibility, and that salamanders from warmer localities are more resilient to elevated temperatures. Second, I performed a study to disentangle the environmental and evolutionary drivers of thermal limits across the geographic range of *P. cinereus*. I found strong support for evolutionary constraints on lower thermal limits, though there was some degree of plasticity in relation to local environmental temperatures. By contrast, upper thermal limits showed little variation across the species’ geographic range and among clades, and far exceeded survival requirements. Third, I combined laboratory experiments, field observations, and population models to explore the role of behavioral thermoregulation in shaping physiological performance in *P. cinereus*. I found that individuals are likely to exploit moist conditions at the cost of reduced performance, and that populations living in poor thermal quality habitats have greater thermoregulatory accuracy. Overall, my work demonstrates significant variation in thermal physiology across the geographic range and among lineages of *P. cinereus* and shows that thermal traits differ in their responsiveness to thermal variability. Together, these results highlight the importance of considering multiple physiological metrics and sampling large geographic areas to understand species’ abundance and distributions, and to assess species’ vulnerability to climate change.
THERMAL PHYSIOLOGY IN A WIDESPREAD LUNGLESS SALAMANDER

by

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Dissertation submitted to the Faculty of the Graduate School of the University of Maryland, College Park, in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2018

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Preface

This dissertation contains an introduction (Chapter 1), three research chapters in manuscript form (Chapters 1-4), and appendices to the chapters (Appendices 1-3). A single bibliography is provided at the end for literature cited throughout the dissertation.
Dedication

To the scientists who value compassion over competition and intellect over impact factors.
Acknowledgments

I am deeply grateful for the support and encouragement that made this dissertation possible. First, I would like to thank my advisor, Alexa Bely, who guided me through professional challenges, no matter how big or small they were. Alexa provided a constructive space for me to develop intellectual as well as emotional skills, and for that I am eternally grateful. I would also like to thank my dissertation committee, who provided critical feedback on my dissertation proposal and challenged me to think through the broader implications of my work.

I had the pleasure of collaborating with many professors, graduate students, and undergraduates on each of my projects. Special thank you to Kristine Grayson who helped facilitate the project described in Chapter 2, who regularly made herself available for long phone conversations about science, and who consistently demonstrated the qualities of a successful professor, collaborator, and mentor. I would also like to thank Caitlin Gabor, Cory Goff, Tori Mezebish, and Lily Thompson who collaborated on the study described in Chapter 2; Matt Watson who provided access to his lab and semimicro bomb calorimeter for processing the samples used in Chapter 4; David Muñoz who ran the mark-recapture models described in Chapter 4; and Sara Via who provided Percival incubators for the physiological and behavioral studies described in Chapters 2-4. The undergraduates and field technicians that I have worked with have been exceptional, and constantly reinvigorate my passion for science. Thank you to Logan McDonald, Sarah Bailey, August Blackman, Kayla Sherman, Andi Levorse, Osmary Medina-Báez, and Sam
Earl for help in the field. And to Tori Mezebish, Matthew Griffin, Melissa Marquez, Hannah Whitakker, Sarah Timko, and Christian Law for assistance in the lab.

Much of my development as a scientist and mentor has occurred at Mountain Lake Biological Station (MLBS), where I spent my summers and performed most of my fieldwork. Special thank you to the staff and residents of MLBS, particularly Butch Brodie, Eric Nagy, Jaime Jones, and Vince Formica, who have supported my science and have helped set me up for a successful career. I’d also like to thank the scientists who have come through the station and left a lasting impact on me and my work, including Caitlin Fisher-Reid, Colleen Ingram, Laurence Frabotta, Christian Cox, Eric Leibgold, and Dave McLeod.

Another irreplaceable source of support and professional development has come from Partners in Amphibian and Reptile Conservation, particularly Priya Nanjappa, Jen Williams, JJ Apodaca, Michelle Christman, Kristin Stanford, and Linda Weir. They have not only empowered me to become a leader in the herpetological conservation community but have also been an endless source of laughter and friendship. My involvement in PARC has kept me going through challenging times, and constantly reminds me of my passion for the animals that drew me into this field.

Finally, I would like to thank my friends and family, who have supported my decisions and graciously engaged with my work, even when it was overly technical and far from their interests. Thanks to my mom, who has done more to shape my personality, and probably my love of reptiles and amphibians, than anyone in this world. And to my dad, who has always demonstrated the value of hard work, a good
sense of humor, and compassion for others. Thank you to Sarah, my fiancé, who has stood by my side through fieldwork catastrophes, kept me calm during existential crises, and even helped me count fruit flies and collect salamander poop on her days off. I am incredibly lucky to have her support in all that I do! And a special thanks to my great friends Elske Tielens, Carly Mulletz Wolz, and Grace DiRenzo who have stuck by my side since my first day of graduate school.

This work was made possible through generous funding by The Explorers Club, MLBS, the Society for the Study of Amphibians and Reptiles, the Society for Integrative and Comparative Biology, and University of Maryland’s Department of Biology, Biological Sciences Graduate Program, and Graduate School.
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Chapter 1: Introduction

Background

Human-accelerated climate change is a threat to biodiversity worldwide, causing population declines and local extinctions (Parmesan and Yohe 2003). Yet, we know shockingly little about the proximate causes of population declines due to climate warming (Cahill et al. 2012). This knowledge gap is likely due to the complex relationships between environmental temperatures, organismal biology, and population ecology. Temperature affects physiological processes at the individual level, adaptation and extinction at the population level, and species interactions and composition at the community level (Peñuelas et al. 2013). These effects are not mutually exclusive; the response at one level of organization usually affects another (Walther 2010). For example, individuals of the marine fish *Zoarces viviparus* become aerobically stressed at temperatures above 22° C and populations decline when daily water temperature exceeds this temperature (Pörtner and Knust 2007). In a more complex case, climate warming reduces the water depth in ponds used by the western toad (*Bufo boreas*), which increases UV-B exposure of embryos (Kiesecker et al. 2001). Increased exposure to UV-B increases sensitivity to a pathogenic fungus present in the community, ultimately causing population declines. Thus, accurate assessment of organismal vulnerability to warming temperatures requires a thorough understanding of thermal physiology and its ecological consequences (Huey et al. 2012).
Physiological responses to temperature depend on the interplay of two major traits: thermal sensitivity and thermoregulation (Angilletta 2009). Thermal sensitivity is the degree to which an individual’s performance (e.g., ingestion rate, digestion efficiency, etc.) depends on temperature, whereas thermoregulation defines an individual’s ability to regulate its internal body temperature. Additionally, thermal limits, or lethal temperatures, often constrain individual activity and species distributions (Buckley et al. 2012; Sunday et al. 2012). Thermal traits are largely determined by evolutionary history and environmental conditions (Hertz et al. 1993; Knies et al. 2009; Vickers et al. 2011; Diamond and Chick 2017). As such, it is common for populations within a single species to have differential responses to temperature (Spicer and Gaston 2009). By identifying and testing current patterns in thermal physiology, we gain insight into the historical factors that shaped species distributions and strengthen our ability to predict future shifts in species abundance and distributions.

Ectotherms are an excellent model for studying thermal physiology because environmental temperatures determine their body temperature, and thus, regulate internal physiological processes (Angilletta 2009). Unlike endotherms, metabolic rates of ectotherms are strongly related to temperature (Buckley et al. 2012). In warmer temperatures, ectotherms have a greater risk of overheating or reducing activity times due to heat avoidance (Kearney et al. 2009; Sinervo et al. 2010). This is likely to threaten individual survival under expected projections of anthropogenic climate warming. Moisture-sensitive ectotherms, such as amphibians, face even greater challenges in that they are sensitive to both warming and drying (Pounds et al.
Despite this dual-threat, efforts to assess species’ vulnerability to climate change generally disregard amphibians because of their complex life history (Huey et al. 2012).

Approximately half of all described amphibian species are in decline, making them the fastest declining tetrapods worldwide (Stuart et al. 2004). They are also the most climate change vulnerable species, with 11-15% of known species being highly vulnerable and threatened with extinction (Foden et al. 2013). Lungless salamanders, family Plethodontidae, are especially vulnerable to climate warming and drying because they “breathe” through permeable skin (Feder 1983). Lunglessness is one of the most distinctive modifications of gas exchange in tetrapods, and has been referred to as an “evolutionary cul-de-sac” because of its ecological and evolutionary limitations (McNab 2002). Skin permeability results in water loss, so plethodontids are at risk of desiccation in dry conditions. Consequently, water loss influences the ways in which plethodontids forage, navigate the landscape, and use energy (Feder 1983).

Despite the physiological limitations of lunglessness, Plethodontidae is the largest family of extant salamanders (450 species in 28 genera) and has adapted to stream, arboreal, terrestrial, fossorial and cave habitats (AmphibiaWeb 2018). The genus Plethodon (55 species) is completely terrestrial and occupies woodland habitats only (AmphibiaWeb 2018). By day, Plethodon salamanders seek refuge beneath cover objects (e.g., rotting logs, rocks, etc.) or burrow belowground (Caruso 2015). Because they cannot dig, they rely on soil crevices and pre-existing burrows for vertical movement (Heatwole 1960). By night, Plethodon emerge atop the leaf litter.
to forage for invertebrates, court potential mates, reproduce, and defend territories (Petranka 1998). They are also facultative climbers and climbing behavior has been suggested as a potential behavioral mechanism of niche partitioning in dense, speciose communities (Mezebish et al. 2018). Nocturnal foraging is strongly related to mass change, indicating that *Plethodon* depend on nighttime activity for food consumption (Liebgold and Dibble 2011). Even at night, *Plethodon* salamanders only forage during and immediately after rain events, which reduces the risk of desiccation (Jaeger 1978).

The eastern red-backed salamander (*Plethodon cinereus*) is the most widely distributed *Plethodon* species in the eastern United States (Figs. 1.1 and 1.2). It occupies more than half of the geographic distribution of the entire genus (1.8 million out of 3.1 million km$^2$) and is the only *Plethodon* species found in the northern-most 1.23 million km$^2$ of the genus’ range (Adams and Church 2011). Several studies have identified genetically distinct groups, or clades, across the geographic range of the species (Highton and Webster 1976; Hass 1985; Radomski 2016). Most recently, a phylogenetic analysis performed by Radomski (2016) recovered 6 well-supported clades within *P. cinereus* and found evidence of climatic niche evolution among these clades. This finding is unusual for plethodontids, which generally exhibit strong phylogenetic niche conservatism, characterized by the retention of similar ecological traits over evolutionary time (Kozak and Wiens 2006).

One reason that *P. cinereus* may deviate from other plethodontids is that they seem to have had greater ecological opportunity (i.e., access to new niche space) after the last glacial maximum (LGM) than their southern relatives, which could have
promoted climatic niche evolution (Harvey and Pagel 1991; Radomski 2016). Greater ecological opportunity may have been facilitated by their low thermal tolerance, relative to other Plethodontid species (Markle 2015). As glaciers retreated, *P. cinereus* rapidly invaded the relatively uninhabited northeastern United States, Southeastern Canada, and Great Lakes region (Highton and Webster 1976). Radomski (2016) suggests that during this range expansion, *P. cinereus* invaded areas with novel climatic regimes, rather than exploiting habitats that matched its current climatic niche. This explanation is supported by evidence that the northernmost clades (i.e., the Pennsylvanian and Northern clades), which extend north of the LGM, had the greatest niche variation and the highest rates of climatic niche evolution relative to clades south of the LGM (Radomski 2016). However, rangewide physiological data are unavailable for *P. cinereus*, so it is unclear whether the fundamental niche of each clade has evolved and facilitates survival in different climatic regimes or whether the fundamental niche remained unchanged as the realized niche expanded (Pearman et al. 2008; Radomski 2016).

*Plethodon cinereus* is also known for its relatively high population densities, which reach 2.8 individuals/m² in Virginia where the average home range is less than 1m² (Mathis 1991). Longevity is rarely measured in terrestrial salamanders, but Leclair and colleagues (2006) report 8 years for female *P. cinereus* and 9 years for males. This long lifespan is surprising considering that males don't become sexually mature until 2-5 years old and females oviposit for the first time at 2-6 years (Petranka 1998; Leclair et al. 2008a). Once they've reached maturity, males reproduce annually and females reproduce once every 1-3 years, depending on the location.
Given their unique biogeography, abundance, and life history, *P. cinereus* makes an especially interesting system for biological inquiry and for understanding intraspecific variation in species’ responses to climate change.

**Dissertation Overview**

My dissertation presents an integrative approach to understanding the role of thermal physiology in shaping organismal biology and potential responses to climate change. Focusing on a single species, *Plethodon cinereus*, I explore the past, present, and future implications of thermal physiology through behavioral, biogeographical, ecological, and evolutionary lenses. The enigmatic success of this dispersal-limited, physiologically-constrained ectotherm drove me to explore two main questions: how does *P. cinereus* thrive across a wide range of temperatures, and does this species possess thermal adaptations that will buffer the ecological consequences of climate change?

In Chapter 2, I investigate geographical variation in physiological responses to elevated temperatures. Specifically, I describe changes in corticosterone (i.e., the dominant amphibian metabolic hormone), ingestion rate, and mass gain after exposing salamanders to a 10°C increase in ambient temperature. In Chapter 3, I use a comparative physiological approach to better understand the historical expansion and climatic niche evolution among clades of *P. cinereus* across their geographic range. Finally, in Chapter 4, I focus on the co-evolution of thermoregulatory behavior and
physiological performance and assess the effectiveness and accuracy of thermoregulatory behavior in the laboratory and field.

\textit{Dissertation Synthesis}

Together, my studies revealed significant variation in thermal physiology across the geographic range and among lineages of \textit{P. cinereus}. I found that individuals collected from warmer localities possess behaviors for coping with the energetic demands of elevated temperatures, whereas those from cooler localities do not. These behaviors included increased ingestion to counterbalance higher metabolic rates experienced in warmer temperatures, as well as more accurate thermoregulation via regulation of activity time to avoid temperatures that decrease performance. Thus, it is possible that the abovementioned behaviors have undergone thermal selection, or exhibit significant developmental or intergenerational plasticity, to maximize performance in warmer environments. It seems that salamanders from cooler sites lack these behavioral strategies for coping with rising temperatures, though it is possible that they will undergo thermal selection for such traits if mean temperatures continue to rise and warm temperature fluctuations become more common.

Additionally, populations varied in lower thermal limits, though these limits appear to be constrained by evolutionary history. Critical thermal minimum, or the temperature at which an individual loses locomotor performance, decreased with environmental temperature at the collection sites, but also differed among phylogenetic clades identified by Radomski (2016). The Pennsylvania clade, in particular, seems to have adapted to the low temperatures experienced throughout its
range, whereas the Northern clade has not. Historical gene flow within the large
Northern clade may have prevented local adaptations to the extreme cold experienced
in the northernmost part of the species’ range. The Southern clade exhibited moderate
thermal minima, which corresponds with the more moderate temperatures they
experience, relative to the two other clades. Given my findings, it appears that the
Pennsylvanian clade is best equipped to deal with an increased frequency of ground
freeze-thaw cycles expected with climate change. However, all clades already
experience temperatures below their lower critical thermal limits in the spring, winter,
and fall. More attention should be given to the potential ecological and evolutionary
consequences of winter weather patterns for salamanders, and ectotherms in general.

On the other hand, populations showed similar rates of change in
corticosterone when exposed to elevated temperatures and did not differ in upper
thermal limits across their geographic range. Salamanders from along a latitudinal
gradient (i.e., Richmond, VA to Millinocket, ME) experienced a similar increase in
corticosterone release rates when exposed to an elevated temperature, though
salamanders from the southernmost site had higher corticosterone release rates
regardless of the temperature. These findings suggest that salamanders from warmer
sites cannot modulate or down-regulate endocrine sensitivity to elevated temperatures
and may be living with chronically elevated corticosterone, which is expected to have
negative effects on reproduction and longevity. I expect that the lack of variation in
upper thermal limits, on the other hand, is less likely to play a role in the ecology of
this species. Critical thermal maximum far exceeded the environmental conditions
experienced by *P. cinereus* and is likely a relic trait that allowed its ancestors to
survive major warming events. Salamanders are likely to avoid temperatures near their upper thermal limits, even under projected climate change.

By considering multiple physiological metrics and sampling a large geographic area, I have identified several behavioral and physiological traits that promote performance, and likely fitness, across a wide range of temperatures in *P. cinereus*. Further, I provide evidence that population- and clade-variation in thermal traits will affect the species’ response to climate change. To best conserve and manage vulnerable taxa, we must incorporate intraspecific variation and evolutionary history in models of species’ responses to climate change.

**Figures**

Fig. 1.1. Photograph of the eastern red-backed salamander (*Plethodon cinereus*), a small-bodied lungless woodland salamander (Photo by: Jonathon Drescher-Lehman).
Fig. 1.2. Geographic range of *P. cinereus*. *Plethodon cinereus* occupies a large geographic range, shown in yellow, spanning throughout eastern North American and into Canada (IUCN 2018).
Chapter 2: Physiological responses to elevated temperature across a latitudinal gradient

Abstract

Widespread species often possess physiological mechanisms for coping with thermal heterogeneity, and uncovering these mechanisms provides insight into species responses to climate change. Here, we examine the effects of elevated temperature on several physiological traits in a widespread terrestrial salamander, *Plethodon cinereus*, from 4 sites across a latitudinal gradient. Using water-borne hormone assays, we found that raising the ambient temperature from 15 to 25°C in an experimental thermal challenge increased corticosterone (CORT) release at a similar rate for salamanders from all sites. However, CORT release rate was higher overall in the warmest, southernmost site. The thermal challenge also affected physiological performance, but the effects differed among sites. Ingestion rate increased in salamanders from the warmer sites but remained the same for those from cooler sites. Mass gain was reduced for most individuals, although this reduction was more dramatic in salamanders from the 2 northernmost, cooler sites. We also found a temperature-dependent relationship between CORT release rate and food conversion efficiency (i.e., the amount of mass gained per unit food ingested). CORT was negatively related to food conversion efficiency at 25°C but was unrelated at 15°C. Thus, the energetic gains of elevated ingestion rates may be counteracted by higher CORT release rates experienced by salamanders in warmer environments. By
integrating multiple physiological metrics, we highlight the complex relationships between temperature and individual responses to climate change.

**Introduction**

Ectothermic organisms may be disproportionately vulnerable to temperature extremes, which are expected to become more common under anthropogenic climate change. The ability of ectotherms to perform physiological tasks is temperature-dependent and co-varies with latitude, which suggests energetic benefits from adaptation to local climates (Addo-Bediako et al. 2000; Deutsch et al. 2008; Terrell et al. 2013). Local adaptations generate geographic patterns in thermal physiology, and these patterns are critical for assessing the vulnerability of species and populations to projected climate change (Huey et al. 2012). For example, populations that are active near, or above, their thermal optimum for performance and that have a limited acclimation capacity are highly sensitive to climate warming (Huey et al. 2012). Further, climate change may be happening too quickly for animals to evolutionarily track temperature shifts through climatic niche evolution (Quintero and Wiens 2013). In this scenario, species that lack physiological plasticity or behavioral responses to fluctuating conditions will be the most susceptible to environmental change.

There is an increasing urgency for biomarkers that can assess organismal responses to changing environments on a broad geographic scale (Wikelski and Cooke 2006; Jeffrey et al. 2015). One method for detecting the effect of environmental stressors is measuring changes in circulating glucocorticoids (GCs) via activation of the hypothalamic-pituitary-adrenal axis (hypothalamic-pituitary-interrenal axis in amphibians; Sapolsky et al. 2000). An acute GC response mobilizes
energy stores, while suppressing growth and reproduction in vertebrates and is a mechanism for maintaining homeostasis (Greenberg and Wingfield 1987; Sapolsky et al. 2000; Romero 2004). The GC response also provides a strong indication of an organism’s physiological response to disturbance (reviewed in Ellis et al. 2012; Dantzer et al. 2014). In a meta-analysis, Dantzer et al. (2014) found that integrated measures of GCs (e.g., fecal, urinary or GC from water-borne hormones) are positively associated with anthropogenic disturbance more so than instantaneous measures of stress (e.g., plasma or salivary GC). Further, using water-borne hormones, Gabor et al. (2018) found that salamanders, *Eurycea tonkawae*, in more urban streams had higher GCs than those in more rural streams.

Acute stressors can trigger increases in the GC response, but animals that are chronically exposed may downregulate GC responses to subsequent stressors (Rich and Romero 2005). This pattern has been shown in individuals that are regularly exposed to elevated temperatures. For example, when exposed to a daily thermal challenge, cane toads (*Rhinella marina*) reduced their GC response (urinary GC) to an acute stressor (i.e., capture and handling; Narayan and Hero 2013). In contrast, fish (creek chub, *Semotilus atromaculatus*) from streams within agricultural areas, which are warmer and have lower dissolved oxygen than forested streams, had similar GC responses when exposed to high temperature conditions in the laboratory compared to those from forested areas (Blevins et al. 2013). In this same study, Blevins and colleagues (2013) showed that fish from agricultural and forested populations differed in physiological performance. Fish from the agricultural area consumed 15% less energy in response to the thermal challenge, relative to fish from the forested area.
These results highlight the need to record multiple physiological metrics and raises questions regarding the interactions between temperature- and GC-induced changes in performance.

Thermal sensitivity of physiological responses often varies among individuals from geographically distinct populations. In addition to hormonal responses, these can include whole-organism physiological performance traits, which determine how well an individual performs a dynamic and ecologically relevant task (Lailvaux and Husak 2014). According to the “hotter is better hypothesis,” species from warmer localities should have higher maximal performance than species from colder localities (Huey and Kingsolver 1989). A core assumption of this hypothesis is that cold-adapted populations cannot overcome the rate-depressing effects of low temperature on biochemical reactions (Hochachka et al. 2002). As a result, warm-adapted populations attain higher maximal performance. Whereas species-level comparisons largely confirm that hotter is better, population-level comparisons have mixed results (Knies et al. 2009; Phillips et al. 2014). A recent study found evidence that hotter is better for energy assimilation (i.e., the amount of energy acquired after accounting for metabolic losses) across salamander populations separated by > 450 km along a latitudinal gradient, but not among populations separated by > 900 m in elevation (Clay and Gifford 2017). In other words, the spatial scale used to test the hotter is better hypothesis matters. A limitation of past physiological studies is that they often measure thermal physiology in single populations or compare extreme ends of the geographic range (e.g., north vs. south or high elevation vs. low elevation; reviewed in Spicer and Gaston 2009). Recent studies of physiological stress across landscapes
have demonstrated the importance of range-wide sampling in identifying populations that are most susceptible to environmental change (Deutsch et al. 2008; Buckley et al. 2014; Crespi et al. 2015).

In this study, we examined the effects of elevated temperature on corticosterone (CORT; the dominant amphibian GC) and physiological performance (i.e., ingestion rate and mass gain) among eastern red-backed salamanders (*Plethodon cinereus*, Green 1818) across a 1,100 km latitudinal gradient (Fig. 2.1). We also explored the relationship between CORT release rates and food conversion efficiency (i.e., the amount of mass gained per unit food ingested), and tested whether this relationship was temperature-dependent. We predicted that CORT would be downregulated in salamanders from warmer sites during an experimental thermal challenge (25°C) relative to a baseline temperature (15°C), because they are more likely to have undergone repeated heat exposure in the wild and could possess an adaptive response to high temperatures. With regard to physiological performance, we predicted that ingestion rate would be greater in salamanders from warmer sites regardless of the temperature treatment, in accordance with the hotter is better hypothesis, and that a greater ingestion rate would reduce mass loss after the thermal challenge. Finally, we predicted that an increase in individual CORT release rates would reduce food conversion efficiency. Our study extends prior work by studying thermal physiology across the geographic range of a widespread species. By integrating multiple physiological metrics, we highlight the complex relationships between temperature and individual responses to climate change.
Methods

Study species

The genus *Plethodon* consists of lungless salamanders (family Plethodontidae) that are restricted to terrestrial habitat, and thus, lack a larval stage (Petranka 1998). The eastern red-backed salamander (*P. cinereus*) is the most widely distributed *Plethodon* species in the eastern United States (Fig. 2.1). It occupies more than half of the geographic distribution of the entire genus (1.8 million out of 3.1 million km²) and is the only *Plethodon* species found in the northernmost 1.23 million km² of the genus’ range (Adams and Church 2011). Even though *P. cinereus* is a model system for plethodontid behavior (Jaeger et al. 2016), we know little about the physiological traits that allow this species to occupy such a large geographic range. Most physiological studies of *P. cinereus* are restricted to single populations (Hutchinson 1961; Heatwole 1962; Merchant 1970; Spotila 1972; Homyack et al. 2010; but see Markle 2015 for a comparative study of critical thermal limits across the southwestern portion of the species range). Here, we compare physiological responses to elevated temperature in *P. cinereus* across a latitudinal gradient from Virginia to Maine (Fig. 2.1).

Salamander collection and husbandry

In August and September 2016, we collected *P. cinereus* from 4 sites spanning 8.3° latitude and > 1,100 km (ME, NY, MD, and VA; Fig. 2.1). Collection occurred prior to breeding season, which occurs from October to December (Petranka 1998). At each site, we hand-captured 16-17 adult salamanders (> 32 mm snout-vent length;
SVL; Sayler 1966) and transported them back to the lab in individually-labelled containers. We based this sample size on a previous study, which found that sample sizes of 15 or 16 salamanders provided enough statistical power to detect a significant difference in CORT release rates (Gabor et al. 2016). We did not discriminate by sex due to collecting limitations, but analyses using independent t-tests indicated that our variables of interest did not differ among the sexes (CORT: \(P = 0.42\); ingestion rate: \(P = 0.22\); mass change: \(P = 0.49\)). To avoid the potentially confounding physiological effects of color polymorphism (Moreno 1989; Fisher-Reid et al. 2013), we only collected individuals that clearly displayed the striped, rather than unstriped, phenotype. At the time of collection, we measured SVL, tail length, and mass, and determined sex using the candling method (described by Gillette and Peterson 2001). After collection, salamanders were transported to temperature-controlled chambers and held at a constant temperature of 15°C, where they underwent an acclimation period of 4 weeks. We housed each salamander individually in a plastic container lined with a moist unbleached paper towel and a crumpled moist paper towel to use as a retreat. Salamanders were fed either 1-3 black soldier fly larvae, 3-5 ¼” crickets, or 15-20 large flightless fruit flies (Drosophila hydei) weekly and sprayed with spring water ad libitum.

Animals were collected with permission from Maine Department of Inland Fisheries and Wildlife (permit #2016-483), Maryland Department of Natural Resources (permit #56409), New York State Department of Environmental Conservation (permit #2007) and Virginia Department of Game and Inland Fisheries (permit #056084). Interstate transport was permitted under a Federal Fish and
Wildlife injurious species permit (permit #MA90136B-0) and vertebrate research was approved by the University of Maryland (protocol FR-15-72) and University of Richmond IACUC (protocol 16-10-001).

**Thermal CORT response experiment**

We examined the GC response of salamanders to elevated temperature using a waterborne CORT assay (Gabor et al. 2013) which provides an integrated measure reflecting an average of blood GCs that have been metabolized and excreted from urine and feces over the time period of the cumulative exposure (Sheriff et al. 2011). We measured CORT release rates from each salamander at a baseline temperature (15°C) and after a thermal challenge (25°C). The baseline temperature treatment was within *P. cinereus’* range of thermal preferences in a laboratory setting (12-22°C) and is a common maintenance temperature for *Plethodon* studies (Feder and Pough 1975; Gabor and Jaeger 1995; Clay and Gifford 2017). We chose to raise the temperature to 25°C for the thermal challenge for several reasons: it is above the thermal preference of this species (Feder and Pough 1975), it reflects a realistically high body temperature in natural conditions (see Chapter 4), it is sublethal (Hutchinson 1961), and it allowed us to simply calculate Q₁₀ temperature coefficients (i.e., the rate at which a physiological response changes with a 10°C increase in temperature) for CORT release rates (McNab 2002).

After acclimating salamanders to the baseline temperature for 4 weeks, we collected the first hormone samples from each salamander using a water-borne assay, described below. Salamanders remained at this temperature for an additional 24 h,
and then we increased the ambient temperature by 2.5°C/day until reaching 25°C. Salamanders remained in this thermally challenging environment for 48 h. After 48 h of exposure, we repeated the water-borne assay at 25°C to collect the hormone sample while maintaining the thermal challenge. All salamanders were offered food 24 h prior to CORT sampling to maintain their weekly feeding schedule and to minimize the potential effects of hunger or feeding frequency on CORT. All salamanders survived this process and did not show external signs of distress or illness.

**Water-borne hormone assays**

To collect water-borne samples for hormone analysis, we placed each salamander individually in 45 mL of bottled spring water in a standard-size petri dish (100 x 15 mm) for 1 h (following Gabor et al. 2016; Fig. 2.2). We also ran blank controls using spring water samples (3 different samples due to different sample times) and subtracted the relevant values from the CORT release rates of each salamander (spring water CORT ranged from 2.61-8.79 pg/ml of sample water). All sampling events were scheduled between 1500 and 1700 h to avoid variation due to circadian fluctuations of CORT (Dunn et al. 1972). We placed a piece of mesh screen in the bottom of each petri dish to aid in removing salamanders, while minimizing sample loss (Fig. 2.2). After 1 h, we lifted the screen out of each petri dish to transfer the salamander back to its housing container while leaving the water sample in the dish. We poured the water sample from each petri dish into a labelled Falcon tube, and immediately stored all samples in a -20°C freezer (Ellis et al. 2004). The petri dishes
and mesh lining were cleaned with 95% EtoH and rinsed with spring water before use. We extracted hormones from water following Gabor et al. (2016). We re-suspended the hormone residue with 95% EIA buffer and 5% ethanol for a total of 220 μl. We measured CORT in duplicate for all samples using a CORT enzyme-immuno assay (EIA) kit (Cayman Chemical Company, Inc, Ann Arbor, Michigan, USA) on a spectrophotometer plate reader set to 405 nm (Biotek ELX 800). We validated the use of water-borne CORT collection methods from *P. cinereus* on Cayman Chemical EIA plates using a pooled sample of hormones from 10 non-experimental animals (following Gabor et al. 2016). We assessed parallelism of the serial dilution curve (1:1-1:32) using the pooled sample. The CORT dilution curve was not significantly different from the standard curve (comparison of slopes, *t*9 = -0.894, *P* = 0.39). To determine quantitative recovery, we spiked the pooled sample with each of eight standards in addition to the un-spiked pooled sample. The minimum observed recovery was 89%. We found a linear relationship between observed and expected slopes (∝ = 1.2, *F*1,6 = 457.38, *R*2 = 0.99, *P* < 0.001). Using a pooled control sample run in quadruplicate on each plate, our intra-plate variation on 5 plates ranged from 0.51-13.7% and the overall inter-plate variation was 15.7%. The sensitivity of the CORT EIA plates ranged between 37.5 and 1,004.3 pg/ml on average.

**Physiological performance experiment**

In addition to GC response, we were interested in whether temperature differentially affected ingestion rate and mass gain among sites. After collecting the second water-
borne CORT sample from each individual after the thermal challenge, we continued to expose salamanders to 25°C and fasted them for 10 days to ensure the clearing of gut contents. Following the fasting period, we began a controlled feeding trial in which we offered 50 fruit flies to each salamander, recorded the number of flies remaining after 24 h, and replenished flies that were eaten. We repeated this procedure (counting flies and replenishing) for 5 consecutive days, recorded the number of remaining flies on day 6, and removed all leftover flies. We calculated ingestion rate as the total number of flies consumed during each trial, corrected for salamander mass (g), and divided by 5 days. To calculate mass gain, we weighed salamanders 24 h before and 48 h after each 5-day controlled feeding trial. Prior to each measurement, we placed salamanders in water for approximately 30 seconds and gently patted them dry with a paper towel to minimize variation in water weight (Fraser 1980). To correct for among-individual variation in initial body weight, we calculated percent change in mass. After completing the feeding trial at 25°C, we acclimated salamanders back to 15°C by decreasing the temperature 2.5°C/day and fed them a maintenance ration of fruit flies. We then acclimated and fasted them for 10 days and repeated the controlled feeding trial. In total, we measured ingestion rate and mass gain for 8 salamanders from each site in each temperature (n = 32). There was a single mortality from the NY population during the physiological performance experiment, so we removed that individual from the performance dataset. Due to differences in the availability of experimental salamanders, a different set of VA salamanders was used for CORT sampling than those used for controlled feeding trials. Thus, we excluded the VA salamanders for analyses that required paired CORT
and performance data but used VA salamanders in population-level comparisons of performance. Overall, we had paired CORT and performance data for 23 salamanders representing 3 sites: MD ($n = 8$), ME ($n = 8$), and NY ($n = 7$).

Analyses

Following Gabor et al. (2016), we multiplied CORT (pg/ml) by 0.22 ml (the volume of the resuspension solution) to account for resuspension and divided by the mass of each individual (g) to obtain standardized CORT in pg/g/h. CORT data were In-transformed for statistical analyses. For all analyses, we used the environmental temperature experienced at each collection site (described below) as an explanatory variable, rather than using site as a categorical variable. We did this for two reasons. First, we were interested in whether salamander populations exhibit physiological adaptations or acclimation based on the temperatures they experience in the wild. Second, differences in thermal conditions between the collection sites were not evenly distributed, therefore it would be inappropriate to simply rank them based on latitudinal or elevational distribution.

To obtain climatological data for our field sites, we downloaded daily maximum ground surface temperatures for each collection site from 1980-2015 from NASA’s Daily Surface Weather and Climatological Summaries (DAYMET) database (Thornton et al. 1997). We chose to use ground surface temperature, rather than air temperature, because $P. \text{cinereus}$ is a small salamander that spends the majority of its time on the forest floor. Further, we chose maximum daily temperature because we were interested in relative heat exposure at each of the field sites. We used mean daily
maximum temperature, averaged over a 35-yr period, as a representative site temperature for all analyses. Hereafter, we refer to our metric for site temperature as the site heat value to avoid confusion with temperature treatments in the laboratory.

All models were run using packages nlme (version 3.1-131) and stats (version 3.4.0) in R statistical software (Development Core Team, 2016). To test whether CORT release rate was related to site heat value and whether it differed between the 15 and 25°C temperature treatments, we used a linear mixed-effects (LME) model fit by maximum likelihood. The model included site heat value, temperature treatment, and the interaction between site heat value and temperature treatment as fixed effects. We nested salamander within temperature treatment as a random effect to account for the repeated sampling of each salamander in 15 and 25°C. We were also interested in whether the relative change in CORT from 15 to 25°C varied among sites. Thus, we calculated a $Q_{10}$ temperature coefficient for each salamander as the rate at which CORT changed with the 10°C increase in temperature during the thermal challenge (Eqn. 1).

\[
Q_{10} = \ln(CORT_{25°C}) - \ln(CORT_{15°C}) \quad (Eqn. \ 1)
\]

To determine whether $Q_{10}$ varied among sites, we fit a linear model with $Q_{10}$ as the response variable and site heat value as the explanatory variable. Additionally, we used a generalized linear mixed effects (GLM) model with a binomial error structure to determine whether the probability of having a positive or negative $Q_{10}$ value was related to site heat value. A positive $Q_{10}$ value indicates an increase in CORT release rate after the thermal challenge relative to baseline CORT release rate, whereas a
negative $Q_{10}$ value indicates a decrease in CORT release rate after the thermal challenge.

We compared ingestion rate and mass gain among sites using LME models fit by maximum likelihood. The models included site heat value, temperature treatment, and the interaction between these terms as fixed effects and either ingestion rate or mass gain as the response variable. We ran an additional analysis to determine whether food conversion efficiency varied with CORT release rates at the individual level, and whether the strength of this relationship was affected by temperature treatment. Food conversion efficiency has long been used as a performance metric in agricultural and fisheries sciences, and is sensitive to temperature (Brett et al. 1969; Bedford and Classen 1992; Imsland et al. 2000; Jonassen et al. 2000; Handeland et al. 2008). As an index of food conversion efficiency, we obtained residual values from a linear regression of mass gain on total ingestion (i.e., the total number of flies consumed during a feeding trial), with temperature treatment as an interactive effect ($R^2 = 0.30; df = 42; P < 0.001$). Thus, food conversion efficiency index reflects the amount of mass gained per unit ingestion for each individual relative to the mean in each temperature treatment. To determine whether CORT release rates and temperature are interactively related to food conversion efficiency, we ran an LMM with temperature treatment, CORT, and the interaction between temperature and CORT as the explanatory variables and food conversion efficiency as the response variable. For all LMMs described above, we nested salamander within temperature treatment as a random effect to account for the repeated sampling of each salamander in 15 and 25°C. Model estimates are reported as mean ± standard error of the mean.
**Results**

**Thermal CORT response experiment**

We found that overall salamander CORT release rates were positively related to site heat value \( \beta = 0.12 \pm 0.02; \chi^2 = 33.07; df = 63; P < 0.001 \) and were significantly higher after the thermal challenge than at the baseline temperature \( \chi^2 = 33.91; P < 0.001 \); Fig. 2.3A). On average, across all sites, the thermal challenge raised CORT release rates by \( 0.71 \pm 0.41 \text{ pg/g/h} \). The interaction term (site heat value*temperature treatment) was not significant \( \chi^2 = 0.08; P = 0.78 \), meaning that CORT release rate responded similarly to the thermal challenge across sites. Removing the random effects (individual nested within temperature treatment) significantly decreased the goodness of fit, as indicated by a likelihood ratio test \( P < 0.001 \), suggesting that individual was an important source of variation in our data.

To better understand the effects of thermal stressors on individual CORT release rates, we calculated \( Q_{10} \) values for each salamander. A linear model determined that site heat value was not a significant predictor of \( Q_{10} \) \( (df = 63; P = 0.91) \). Individual \( Q_{10} \) values varied widely, with a mean of 0.54 and standard deviation of 0.76 (Fig. 2.3B). Overall, 77% (50/65) of individuals had a positive \( Q_{10} \) value and experienced a \( 0.93 \pm 0.09 \) rate of increase in CORT release after the thermal challenge, whereas the other 23% (15/65) had a negative \( Q_{10} \) value and experienced a \( 0.47 \pm 0.10 \) rate of decrease in CORT release after the thermal challenge. The probability of an individual having a positive or negative \( Q_{10} \) value was not significantly related to site heat value \( z = 0.04; df = 63; P = 0.97 \).
Physiological performance experiment

We found an interactive effect of site heat value and temperature treatment on ingestion rate ($\chi^2 = 33.02; df = 63; P < 0.001; \text{Fig. 2.4A}$). The slope of the relationship between ingestion rate and site heat value was 5x greater in the 25°C treatment than in the 15°C treatment ($\beta_{15^\circ} = 0.38 \pm 0.25; \beta_{25^\circ} = 1.91 \pm 0.28$). At 25°C, salamanders from the warmest sites (MD and VA) ingested an average of 32.77 ± 5.68 and 33.73 ± 7.28 flies/g/day, respectively, while salamanders from the coolest sites (ME and NY) ingested an average of 14.65 ± 4.17 and 19.04 ± 7.76 flies/g/day, respectively. Similarly, we found a significant interactive effect of site heat value and temperature treatment on mass gain ($\chi^2 = 3.96; df = 63; P = 0.047; \text{Fig. 2.4B}$). The slope of the relationship between mass gain and site heat value was negative in the 15°C treatment but positive in the 25°C treatment ($\beta_{15^\circ} = -0.32 \pm 0.25; \beta_{25^\circ} = 0.34 \pm 0.34$). On average, the mass of individuals increased by 6.85 ± 5.25% in the 15°C treatment and 0.96 ± 6.38% in the 25°C treatment. Across all sites, 41% (13/32) of salamanders lost weight in the 25°C treatment, whereas only 9% (3/32) of salamanders lost weight in the 15°C treatment.

To explore the association between CORT release rates and physiological performance, we tested the relationship between CORT and food conversion efficiency index in salamanders that underwent hormone sampling and controlled feeding trials. We found that CORT release rate and temperature treatment were interactively related to food conversion efficiency index ($\chi^2 = 6.89; df = 20; P = 0.009; \text{Figs. 2.5A and 2.5B}$). In the 25°C treatment, an increase in CORT release rate
was related to a decrease in food conversion efficiency, indicating that individuals with higher CORT release stored less energy per unit ingestion than individuals with a lower CORT release rate ($\beta_{25^\circ} = -7.48 \pm 2.89$). In the 15°C treatment, the relationship between CORT release rate and food conversion efficiency was virtually absent ($\beta_{15^\circ} = -0.23 \pm 1.67$).

**Discussion**

Our results suggest that thermal flexibility differs between physiological traits and across a latitudinal gradient in *P. cinereus*. The ability of individuals to adjust CORT and performance may result in an improved ability to maintain homeostasis and may allow populations to persist across a range of environmental conditions. Using an integrated measure, we found that CORT release rates were positively related to site heat value and were consistently elevated when salamanders were exposed to a thermal challenge. Contrary to our prediction that CORT would be downregulated in salamanders from warmer sites during an experimental thermal challenge, the rate at which CORT release increased from 15 to 25°C did not vary among sites. This result suggests that salamanders from these sites are unable to downregulate or modulate the GC response when exposed to an acute thermal stressor regardless of their geographic origin. As for performance, salamanders from warmer sites responded strongly to the thermal challenge by increasing ingestion rate, whereas salamanders from cooler sites did not. The thermal challenge reduced mass gain in all 4 sites, though this reduction was more dramatic for salamanders from cooler sites. Finally, we found a temperature-dependent relationship between CORT release rates and food conversion...
efficiency where salamanders with higher CORT release had lower food conversion efficiency at 25°C but CORT release was unrelated to food conversion at 15°C. Thus, the energetic gains of elevated ingestion rates may be counteracted by higher CORT release rates experienced by salamanders exposed to an elevated temperature.

When faced with a thermal challenge, salamanders from all collection sites experienced elevated CORT release rates. This result confirms that body temperature influences CORT release rates in *P. cinereus* and that CORT release rates are greater, on average, in warmer temperatures. This pattern is unsurprising, as CORT is primarily a metabolic hormone, and the metabolic rate of *P. cinereus* increases exponentially with temperature, after accounting for variation in body size (Sapolsky et al. 2000; Homyack et al. 2010). Very few studies have measured the effects of temperature on CORT responses in amphibians, but our results are similar to those found in *R. marina* (Narayan et al. 2012). Narayan and colleagues (2012) found that although urinary CORT metabolite concentrations (another integrated measure) rose during a 24 h acclimation period, *R. marina* consistently exhibited higher CORT at 25°C relative to 15°C. However, our study also revealed geographic variation in baseline CORT. CORT release rates increased as site heat value increased, indicating that temperatures experienced in the wild may have long-lasting effects on salamander CORT. The observed trend was largely driven by elevated CORT release rates in salamanders from the southernmost, warmest collection site (Richmond, VA). CORT is released in response to stressors to mobilize energy stores (Sapolsky et al. 2000). If salamanders living near the southern range-edge have higher body temperatures and therefore higher metabolic rates, on average, then elevated CORT
may be an adaptive physiological response to mobilize energy in warm environments. Additional studies with repeated measures of CORT across temperatures to obtain reaction norms would provide insight into whether natural selection has optimized this physiological response.

Salamanders from all of our study sites experienced a similar increase in CORT release rates after experiencing the thermal challenge. This finding was surprising, as amphibians have been shown to modulate or down-regulate endocrine sensitivity to acute thermal stressors after repeated exposure to high temperatures in the laboratory (Narayan and Hero 2014). We expected that populations towards the southern range edge experience repeated exposure to heat in natural conditions and would therefore down-regulate CORT in response to an elevated temperature. One possible explanation for this discrepancy is that salamanders can avoid extreme heat and desiccation in the wild by retreating belowground (Taub 1961). In Richmond, Virginia, *P. cinereus* retreat belowground from May to September (Grayson, unpublished data). Behavioral avoidance of high temperatures may provide an immediate escape from lethal temperatures, as well as ultimately weakening selective pressures and reducing local adaptation to environmental temperatures (Buckley et al. 2015).

We did, however, find strong individual variation in $Q_{10}$ temperature coefficients. Of 65 salamanders used in the CORT experiment, 23% experienced a decrease in CORT release after the thermal challenge. Such decrease in CORT release rates with increasing temperature has also been shown in reptiles, specifically the Children’s python (*Antaresia children*; Dupoué et al. 2013). Researchers
suggested that snakes may release more CORT at low temperatures as a means of coping with suboptimal temperatures. It is possible that salamanders with higher thermal optima experience greater physiological consequences at lower temperatures, and potentially respond to that stress with an increase in CORT. Regardless of the cause, flexibility in $Q_{10}$ temperature coefficients may act as a basis for future plasticity or local adaptation to climate change if selective pressures become stronger (Ghalambor et al. 2007; Urban et al. 2013).

Our study revealed geographic variation in thermal performance in accordance with the hotter is better hypothesis (Huey and Kingsolver 1989; Angilletta 2009). When exposed to a thermal challenge, *P. cinereus* from northern populations maintained low ingestion rates and consequently lost mass. In contrast, the southernmost populations effectively responded to the thermal challenge by capturing and ingesting more prey thereby counteracting energy loss in warmer temperatures. This pattern may be explained by differences in life history strategies of *P. cinereus* across their range. Salamanders from colder localities endure longer winters and a shorter growing season than those from warmer localities. In the 15°C treatment, *P. cinereus* from the northernmost site (Millinocket, ME) gained the most weight while consuming the least amount of energy. This result suggests that the Maine population may have experienced depressed metabolic rates or allocated more energy towards mass gain (i.e., growth and fat storage) at 15°C relative to the other populations. There is evidence for both strategies, metabolic depression and fat storage, in temperate salamanders. When acclimated to warmer temperatures, salamanders may use metabolic depression as a mechanism for coping with thermal stressors (Bernardo
and Spotila 2006; Markle 2015). Although metabolic depression has not been explicitly tested as a means of maximizing fat storage during the limited growing season in cold-adapted salamanders, it plays an important role in torpor, hibernation, and estivation in other taxa (Guppy and Withers 1999). In addition to potential metabolic depression, *P. cinereus* from high elevations are known to allocate more energy towards tail fat storage than those from low elevations (Takahashi and Pauley 2010). Although salamanders from Maine seem to possess successful strategies for coping with a shorter growing season, they suffered the greatest loss of mass when exposed to an elevated temperature.

An important precursor to using an integrated measure of CORT release rates as a biomarker of stress is linking CORT with performance and fitness consequences. We provide evidence for such a link in terms of a temperature-dependent relationship between CORT release rates and whole-organism physiological performance. CORT release rates were unrelated to food conversion efficiency index in the 15°C treatment but were negatively related to food conversion efficiency index in the 25°C treatment. Thus, it seems that elevated temperatures may have greater ecological consequences for individuals with higher CORT release rates. We propose that the increased metabolic demands imposed by CORT and thermal stressors can overwhelm an individual’s ability to maintain a positive energy budget. This idea is supported by previous studies of metabolic rates in plethodontid salamanders. In *P. cinereus*, metabolic rate increases exponentially with increases in temperature (Homyack et al. 2010). Red-legged salamanders (*Plethodon shermani*) with chronically elevated plasma CORT also have higher metabolic rates (Wack et al. 2012). To date, there is
mixed evidence for a relationship between CORT and performance in lungless salamanders. Salamanders with chronically elevated CORT have a weaker immune response (i.e., slower wound healing) than control individuals (Thomas and Woodley 2015). A suppressed immune response may be a result of energetic tradeoffs, where chronically stressed individuals allocate more energy towards physiological maintenance than immune response (Korfel et al. 2015). By contrast, increased levels of plasma CORT did not affect locomotor performance in Allegheny dusky salamanders (*Desmognathus ochrophaeus*), though other stressors (i.e., handling and low pH) reduced performance (Ricciardella et al. 2010; Woodley et al. 2014). More research on geographic patterns of environmental stressors on physiological performance is needed to understand the mechanisms underlying the observed variation in performance and its relationship to CORT.

Environmental disturbances ultimately affect fitness in wild populations through changes in individual physiology and performance (Jeffrey et al. 2015). Thus, understanding cause-and-effect relationships between environmental disturbances and animal physiology allows us to make predictions about population-level responses to environmental change (Wikelski and Cooke 2006; Cooke and O’Connor 2010). Our results suggest that southern populations of *P. cinereus* may be more resilient to climate warming than northern populations because they exhibit greater flexibility in performance (i.e ingestion rate) when exposed to high temperatures. Although salamander CORT release rates responded similarly to an elevated temperature among our study sites, salamanders from the southern sites were able to increase ingestion rate and thereby compensate for increased metabolic demands. This pattern
seems counterintuitive, when considering the longstanding assumption that populations near southern range edges are living at or near their physiological limits (Sexton et al. 2009b; Cahill et al. 2014; Micheletti and Storfer 2015). However, a landscape-level study of physiology and disease susceptibility in another widely distributed amphibian followed a similar pattern. Wood frogs (*Lithobates sylvaticus*) living at the center of their eastern range had a higher prevalence of Ranavirus than those towards the peripheries (Crespi et al. 2015). This result was contrary to the prediction that periphery populations would be more susceptible to disease because they are already experiencing greater physiological stress. Taken together, our results and previous studies suggest that peripheral populations may be more resilient to environmental change when they possess flexible physiological responses for coping with physiological stressors (Herstoff and Urban 2013).
Fig. 2.1. Salamander collection sites in the eastern United States. The coloration of the points indicates the site heat value, a measure of daily maximum surface temperature averaged over 35 years. We collected 16-17 salamanders from each site.
Fig. 2.2. Water-borne CORT collection procedure. We collected water-borne CORT samples by placing each salamander in 45 mL of spring water in a petri dish for 1 h. We lined the bottom of each petri dish with mesh screen to provide a textured surface for the salamanders to stand on, and to easily remove the salamander from the petri dish while leaving the water sample behind for collection.
Fig. 2.3. CORT response to an elevated temperature. (A) Mean CORT release rates at 15°C \((n = 65)\) and 25°C \((n = 65)\) for each collection site. Error bars represent standard error of the mean and point colors indicate the site heat value. Salamander CORT release rates were positively related to site heat value \((P < 0.001)\) and were significantly higher after the thermal challenge than at the baseline temperature \((P < 0.001)\). (B) Lack of relationship between site heat value and \(Q_{10}\) temperature coefficients, or the rate at which CORT changed with a 10°C increase in temperature \((n = 65)\). A linear model determined that site heat value was not a significant predictor of \(Q_{10}\) \((P = 0.91)\).
Fig. 2.4. Variation in thermal performance (i.e., ingestion rate and mass gain) among collection sites. Points represent mean ingestion rate (A) and change in mass (B) for salamanders from each collection site in 15°C ($n = 32$) and 25°C ($n = 32$) temperature treatments. Error bars represent standard error of the mean and point colors indicate the site heat value. Using LME models, we found an interactive effect of site heat value and temperature treatment on ingestion rate ($P < 0.001$) and mass gain ($P = 0.047$).
Fig. 2.5. Relationship between individual CORT release rate and food conversion efficiency index in sites with paired hormone and performance data (ME, NY, and MD). Temperature treatments are shown in separate panels for clarity with 15°C above (A; n = 23) and 25°C below (B; n = 23). Regression lines and 95% confidence intervals were fit using an LME model with salamander nested within temperature treatment as a random effect. CORT release rates and temperature treatment were interactively related to food conversion efficiency index ($P = 0.009$).
Chapter 3: Disentangling environmental and evolutionary drivers of thermal limits

Abstract

Ectotherms generally exhibit geographic variation in thermal limits, which can be shaped by two major factors: current climatic variation and evolutionary history. The degree to which each of these factors has shaped thermal limits is likely to play a role in species’ responses to projected climate change. When present, intraspecific variation in thermal limits can significantly expand the amount of suitable habitat available to a species. The eastern red-backed salamander (*Plethodon cinereus*) is a widely-distributed lungless salamander composed of six mtDNA clades, which differ in their climatic niche. Here, I compared the relative effects of current environmental temperatures and phylogenetic history on thermal limits across the geographic range of *P. cinereus*. I found strong support for evolutionary constraints on lower thermal limits, though there appears to be some degree of plasticity in relation to local environmental temperatures. In contrast, upper thermal limits and thermal tolerance breadth showed little variation across the species’ geographic range and among clades. Finally, I found that thermal limits confer greater protection from heat exposure than cold exposure in this species, and that thermal safety margins are narrower in the fall than the spring. These findings suggest that warmer fall temperatures may reduce temperature-induced mortality, but increased exposure to freezing temperatures as a result of winter warming and snow melt may intensify overwinter mortality rates. Overall, this study adds to our knowledge of historical
range expansion and climatic niche evolution in *P. cinereus*, which has largely been inferred in the absence of physiological evidence and highlights the importance of incorporating intraspecific variation and evolutionary history in models of species’ responses to climate change.

**Introduction**

Thermal limits play a major role in determining species distributions in space and time. By defining the range of temperatures in which a species can survive and reproduce, thermal limits constrain the location and range of suitable habitats (Kearney and Porter 2004). As such, thermal limits have been used to predict changes in species abundance and distribution in response to climate change (Pörtner and Knust 2007; Sunday et al. 2012; Buckley et al. 2015). In fact, one of the most common ecological responses to climate change involves a shift in temporal or spatial occurrence, which is directly linked to the expansion or reduction of suitable temperatures (Parmesan and Yohe 2003; Parmesan 2006). Given the major ecological and conservation implications of thermal limits, there is a growing interest in understanding the underlying factors that shape these traits.

Ectotherms often exhibit geographic variation in thermal limits (Spicer and Gaston 2009). Such variation may be shaped by geographic variation in climate, which can either act as an environmental filter to determine which organisms can colonize a given thermal habitat or niche space, or as a selective pressure that drives local thermal adaptations (Diamond 1975; Blanquart et al. 2013). On a global scale, the thermal tolerance breadth (TTB) of terrestrial ectotherms generally increases with
latitude (Sunday et al. 2011). This pattern is largely driven by a decrease in critical thermal minimum (CTmin) at higher latitudes, which corresponds with the low temperatures experienced by temperate ectotherms and may be the result of environmental filtering or local adaptation (Sunday et al. 2011). Critical thermal maximum (CTmax), on the other hand, is fairly conserved across a global spatial scale (Sunday et al. 2011). As a result, temperate ectotherms often have a CTmax that far exceeds their environmental temperatures, giving them greater thermal safety (i.e., an excess of heat tolerance) than tropical ectotherms (Deutsch et al. 2008; Sunday et al. 2014). Sunday and colleagues (2011) proposed three explanations for the near absence of spatial patterns in upper thermal tolerances in terrestrial ectotherms: (1) CTmax reflects warm temperatures experienced in the field, which is similar across latitudes, unlike CTmin which dramatically decreases with latitude; (2) thermoregulatory behavior, specifically basking, decouples body temperatures from environmental temperatures so that body temperatures are constant across latitudes; and (3) CTmax is evolutionarily conserved, and often exceeds survival requirements. The range of explanations described above demonstrates the importance of considering both ecological and evolutionary drivers of thermal limits to better understand their relative importance in shaping physiological traits and ecological outcomes.

Physiological patterns also manifest as a result of shared evolutionary history, where taxa that live in a particular area share genetic or developmental characteristics that alter the expression of physiological traits (Hoffmann et al. 2013). Further, evolutionary processes can differentially act upon CTmin and CTmax, so it is
important to consider both traits independently (Diamond and Chick 2017). In a meta-analysis of 254 lizard species from 34 families, Grigg and Buckley (2012) found evidence of phylogenetic niche conservatism for CTmax and TTB, but not CTmin. A separate meta-analysis of 148 ant species revealed the reverse pattern: evolutionary history was a strong driver of variation CTmax, and a weaker, though significant, driver of variation in CTmin (Diamond and Chick 2017). Both studies found that CTmin exhibited phylogenetic and spatial variation (Grigg and Buckley 2013; Diamond and Chick 2017). Although the aforementioned studies take critical steps towards understanding large-scale patterns in thermal limits and performance, they do not account for physiological variation within species.

Substantially diverged lineages within species provide an ideal system for exploring the drivers of physiological variation. This assertion is especially true when lineages exhibit strong phylogeographic structure (hereafter referred to as phylogeographic lineages), suggesting a long history of isolation from one another (Pearman et al. 2010). For example, phylogeographic lineages have been useful for testing the physiological assumptions underlying the center-periphery hypothesis of species distributions (Lawton 1993; Moritz et al. 2012). In three species of tropical ground-dwelling skinks, lineages that occur towards the center of the species’ range have a lower CTmin than peripheral lineages, consistent with the idea that central lineages experience lower temperatures on average (Moritz et al. 2012). In contrast, CTmax did not differ between peripheral and central lineages, indicating that peripheral populations have not evolved higher CTmax to cope with increased heat exposure (Moritz et al. 2012). In this way, phylogeographic lineages can be used to
explore geographic variation in thermal limits within widespread species and may reveal intraspecific variation in species’ responses to climate change.

Phylogeographic variation in thermal limits can have significant effects on models of current and future species distributions. Models that incorporate intraspecific variability often project greater tolerance to climate change than models that use species’ trait means (Hoffmann and Sgrò 2011; Oney et al. 2013; Riddel and Sears 2015; Sgrò et al. 2016). Similarly, models that incorporate phylogeographic structure generally predict larger areas of suitable climatic conditions, and suggest that different subtaxa (i.e., subspecies or genetically distinct clades) will respond differently to projected climate change (Pearman et al. 2010). Such models illustrate the importance of considering pre-existing local adaptation or plasticity in thermal traits. When present, these “pre-adaptations” allow populations to respond more favorably to novel temperatures than we would otherwise expect (Herstoff and Urban 2013). Unfortunately, we lack exhaustive rangewide physiological data for most ectothermic species, making it difficult to thoroughly assess species’ vulnerability to climate change (Huey et al. 2012).

One group of ectotherms that are particularly vulnerable to climate change, and exhibit substantial phylogeographic divergence, is the salamander family Plethodontidae (Shepard and Burbrink 2009; Highton 2013; Thesing et al. 2015; Radomski 2016). Plethodontids are expected to suffer major range reductions via the loss of climatic niche space under projected climate change (Milanovich et al. 2010; Sutton et al. 2014). Further, little is known about geographic variation in plethodontid physiology (reviewed in Gifford 2016). Most of our knowledge of plethodontid
thermal physiology comes from studies of single populations that were performed in the second half of the 20th century (Hutchinson 1961; Merchant 1970; Feder and Pough 1975; Feder 1976; Feder 1983; Storey and Storey 1986). There has been a recent resurgence of plethodontid physiology studies, which highlight the importance of using a multi-population approach, but have not yet explicitly focused on intraspecific variation in thermal limits (Winters and Gifford 2013; Young and Gifford 2013; Careau et al. 2014; Markle 2015; Riddel and Sears 2015; Clay and Gifford 2017).

Here, I performed a comparative physiological study to disentangle the environmental and evolutionary drivers of thermal limits in the eastern red-backed salamander (*Plethodon cinereus*). This species persists across a wide range of temperatures and is composed of six mtDNA clades, which differ in their climatic niches (Radomski 2016). However, rangewide physiological data are unavailable for *P. cinereus*, so it is unclear whether the fundamental niche of each clade has evolved and facilitates survival in different climatic regimes or whether the fundamental niche remained unchanged as the realized niche expanded (Pearman et al. 2008; Radomski 2016). In this study, I was specifically interested in whether CTmin, CTmax, and TTB differ among phylogeographic lineages of *P. cinereus* and to what extent these physiological traits were related to environmental temperatures. I predicted that CTmax would be conserved among clades and across the species’ geographic range, whereas CTmin would decrease with environmental temperature, in accordance with previous studies of most other ectothermic taxa (Sunday et al. 2011; Grigg and Buckley 2013). Further, I expected that salamanders living near the northern and
southern range edges to be living close to their lower and upper thermal limits, respectively, and therefore to have smaller thermal safety margins (defined herein as the temperature buffer between thermal limits and ambient temperature) than salamanders from more central sites (Sagarin and Gaines 2002; Hampe and Petit 2005). This study expands upon our understanding of thermal limits, sheds light on the evolutionary history of *P. cinereus*, and demonstrates the importance of considering evolutionary history within a species when assessing spatial patterns of physiology and ecological responses to climate change.

**Methods**

*Salamander collection and captivity*

I collected 105 sexually mature salamanders (>32mm snout-vent length; SVL; Sayler 1966) from 13 sites across the species’ geographic range, spanning a total distance of 10.1° latitude, 12.6° longitude, and 1468.7 m in elevation (Fig. 3.1; Table 3.1). Together, these sites represent three of the largest clades identified by Radomski (2016) – the Northern (*n* = 5 sites) and Pennsylvanian (*n* = 3 sites) clades, which extend north of the last glacial maximum (LGM), and the Southern (*n* = 5 sites) clade, which is restricted to areas south of the LGM. Collection sites were all within protected and/or managed natural areas, had historical occurrence data for *P. cinereus*, and captured a wide range of environmental temperatures within and among clades. Most salamanders were collected between June and September 2016, but a hurricane delayed sampling at the southernmost site in North Carolina until December. At the time of collection, I measured SVL, tail length, and mass, and
determined sex using the candling method (described by Gillette and Peterson 2001). After collection, salamanders were transported to temperature-controlled chambers and held at a constant temperature of 15°C, where they underwent an acclimation period of 14-30 days. I housed each salamander individually in a plastic container lined with a moist unbleached paper towel and a crumpled moist paper towel to use as a retreat. Salamanders were fed 15-20 large flightless fruit flies (Drosophila hydei) or 3-5 ¼” crickets weekly, and 5-7 days before each measurement to standardize the effects of hunger and digestion among individuals.

**Critical thermal limits measurements**

Critical thermal limits have been defined as the thermal points at which locomotor activity becomes disorganized and the animal loses its ability to escape from conditions that ultimately result in death (Cowles and Bogert 1944). These limits act as ecological endpoints, and allow more precise measurements than trying to estimate the precise moment of death (Feder 1992). To measure critical thermal limits, each salamander was placed in a metal chamber (6” diameter) lightly sprayed with water to prevent desiccation. The chamber was submerged in a water bath filled with approximately 950 ml of water and held at 15°C. To measure CTmin, I slowly added ~250 ml of ice to the water bath to decrease the temperature at a constant rate of approximately 1°C/min and recorded the salamander’s body temperature every minute using an infrared temperature gun (adapted from Brusch et al. 2015). To reach negative temperatures, I added small amounts of NaCl to the water bath once the temperature approached 0°C.
I measured CTmin as the lowest temperature experienced before loss of righting response, which I tested each minute by flipping the salamander on its dorsal side using forceps. Motor function quickly diminished near the thermal limits, so I increased the frequency of righting ability tests to approximately 4 tests/min once the salamander exhibited signs of impairment (e.g., muscle spasms and loss of coordination). Once the salamander lost righting ability, I recorded body temperature as CTmin using an infrared temperature gun aimed at the cranial region and immediately transferred the animal to a recovery chamber. After a 10-14 day resting period, I repeated this procedure for CTmax while raising, rather than lowering the temperature by adding hot water (100°C) to the water bath. I measured CTmax as the highest temperature experienced before loss of righting ability. Salamanders were monitored for health throughout the experiment and for 48-hr after and did not show signs of enduring physical stress or mortality. Overall, I measured CTmin for all 105 individuals and CTmax for 89 individuals (Table 4.1). TTB was calculated as the difference between CTmin and CTmax.

Climate data
I downloaded daily minimum and maximum surface temperatures for each of the collection sites from NASA’s Daily Surface Weather and Climatological Summaries (DAYMET) database (Thornton et al. 1997). I was interested in whether the frequency of low and high temperatures acts as a selective pressure or environmental filter on thermal limits, so I calculated indices of exposure to cold and heat events as follows. I calculated the mean number of days when the temperature dropped below
5°C (cold exposure index) and the mean number of days when the temperature exceeded 25°C (heat exposure index) for each year in the database (1980 – 2015). For TTB, I was more interested in whether the range of annual temperatures acts as a selective pressure or environmental filter. Thus, I also calculated annual thermal range as the difference between the mean maximum and minimum annual temperatures at each site from 1980 – 2015. Annual thermal range was strongly correlated with latitude (Pearson’s r = 0.96; t = 32.97; df = 104; P < 0.001), but not elevation (Pearson’s r = -0.14; t = -1.39; df = 104; P = 0.17) at my field sites.

**Statistical analyses**

All models were run using R statistical software (Development Core Team, 2017). First, I ran a Pearson’s correlation test to determine whether critical thermal limits (CTmin and CTmax) were correlated, and if so, to calculate the strength and direction of the relationship. I also ran an initial series of ANOVAs to determine whether sex was a significant predictor of thermal limits and breadth. The physiological traits of interest did not differ between females and males within each clade, including CTmin (F = 0.05; df = 1; P = 0.82), CTmax (F = 0.25; df = 1; P = 0.62), and TTB (F = 0.68; df = 1; P = 0.41). Thus, sex was not included in the following models.

To determine the effects of evolutionary history (clade) and environmental conditions (local temperature) on critical thermal limits, I fit ANCOVA models for CTmin, CTmax, and TTB. For models of lower and upper thermal limits, explanatory variables included clade (i.e., Northern, Pennsylvanian, or Southern), cold or heat exposure index (for CTmin and CTmax, respectively), and the interaction term
(clade*cold or heat exposure index). For TTB, explanatory variables included clade, annual thermal range, and the interaction term (clade*thermal range). For models where clade was a significant predictor of the response variable, I ran a Tukey posthoc test to determine which clades differed from one another.

In addition to understanding the drivers of thermal limits, I was interested in how often salamanders are potentially exposed to critical temperatures at each of my study sites. Thus, I calculated upper thermal safety margins as the difference between CTmax and daily maximum temperature and lower thermal safety margins as the difference between CTmin and daily minimum temperature for each day from 1980-2015. Larger thermal safety margins indicate a greater buffer between thermal limits and ambient temperature, and therefore, a lower risk of experiencing lethal temperatures in the wild. I chose to analyze thermal safety margins during the spring and the fall months, only, because *P. cinereus* are active during spring and fall across its geographic range, whereas they are inactive during the summer months at the warmer sites and during the winter months at the colder sites (Sayler 1966; Petranka 1998; Leclair et al. 2008b; Woolbright and Martin 2014). I performed separate ANCOVA and Tukey posthoc analyses for upper and lower thermal safety margins during the fall and spring by including thermal safety margin as the response variable and collection site as the explanatory variable.

**Results**

I found that CTmin and CTmax were not correlated, suggesting that upper and lower thermal limits may be subject to differential evolutionary processes at these sites ($r =$
0.19; \( t = 1.83; df = 87; P = 0.07 \). CTmin differed among clades \( (df = 2; F = 3.54; P = 0.03; \text{Fig. 3.2A}) \) and was negatively related to the cold exposure index \( (df = 1; F = 5.12; P = 0.03; \text{Fig. 3.2B}) \). In other words, CTmin decreased, or got colder, as sites experienced a greater frequency of days below 5°C \( (\beta = -0.01; \text{Fig. 3.2B}) \). Clade and cold exposure index did not interactively affect CTmin, suggesting that the relationship between CTmin and the frequency of cold events is consistent across clades \( (df = 2; F = 2.49; P = 0.09) \). The Tukey posthoc test revealed that CTmin was significantly different among all 3 clades, so that salamanders from the Pennsylvanian clade tolerated the coldest temperatures \(-2.11 \pm 1.10°C\), followed by salamanders from the Southern clade \(-0.72 \pm 1.03°C\) and then the Northern clade \(-0.18 \pm 0.92°C; \text{Fig. 3.2A})\). Among individuals, CTmin ranged from -3.60°C in a salamander from Philipsburg, PA (PA; Pennsylvania clade) to 2.10°C in a salamander from Raleigh, NC (NC; Northern clade). On the other hand, I found that CTmax did not vary among clades \( (df = 2; F = 0.40; P = 0.67; \text{Fig. 3.3A}) \) and was not related to the heat exposure index \( (df = 1; F = 2.14; P = 0.15; \text{Fig. 3.3B}) \). The interaction term was also nonsignificant \( (df = 2; F = 1.87; P = 0.16) \). Among individuals, CTmax ranged from 31.10°C in a salamander from Hunter, NY (HUN; Northern clade) to 37.70°C in a salamander from Blacksburg, VA (BB; Southern clade).

TTB did not differ among clades \( (df = 2; F = 0.16; P = 0.85; \text{Fig. 3.4A}) \) and was not related to annual thermal range \( (df = 1; F = 0.06; P = 0.89; \text{Fig. 3.4B}) \). The interaction term was also nonsignificant \( (df = 2; F = 0.80; P = 0.87) \). I found that on average, TTB was 34.80 ± 1.85°C, which was much narrower than the annual thermal range across collection sites, 52.87 ± 3.59°C. Although clade was a non-significant
predictor of TTB, I observed that the mean TTB was lower for the Northern clade (33.90 ± 1.43°C) than the Pennsylvanian (35.88 ± 1.34°C) and Southern clades (35.26 ± 2.01°C; Fig. 3.4A).

After analyzing thermal limits and breadth, I calculated upper and lower thermal safety margins during the spring and fall to understand whether the risk of experiencing lethal temperatures varies among sites. I found that upper thermal safety margins differed among sites during the fall ($df = 12; F = 5,993.9; P < 0.001$; Fig. 3.5A) and spring ($df = 12; F = 6,770; P < 0.001$; Fig. 3.5B). Salamanders were generally well-buffered from CTmax, but upper thermal safety margins were higher on average during the fall (21.94 ± 7.75°C) than the spring (15.89 ± 7.22°C), indicating that *P. cinereus* is strongly buffered from lethal heat exposure during fall activity and slightly less buffered from CTmax during spring activity. Salamanders from the northernmost site (ME; Northern clade) had the greatest buffer from CTmax in both seasons (25.21 ± 7.77°C in the fall and 18.41 ± 7.62°C in the spring), while salamanders from the southernmost site (NC; Northern clade) had the weakest buffer from CTmax in both seasons (13.40 ± 6.21°C in the fall and 7.51 ± 5.54°C in the spring).

Lower thermal safety margins also differed among sites during the fall ($df = 12; F = 497.68; P < 0.001$; Fig. 3.5C) and spring ($df = 12; F = 3,938.1; P < 0.001$; Fig. 3.5D). Mean lower thermal safety margins were much smaller than upper margins (5.53 ± 4.20°C in the fall and 7.59 ± 5.06°C in the spring), indicating that salamanders are more likely to experience temperatures below CTmin than temperatures above CTmax during spring and fall. While CTmin varied among sites,
it did not vary such that the lower safety margin remained constant across sites.

Salamanders from the southernmost site (NC; Northern clade) had the greatest buffer from CTmin in both seasons (13.40 ± 6.21°C in the fall and 7.51 ± 5.54°C in the spring), while the northernmost site (ME; Northern clade) had the weakest buffer from CTmin in the spring (5.50 ± 4.18°C) and a mid-latitude site (ALL; Pennsylvanian clade) had the weakest buffer from CTmin in the fall (4.78 ± 3.96°C) despite having a lower CTmin than all other sites.

Discussion

In this study, I explored the evolutionary and environmental factors underlying variation in thermal tolerance among phylogeographic lineages of a widespread terrestrial ectotherm. I found support for evolutionary constraints on CTmin, though there appears to be some degree of plasticity in relation to local environmental temperatures. In contrast, CTmax showed little variation across the species’ geographic range and among clades, and far exceeded survival requirements, similar to findings in other terrestrial ectotherms (Sunday et al. 2011; Grigg and Buckley 2013). TTB was consistently wide across the species range and among clades, suggesting that clade range size is not related to TTB. This relationship differs from plethodontid species’ range sizes, which are positively related to TTB (Markle 2015). Finally, I found that thermal limits confer greater protection from heat exposure than cold exposure in *P. cinereus*, and that lower thermal safety margins are narrower in the fall than the spring. These findings suggest that warmer fall temperatures may reduce temperature-induced mortality, but increased exposure to freezing
temperatures as a result of winter warming and snow melt may intensify overwinter mortality rates (Williams et al. 2014b). My results add to our knowledge of historical range expansion and climatic niche evolution in *P. cinereus*, which has largely been inferred in the absence of physiological evidence (Highton and Webster 1976; Hass 1985; Radomski 2016). Further, my results highlight the importance of incorporating intraspecific variation and evolutionary history in models of species’ responses to climate change.

Evolutionary history accounted for most of the variation in CTmin in *P. cinereus*. Given that clades north of the LGM exploited colder environments and underwent rapid climatic niche evolution (Radomski 2016), I predicted that CTmin would differ among clades such that the Northern clade would have the lowest CTmin, followed by the Pennsylvanian clade, and then the Southern clade. However, I found a much different pattern—salamanders from sites within the Pennsylvanian clade had a notably lower CTmin than salamanders from all other sites, indicating that the Pennsylvania clade is the most cold-tolerant lineage. This finding may be explained by the geographic distribution of the Pennsylvanian clade, which occurs in the Allegheny Plateau Physiographic Province and is geographically separated from the Northern and Southern clades by major waterways (i.e., Conococheague Creek and the New River, respectively; Radomski 2016). Because of its location, the Pennsylvanian clade is exposed to consistently harsh winters and moderate summer temperatures (Whitney 1990). This condition differs from the Northern and Southern clades which were characterized by more variable (Northern clade) or more moderate (Southern clade) cold exposure indices. I propose that the combination of geographic
isolation and consistently strong selective pressure for cold tolerance may have shaped the surprisingly low thermal limits of the Pennsylvanian clade (Moritz et al. 2012; Valladares et al. 2014).

CTmin was negatively related to the cold exposure index in *P. cinereus*. This finding is similar to the pattern observed in studies of other terrestrial ectotherms (Sunday et al. 2011; Grigg and Buckley 2013; Diamond and Chick 2017). However, this relationship accounted for much less variation in CTmin than clade identity. The relatively weak relationship between environmental temperatures and CTmin may be a result of weak local adaptation or environmental filtering, particularly in the Northern clade (Kellermann et al. 2012; Grigg and Buckley 2013). Salamanders from the coldest sites in the Northern clade exhibited slightly lower CTmin than those from the warmest sites in the clade, but they still could not tolerate temperatures as low as those in the Pennsylvanian clade. Weak, or absent, adaptation to the local environment may be the result of historical gene flow (reviewed in Gaston 2009). If true, strong gene flow may have swamped the effects of selection for lower CTmin through the introduction of alleles from more southern populations (Bridle and Vines 2007; Sexton et al. 2009a). Alternatively, behavioral avoidance of cold temperatures may have reduced selective pressure for lower thermal limits (Buckley et al. 2015). While behavioral thermoregulation has been shown to reduce adaptive capacity in reptiles, *P. cinereus* are weak thermoregulators and can be found under rocks and logs with body temperatures at, or near 0°C (Chapter 4; pers. obs.). Additionally, sites toward the southern edge of the Northern clade’s range can experience up to 40% overwinter mortality rates, suggesting that winter conditions may severely impact
survival, and therefore fitness (Muñoz et al. 2016). Thus, historic gene flow, rather than behavioral avoidance of low temperatures, is a potential explanation for weak variation in CTmin within the Northern clade.

Upper thermal limits were conserved among clades and were unrelated to environmental conditions in *P. cinereus*, similar to previous findings in a wide range of ectothermic taxa (Sunday et al. 2011; Moritz et al. 2012; Grigg and Buckley 2013; Markle 2015). My results support Sunday and colleagues (2011) third explanation for the near absence of spatial patterns in upper thermal tolerances in terrestrial ectotherms, that CTmax is conserved among clades of *P. cinereus* and exceeds survival requirements. *Plethodon cinereus* has surprisingly high upper thermal limits, which far exceed environmental temperatures during times of aboveground activity (Chapter 4; Fig. 3.5). The divergence of many extant plethodontids coincided with major global warming events during the late Cretaceous Period and the Paleocene-Eocene thermal optimum (Vieites et al. 2007). Thus, it is likely that high thermal limits were once beneficial in plethodontids, as well as other ectotherms that underwent similar radiations during these time periods. If true, it is reasonable to suspect that the maintenance of CTmax is not energetically costly to maintain (Feder 1983). My results do not support the two alternative explanations posed by Sunday et al. (2011). Upper thermal limits do not seem to reflect the range of temperatures experienced in my study species, as demonstrated by salamanders from five collection sites that experience less than 50 days/year above 25°C, on average, but still have a CTmax above 30°C. Further, the life history of *P. cinereus* does not support the idea that thermoregulatory behavior allows individuals to maintain high
temperatures regardless of their locality. Although this phenomena has been reported in basking reptiles (Buckley et al. 2015) and some insects (Chown 2001), *P. cinereus* maintains active body temperatures far below their CTmax (Chapter 4).

TTB did not differ among clades and was unrelated to annual thermal range in *P. cinereus*. This result differs from patterns of TTB in plethodontids, in which individuals from sites with greater thermal seasonality and species with larger geographic ranges exhibit a wider thermal tolerance (Markle 2015). My results suggest a lack of relationship between clade range size and TTB, as the largest clade (Northern) has the narrowest TTB. However, thermal limits only varied slightly among sites within the Northern clade, resulting in a lack of relationship between environmental temperatures and TTB. Mean TTB observed in this study was within 1°C of the mean TTB reported by Markle (2015), suggesting that TTB may be a repeatable trait, though future studies should explicitly test the repeatability of thermal limits in this species (Nespolo and Franco 2007). However, both studies were constrained to sexually mature individuals, so we cannot rule out the possibility that juvenile thermal tolerance varies from adults and potentially varies predictably across the species’ range (Miller et al. 2013). Juvenile plethodontids are also expected to incur severe consequences from exposure to extreme temperatures, as their surface-to-volume ratio is related to elevated evaporative water loss, and may more likely than adults to adjust surface activity to avoid suboptimal conditions (Grover 1998; Peterman and Semlitsch 2014). Future studies should explore the repeatability of TTB within and among age classes, as well as the ecological consequences of TTB in embryos, hatchings, and sexually immature individuals.
Finally, I expected that salamanders living near the northern and southern range edges would have narrower thermal safety margins than salamanders from more central sites. While this prediction was somewhat true, my results uncover a more complex story. First, *P. cinereus* appears to be at greater risk of freezing, rather than overheating during the seasons when they are most active (i.e., fall and spring). Second, this risk differs among seasons and among sites. For example, lower thermal safety margins were narrower in the fall than in the spring and provided the weakest buffer from cold temperatures for salamanders from Millinocket, ME in the spring and Salamanca, NY in the fall. Interestingly, *P. cinereus* is highly active at northern (Canada: Leclair et al. 2008b) and southern (Maryland: Sayler 1966; Muñoz et al. 2016 and Virginia: Grayson, unpublished data) sites within the Northern clade in the fall, despite the risk of freezing. In contrast, high elevation populations within the Southern clade are more likely to exploit warmer temperatures in the summer and early fall, potentially incurring a greater survival advantage (H. Wilbur, unpublished data; Chapter 4). While the risk of freezing increases in fall, salamanders may benefit from performance advantages (i.e., lower metabolic costs and lower CORT release rates) and a lower risk of desiccation brought on by evaporative water loss related to the lower mean temperatures (Homyack et al. 2010; Peterman and Semlitsch 2014; Chapter 2). To better understand the ecological consequences of thermal safety margins, we require a rangewide perspective of population dynamics in relation to seasonal temperatures in this species.

My results contradict those of Markle (2015) who found that critical thermal limits did not vary substantially among populations of *P. cinereus*. Two major
differences in our sampling design may account for this disparity. First, my study was focused solely on *P. cinereus*, which allowed me to cover a much larger geographic range when measuring thermal limits. Markle was comparing thermal limits among plethodontid species, and therefore restricted collection sites to the southern Appalachian Mountains, a global hotspot for plethodontid diversity (Petranka 1998). By including thermally “extreme” localities towards the species’ northern and southern range-edge (e.g., ME and NC), I captured a wider range of environmental temperatures. Second, Markle’s (2015) study occurred prior to Radomski’s (2016) identification of lineages within *P. cinereus*, and therefore did not account for among-clade variation in thermal limits. Without accounting for among-clade variation in CTmin, the regression between environmental temperature and CTmin becomes much weaker, and potentially absent. Future studies of thermal physiology in *P. cinereus* should maximize geographic sampling efforts and incorporate phylogeographic structure in the study design.

My study highlights the importance of incorporating geographic and phylogenetic variation in thermal physiology in projections of *Plethodon cinereus*’ response to climate change. Projections of plethodontid responses to climate change are mostly correlative (Milanovich et al. 2010; Sutton et al. 2014), though a recent study demonstrated the ecological role of intraspecific variation for cutaneous resistance to water loss (Riddel and Sears 2015). Riddel and Sears (2015) found that variation in resistance to water loss along an elevation gradient allowed two *Plethodon* species to double their potential activity at low elevations. It would be relatively simple to apply Riddel and Sears’ mechanistic approach to estimate
salamander activity and energetics while incorporating variation in thermal limits and performance. In fact, a pre-existing mechanistic model already exists for plethodontids, but does not yet allow for physiological variation (Gifford and Kozak 2012; Peterman and Gade 2017). Expanding the biophysical model of Gifford and Kozak (2012) to allow for intraspecific variation will be a key step in assessing population vulnerability to climate change in Plethodon salamanders.

Much attention has been given to the ecological impacts of warmer temperatures during growing seasons, rather than changes in winter temperatures (Williams et al. 2014b). My results highlight the potential threat of thermal shifts during the colder months when *P. cinereus* already has the narrowest thermal safety margins. Winter warming may affect *P. cinereus*, as well as other ground dwelling ectotherms, in two major ways. First, the combination of warmer air temperatures and reduced snow cover is expected to intensify soil freeze-thaw cycles, potentially exposing *P. cinereus* to lethal temperatures even when they are overwintering belowground (Brown and DeGaetano 2011). Second, winter warming may increase energetic costs and lead to mortality or a reduction in the amount of energy allocated towards critical biological functions such as immune response (Raffel et al. 2006; McCoy et al. 2017), reproduction (Irwin and Lee 2016), or growth (Byström et al. 2006). As my work emphasizes, phylogeographic lineages within species may differ in their thermal traits, and therefore differ in their response to overwinter survival and energetics. A similar pattern has been demonstrated amongst ecotypes of a Lepidopteran species, *Hyphantria cunea*, which has higher winter survival in natal compared with non-natal winter environments (Williams et al. 2014a). Together, this
work points towards the importance of considering cross-seasonal impacts of climate change, especially for ground-dwelling temperate ectotherms.
Tables

Table 3.1. Salamander collection sites and sample sizes ($n$) for critical thermal minimum (CTmin) and critical thermal maximum (CTmax) measurements. Field sites captured 3 of the largest phylogenetic clades within *P. cinereus* (previously described by Radomski 2016): Northern (N), Pennsylvanian (PA), and Southern (S).

<table>
<thead>
<tr>
<th>Clade</th>
<th>Site</th>
<th>Location</th>
<th>Sample size, $n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>CFSP</td>
<td>Cunningham Falls State Park, Thurmont, MD</td>
<td>8 8</td>
</tr>
<tr>
<td>N</td>
<td>HUN</td>
<td>Hunter Mountain, Hunter, NY</td>
<td>8 8</td>
</tr>
<tr>
<td>N</td>
<td>ME</td>
<td>Debsconaeg Lakes Wilderness Area, Millinocket, ME</td>
<td>8 8</td>
</tr>
<tr>
<td>N</td>
<td>NC</td>
<td>Swift Creek Bluffs Nature Conservancy, Raleigh, NC</td>
<td>8 5</td>
</tr>
<tr>
<td>N</td>
<td>RIC</td>
<td>James River Park System, Richmond, VA</td>
<td>8 8</td>
</tr>
<tr>
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<td>ALL</td>
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<td>8 6</td>
</tr>
<tr>
<td>PA</td>
<td>PA</td>
<td>Black Moshannon State Park, Philipsburg, PA</td>
<td>8 4</td>
</tr>
<tr>
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<td>Dan’s Mountain State Park, Lonaconing, MD</td>
<td>8 5</td>
</tr>
<tr>
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<td>BB</td>
<td>Pandapas Pond Recreation Area, Blacksburg, VA</td>
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<td>CHF</td>
<td>Cherokee Flats Day Use Area, Pembroke, VA</td>
<td>8 9</td>
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<td>MLBS</td>
<td>Mountain Lake Biological Station, Pembroke, VA</td>
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<tr>
<td>S</td>
<td>ROG</td>
<td>Mt. Rogers National Recreation Area, VA</td>
<td>8 6</td>
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<tr>
<td>S</td>
<td>WR</td>
<td>Wind Rock, George Washington and Jefferson National Forests, Pembroke, VA</td>
<td>8 8</td>
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</table>

Total: 105 89
Fig. 3.1. Map of salamander collection sites in the eastern United States. Each circle represents a single collection site and the triangle in southwestern Virginia represents four collection sites along an elevation gradient (650-1,250m asl). Phylogenetic clades are based on Radomski (2016).
Fig. 3.2. Critical thermal minimum (CTmin) for *P. cinereus* from 13 sites across their geographic range. (A) CTmin was significantly different among clades, depicted as mean CTmin for each clade with standard error bars. (B) CTmin was also related to cold exposure index, calculated as the mean number of annual days when the temperature fell below 5° C from 1980 – 2015. For both panels, point colors represent clade (dark blue = Northern, light blue = Pennsylvanian, and green = Southern).
Fig. 3.3. Critical thermal maximum (CTmax) for *P. cinereus* from 13 sites across their geographic range. (A) CTmax did not vary among clades, depicted as mean CTmax for each clade with standard error bars. (B) CTmax was not related to heat exposure index, calculated as the mean number of annual days when the temperature exceeded 25°C from 1980 – 2015. For both panels, point colors represent clade (dark blue = Northern, light blue = Pennsylvanian, and green = Southern).
Fig. 3.4. Thermal tolerance breadths (TTB) for *P. cinereus* from 13 sites across their geographic range. (A) TTB did not vary among clades, depicted as mean TTB for each clade with standard error bars. (B) TTB was not related to annual thermal range, calculated as the difference between the mean maximum and minimum mean annual temperatures at each site from 1980 – 2015. For both panels, point colors represent clade (dark blue = Northern, light blue = Pennsylvanian, and green = Southern).
Fig. 3.5. Thermal safety margins (TSM) during fall and spring in *P. cinereus* from 13 sites across their geographic range. Larger TSMs represent a greater buffer from critical thermal temperatures, and therefore, a lower risk of heat- or cold-induced mortality. Bar colors represent clade (dark blue = Northern, light blue = Pennsylvanian, and green = Southern); error bars represent standard error, and lowercase letters represent statistical groups as indicated by a Tukey posthoc test. Refer to Table 3.1 for collection site codes and information.
Chapter 4: Moisture and thermal quality influence behavioral thermoregulation in *Plethodon cinereus*

*Abstract*

Many ectotherms use behavioral thermoregulation as a means of coping with thermal heterogeneity. Such strategies allow individuals to modify their body temperature to optimize performance and avoid the negative consequences of suboptimal environmental temperatures. However, the degree to which ectotherms can accurately and effectively thermoregulate varies among species and populations. In this study we investigated the role of thermoregulatory behavior in shaping physiological performance across a range of environmental conditions (i.e., moisture and thermal quality of the habitat) in the eastern red-backed salamander, *Plethodon cinereus*. We found that *P. cinereus* does not prefer temperatures that optimize performance in a laboratory setting, rather that they regulate activity time to optimize performance in the field. In both lab and field settings, environmental moisture affected thermoregulatory behavior. Salamanders preferred temperatures farther from thermal optima in dry conditions in the lab, suggesting a tradeoff between performance and hydration. In the field, rain decreased thermoregulatory effectiveness, suggesting that opportunistic foraging may incur long-term performance costs. Finally, we found that salamanders from poor thermal quality sites were more accurate thermoregulators than those from moderate thermal quality sites. Overall, our results suggest that *P. cinereus* exhibits relatively weak behavioral thermoregulation, though salamanders
from poorer thermal quality habitats display higher acuity for regulating body
temperatures, which in turn promotes greater performance. This study expands upon
our knowledge of thermal performance and behavior in ectotherms and sheds light on
the thermal strategies that allow widespread species to exist across a wide range of
temperatures.

Introduction

Widespread species must overcome the challenge of living across a wide range of
climatic conditions. This statement is especially true for widespread ectotherms,
because environmental temperatures determine their physiological rates, and in turn,
influence their survival and distributions (Kearney and Porter 2004; Bozinovic et al.
2011a; Bozinovic et al. 2011b). However, a wide geographic range does not equate to
a wide thermal niche (Grigg and Buckley, 2013; Hof et al., 2010). Many ectotherms
use behavioral strategies to maintain a narrower range of body temperatures than they
would otherwise experience in their environment. Thermoregulatory behaviors take
several forms, including microclimate selection, posture modification, and activity
time regulation (reviewed in Huey & Slatkin, 1976). Regardless of the strategy,
ectotherms thermoregulate with similar goals, to minimize exposure to harmful
temperatures and to maximize physiological performance.

The shape of the relationship between physiological performance and
temperature is nearly universal among ectotherms – performance increases with
temperature until it reaches a peak, or thermal optimum ($T_{opt}$; see Table 4.1 for all
abbreviations used in this chapter), and then rapidly decreases until reaching an upper

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thermal limit (Angilletta, 2006; Angilletta, Niewiarowski, & Navas, 2002; Bulté & Blouin-Demers, 2006). Ectotherms that maintain a set range of body temperatures often prefer temperatures within their range of thermal optima (Angilletta, Hill, & Robson, 2002; Angilletta, 2001; Bonino et al., 2011; Dorcas, Peterson, & Flint, 1997; Gaby et al., 2011; Huey & Bennett, 1987; Lelièvre et al., 2011; Lelièvre, Le Hénanff, Blouin-Demers, Naulleau, & Lourdais, 2010). Animals may select optimal temperatures for performance when behavior has coevolved with whole-organism performance traits, which determine how well an individual performs a dynamic and ecologically relevant task (Lailvaux and Husak, 2014). This concept, that the behavior and performance of ectotherms should coevolve to optimize thermal performance, is described by the thermal coadaptation hypothesis (Angilletta et al., 2006; Angilletta, 2009; Garland, Huey, & Bennett, 1991; Huey & Bennett, 1987).

Given the rapid decrease in performance at temperatures above $T_{opt}$, it is beneficial to maintain body temperatures slightly below, rather than equal to or greater than, $T_{opt}$ (Martin and Huey 2008). This model of behavioral thermoregulation, the “asymmetry model” or Jensen’s Inequality, is especially useful for inaccurate thermoregulators, which are exposed to a wide range of temperatures in heterogeneous environments and are therefore at a greater risk of rapid declines in performance when exposed to temperatures above $T_{opt}$ (Martin and Huey 2008). The asymmetry model has largely been used to describe reptilian taxa that maintain body temperatures within 3°C of their $T_{opt}$, and it is unclear whether non-reptilian taxa, particularly cold-adapted ectotherms, conform to the thermal coadaptation hypothesis.
and the asymmetry model of thermoregulation (Gaby et al. 2011; Gvoždík 2015; Halliday and Blouin-Demers 2015).

The extent to which an ectotherm can maintain temperatures that maximize performance has ecological and evolutionary consequences. When thermal plasticity is limited, or absent, behavioral thermoregulation might be the only immediate means of evading unfavorable temperatures (Gunderson and Stillman 2015). For dispersal-limited species, behavioral thermoregulation may buffer the consequences of anthropogenic climate change (Kearney, Shine, & Porter, 2009; Ruiz-Aravena et al., 2014). However, the potential for behavioral thermoregulation to promote species survival in warming climates depends on two major factors. First, the species must effectively thermoregulate within a set range of temperatures to optimize performance (Huey et al., 2012). Second, preferred temperatures must be available as microhabitats or thermal refugia (Kearney et al. 2009). Even when these conditions are met, behavioral thermoregulation may actually reduce a species’ ability to persist in a warming climate. By narrowing the range of body temperatures an organism experiences, thermoregulatory behaviors reduce the strength of thermal selection in natural environments, thereby limiting local adaptation (Buckley et al. 2015). To make accurate predictions about species’ responses to climate change, we must understand the adaptive potential of behavioral thermoregulation in vulnerable taxa.

Little is known about thermal coadaptation in amphibians, which are among the most vulnerable taxa to environmental disturbances and climate change (Stuart et al. 2004; Foden et al. 2013). As most amphibians are ecologically constrained by evaporative water loss, the immediate costs of desiccation in dry environments may
outweigh the long-term benefits of maximizing thermal performance (Jørgensen 1997). To date, the thermal coadaptation hypothesis has only been validated in two amphibians, *Eurycea cirrigera* and *E. wilderae*, which prefer temperatures that maximize performance and that correspond with environmental temperatures within their respective geographic ranges (Strickland et al. 2016). In contrast, the common frog (*Rana temporaria*) prefers temperatures that reflect a tradeoff between minimizing water loss and maximizing performance (Köhler et al. 2011) and green frogs (*Lithobates clamitans*) prefer moist environments regardless of thermal optima (Mitchell and Bergmann 2015). In a more extreme case, European newts prefer temperatures 8°C below $T_{\text{opt}}$ (Gvoždík 2015). As the aforementioned studies illustrate, there is mixed evidence for thermal coadaptation in amphibians. Further, we have yet to test the hypothesis in terrestrial amphibians, which generally experience a wider range of temperatures and a greater threat of desiccation than their aquatic relatives.

Here, we combined laboratory experiments, field observations, and population models to explore the role of environmental conditions (i.e., moisture and thermal quality of the habitat) in shaping physiological performance in a terrestrial salamander, *Plethodon cinereus*. First, we performed a laboratory experiment to test the thermal coadaptation hypothesis in wet and dry moisture treatments. We hypothesized that $T_{\text{opt}}$ would be relatively low in this species, as they are the northernmost *Plethodon* salamander, and that a low $T_{\text{opt}}$ would allow them to choose optimal temperatures in wet and dry conditions without suffering from exacerbated evaporative water loss. Then, we used field observations from a 3-year mark-
recapture study, along with population models, to compare the accuracy and effectiveness of thermoregulatory strategies (i.e., regulation of surface activity or microhabitat selection) across an elevation gradient (650-1,250m asl). Conducting this study along an elevation gradient allowed us to determine whether behavioral thermoregulation has arisen in certain populations in response to local temperatures, either through local adaptation or developmental plasticity. We predicted that salamanders from warmer sites would thermoregulate more effectively than those from cool sites to reduce their risk of excess energy expenditure and overheating, which are associated with warmer temperatures. This study expands upon our knowledge of thermal performance and behavior in ectotherms and sheds light on the thermal strategies that allow widespread species to exist across a wide range of temperatures.

Methods

Thermal performance experiment

We hand-captured 40 adult (>32mm snout-vent length; SVL; Sayler 1966) male salamanders from a single field site (Wind Rock; Table 4.2) in October 2015 and transported them back to the lab in individually-labelled containers. To avoid the potentially confounding physiological effects of color polymorphism, we only collected individuals that clearly displayed the striped, rather than unstriped, phenotype (Moreno 1989; Fisher-Reid et al. 2013). At the time of collection, we measured SVL, tail length, and mass, and determined sex using the candling method (described by Gillette & Peterson, 2001). After collection, salamanders were
transported to temperature-controlled chambers and held at a constant temperature of 15°C, where they underwent an acclimation period of 4 weeks. We housed each salamander individually in a plastic container lined with a moistened unbleached paper towel and a crumpled moist paper towel to use as a retreat. Salamanders were fed 15-20 large flightless fruit flies (*Drosophila hydei*) weekly and sprayed with spring water as necessary.

Following acclimation, we measured thermal performance as energy assimilation at 5, 10, 15, 20, and 25°C. Energy assimilation determines the amount of energy available for growth and reproduction, and is therefore tied to individual fitness (van der Meer 2006). This performance trait has also been shown to be more sensitive to temperature than other commonly used performance metrics, such as sprint speed and endurance (Angilletta et al. 2002a). Here, we define energy assimilation as:

\[
\text{Energy assimilation} = E_A - (E_F + E_S)
\]

where \(E_A\) is the energy acquired through ingestion, \(E_F\) is the energy lost as feces, and \(E_S\) is the energy lost as shed skin. As salamanders shed skin more frequently at higher temperatures, we chose to quantify \(E_S\) to account for variation in energy expenditure among temperatures (Merchant 1970).

To measure energy assimilation, we performed controlled feeding trials following the protocol of Clay and Gifford (2017; Fig. 2.1). During each trial, we offered 50 large fruit flies (*Drosophila hydei*) to each salamander, recorded the
number of flies remaining after 24 h, and replenished the flies that were eaten. We repeated this procedure (i.e., counting flies and replenishing) for 5 consecutive days, recorded the number of remaining flies on the 6th day, and removed all leftover flies. We collected feces and shed skin during each trial and continued until the digestive tract was clear, as determined by 3-5 days without further fecal production. After fecal collection, we transferred salamanders to the next experimental temperature and acclimated them for 7-10 days prior to the next trial. During this time, they were not fed. Each salamander was tested at 5, 10, 15, 20, and 25°C. We observed successful foraging behavior and 100% survival at the warmest temperature (25°C), which was surprising given that Merchant (1970) reported little, to no ingestion at 25°C. Thus, we chose to test an additional temperature of 30°C using a subset of salamanders ($n = 8$) and observed 100% mortality within 24 – 72 h. Two other individuals died of natural causes during this experiment.

Using a Parr 6725 semimicro calorimeter, we quantified energy acquired as $E_A$ and energy lost as $E_F$ and $E_S$, combined. By subsampling fruit flies at different times during their adult life stage, we found a mean caloric content of 0.064 kJ/fly. Using this measurement, we calculated $E_A$ as the total number of calories ingested by each salamander at each temperature. Most individual fecal and shed skin samples were too small to process on their own. Instead, we combined all fecal and skin samples for each temperature. We dried each sample at 80°C for 24 – 48 h, and pelletized subsamples for each temperature. As a metric of energy lost, we used the mean energy content of fecal and skin samples, in kJ, at each temperature.
Temperature preference experiment

We measured thermal preference ($T_{\text{pref}}$) using the remaining individuals from the energy assimilation measurements ($n = 30$). To measure $T_{\text{pref}}$, we placed salamanders in linear thermal gradients ranging from approximately 5 to 30°C and recorded body temperatures every 4-6 h. Each gradient consisted of a shallow plastic tray (54 x 27.5 x 3.3 cm) half filled with soil, with heat tape attached below one end of the tray and ice packs below the other. The soil was sieved to remove large particles and insects and dried prior to the study. To stabilize the temperature gradient, we held trays in incubators at 15°C and replaced ice packs as needed. We secured all trays with a plastic lid to prevent salamander escape and control humidity. Additionally, we placed a single sheet of thin foam board (1cm thick) lengthwise along the center of each tray for salamanders to use as refugia. This refugia allowed salamanders to settle between the foam board and the soil, mimicking their natural behavior in the wild. In preliminary trials without refugia, salamanders constantly paced along the walls of the tray, which prevented temperature selection and caused unnecessary stress to the animals. The enclosures were held in a 12:12 light:dark cycle throughout this study.

To determine whether moisture affects $T_{\text{pref}}$, we sprayed half of the enclosures with 150mL of water (wet treatment) and half with 75mL of water (dry treatment). We fed salamanders 12 h prior to each trial and released 5 individuals along the midline of each gradient, allowing them 12 h to acclimate to the thermal gradient. The density of each enclosure was 0.003 salamanders/m$^2$. Feder and Pough (1978) found that density of salamanders, when using 10 individuals (0.001 salamanders/m$^2$), did not affect $T_{\text{pref}}$ in a linear gradient. After 12 h, we recorded body temperatures using
an infrared thermometer 8 times over a 48-h period, at 0900, 1300, 1900, and 2300 h each day. We repeated this procedure twice per individual in each moisture treatment, with at least 24 h between each trial. Salamanders were marked with unique combinations of visual implant elastomers (VIE) to identify individuals for repeat measurements (Heemeyer et al. 2007). VIEs are injected subcutaneously and have no detectable effects on the body condition, mortality, or reproductive development of Plethodontid salamanders (Bailey 2004; Davis and Ovaska 2001). We calculated $T_{\text{pref}}$ as the mean body temperature selected by each salamander and the set-point (or target) temperature range ($T_{\text{set}}$) as the central 50% of all body temperatures selected by each salamander in each moisture treatment (Hertz et al. 1993).

**Mark-recapture field study – microhabitat selection & surface activity**

We performed a mark-recapture field study to determine whether *P. cinereus* uses behavioral thermoregulation to select optimal microhabitats when active and/or regulate surface activity based on environmental conditions. For three consecutive summers (May-August 2014-2016), we performed biweekly nocturnal salamander surveys to capture, measure, and mark surface-active *P. cinereus*. Surveys occurred between the hours of 2000 and 0400. We define surface-active salamanders as those that are visible atop the leaf litter or climbing on microhabitat structures (e.g., rocks, logs, vegetation, etc.). Surveys were conducted at 5 field sites along an elevation gradient on Salt Pond Mountain in Pembroke, Virginia (Table 4.2). At each site, we established 2 transects, at least 100 m apart, and installed 3 permanent 9 m$^2$ plots along each transect. The plots were separated by at least 25 m to reduce the likelihood that
salamanders would move between plots, as *P. cinereus* has a very small home range (<1m²; Mathis 1991).

During each sampling event, a team of 2-3 people visually inspected the plot for salamanders atop the leaf litter. We chose to use nocturnal visual encounter surveys because they have a greater initial encounter probability than other methods commonly used to survey *P. cinereus* (Buderman and Liebgold 2012). They also allowed us to quantify microhabitat selection for active salamanders without manipulating the environment, unlike cover board searches which are used to capture salamanders that are largely inactive and depend on the use of artificial cover objects (Hesed 2012). Upon sighting, we immediately recorded body and substrate temperatures using an infrared temperature gun. After recording body temperature, we hand-captured the salamander, placed it in an individually labeled bag, and placed a marker at the point of capture to ensure the individual was returned to its precise location. Each survey ended after searching for 5 min without finding an additional salamander. We weighed (g), measured (mm), sexed (using the candling method described by Gillette and Peterson, 2001), and marked all individuals with unique combinations of VIEs (Davis and Ovaska 2001). All individuals were released within 48 h of capture.

To understand the role of temperature and moisture in shaping thermoregulatory behavior, we needed further information about the environmental conditions at each field site. Thus, we installed HOBO temperature loggers on the forest floor at each site to record the temperature every 30 min throughout the study and used these data as an estimate of operative temperature (*T_e*). *T_e* is often measured
using a copper replica of the animal to account for thermal properties such as color, shape, and size (Muñoz et al. 2014). In this study, the HOBO loggers provided an appropriate estimate of $T_e$ because *P. cinereus* is a small (<3g) nocturnal ectotherm that remains relatively close to the forest floor. Thus, its body temperature is mostly buffered from radiation and wind, which would otherwise affect $T_e$ of a larger, diurnal ectotherm (Sears et al. 2011). The use of HOBO loggers to estimate $T_e$ is further supported by our field data, which showed that salamander body temperature almost perfectly conforms to the temperature of the substrate it is found on ($\beta = 0.99; R^2 = 0.99; P < 0.001; df = 996; \text{Fig. A1.1}$). To approximate relative precipitation during each survey, we downloaded data from MLBS’ weather station, and calculated the total precipitation on the day of each salamander survey.

After recording field temperatures for 3 years, we characterized thermal quality of the habitat ($d_e$) at each site by modifying the protocol of Hertz et al. 1993. This index, $d_e$, represents the deviation of available temperatures within a habitat from the range of temperatures that optimize performance. Thus, a $d_e$ value equal to 0 is physiologically ideal for the organism. When ectotherms prefer temperatures that optimize performance in a laboratory setting, it is appropriate to calculate $d_e$ as the absolute deviation of $T_e$ from $T_{set}$. Here, we use $T_{opt}$ in place of $T_{set}$ because $T_{set}$ did not reflect optimal performance in this species (see laboratory results below). For each site, we calculated $d_e$ every 30 min between 2000 and 0400 h during the active season of *P. cinereus* throughout the study (Fig. A1.2). The active season is based on observations from a population at Mountain Lake Biological Station (MLBS), in which almost all salamander activity was observed between 1 April and 1 Oct during
a 5-year mark-recapture study (H. Wilbur, unpublished data). We found that \( d_e \) varied significantly among sites (\( df = 34,996; F = 277.58; P < 0.001 \)) and was not necessarily a reflection of elevation. Thus, we used a Tukey’s HSD test to statistically categorize sites by \( d_e \), rather than combining sites by elevation categories. We categorized sites into 3 discrete thermal quality groups for further analysis: superior thermal quality (\( d_e \leq 0.5 \)), moderate thermal quality (\( 0.5 > d_e > 1.0 \)), and poor thermal quality (\( d_e \geq 1.0 \); Table 4.2; Fig. A1.2).

To determine how well surface-active salamanders regulate their body temperatures, we calculated effectiveness of thermoregulation (\( E \)) for each observed individual using the index described by Hertz et al. 1993:  

\[
E = 1 - \left( \frac{\bar{d}_b}{\bar{d}_e} \right)
\]

In this case, \( \bar{d}_b \) is the mean absolute deviation of field active body temperature (\( T_b \)) from \( T_{opt} \) for each mark-recapture plot and \( \bar{d}_e \) is the mean absolute deviation of \( T_e \) from \( T_{opt} \) for each mark-recapture plot during survey events when at least 1 salamander was observed. The metric \( \bar{d}_b \) represents accuracy of thermoregulation, with values closer to 0 indicating higher accuracy, and \( \bar{d}_e \) is the thermal quality index, with values close to 0 indicating optimal environmental temperatures. The index \( E \) ranges between 0 and 1, where a value of 0 indicates thermoconformity and a value of 1 indicates perfect thermoregulation. When the thermal environment matches \( T_{opt} \), thermoregulation is not necessary to optimize performance, and \( E \) is undefined.

**Analyses**

**Thermal performance experiment**
To quantify $T_{\text{opt}}$, we fit individual thermal performance curves for energy assimilation using the following function:

$$\text{Energy assimilation} \sim P_{\text{max}} \left[ \left( \frac{T_{\text{max}} - T}{T_{\text{max}} - T_{\text{opt}}} \right) \left( \frac{T}{T_{\text{opt}}} \right)^{T_{\text{opt}} / T_{\text{max}} - T_{\text{opt}}} \right]$$

where $P_{\text{max}}$ is maximum performance, $T_{\text{opt}}$ is thermal optimum, $T_{\text{max}}$ is thermal maximum, and $T$ is the experimental temperature during each energy assimilation trial. This model fit the data better than several competing models (Table A1.1). We fit curves using a nonlinear mixed effects (NLME) model with individual as a random effect to estimate the parameters of interest, $P_{\text{max}}$ and $T_{\text{opt}}$, for each salamander.

Typically, researchers use critical thermal limits as a constraint on the upper and lower bounds of the thermal performance curve. However, critical thermal limits represent the temperature at which salamanders lose righting ability, and likely overestimate the maximum temperature at which a salamander can capture prey items and survive for extended periods of time. In this population, the mean critical thermal maximum was 33.5°C (Chapter 3), but salamanders experienced 100% mortality when held at 30°C for 48 h for feeding trials. Thus, we constrained the upper thermal limit, or $T_{\text{max}}$, to 30°C. Likewise, we assumed that salamanders would die at a constant temperature of 0°C as they are freeze intolerant (Storey and Storey 1986), although the mean critical thermal minimum in this population was -1.05°C (Chapter 3). Therefore, we provided an energy assimilation value of 0 kJ/g/day for each salamander at 0°C. Analyses were performed using packages nlme (version 3.1-131) and AICcmodavg (version 2.1.0) in R statistical software (Development Core Team, 2016).
Temperature preference experiment

To determine whether $T_{\text{pref}}$ was related to $T_{\text{opt}}$ and whether this relationship was affected by moisture, we fit a linear mixed effects (LME) model using a maximum likelihood approach with $T_b$ as the response variable, $T_{\text{opt}}$, moisture treatment, and the interaction between the two as fixed effects, and individual as a random effect. The model was run using package *nlme* (version 3.1-131) in *R* statistical software (Development Core Team, 2016).

Mark-recapture field study – microhabitat selection

To explore thermoregulatory effectiveness of active salamanders in relation to available microclimates, we calculated $T_e$, $T_b$, $\bar{d}_e$, $\bar{d}_b$, and $E$ for sites within each thermal quality category (i.e., poor, moderate, and superior). For $\bar{d}_e$ and $\bar{d}_b$, we used the range of $T_{\text{opt}}$’s estimated for salamanders from 3 of our field sites (Horsenettle Trail, John’s Creek Trail, and Wind Rock): 17.81 – 21.59°C. In a separate study, we found that $T_{\text{opt}}$ did not vary among these 3 sites, so it was appropriate to combine thermal performance data (See Appendices 2 and 3). For each metric, we fit an LME model by maximum likelihood with month (as a quadratic term), thermal quality category, and the interaction term as explanatory variables, the respective metric as the response variable, and year as a random effect. To determine whether thermoregulatory effectiveness was influenced by environmental moisture, we ran a separate LME model with total rainfall (on the day of the survey, mm) as an explanatory variable for $E$ and adjusted the $P$-values using a Bonferroni correction.
Analyses were performed using packages *nlme* (version 3.1-131) and *stats* (version 3.4.0) in R statistical software (Development Core Team, 2016).

**Mark-recapture field study – surface activity**

We used a mark-recapture analysis to estimate surface activity as a function of environmental conditions (i.e., $T_e$, precipitation, and $d_e$). Mark-recapture data were analyzed using “Higgins p and c” closed robust design models in program MARK (White and Burnham 1999) and closely follows methodology of a similar *P. cinereus* study in Maryland (Muñoz et al. 2016). Robust design models estimate temporary emigration from survey sites between primary periods (survey seasons), apparent survival between primary periods, abundance within primary periods, and detection probability of secondary occasions (surveys; Kendall et al. 1997). We used AIC to determine which model structures (e.g., time-varying vs. intercept only) best described the structure of nuisance parameters (Table A1.2). Similar to other plethodontid studies, temporary emigration, $\gamma$, was best modeled as random movement (Bailey et al. 2004). It was also modeled as constant across time and sites (i.e., intercept only). Survival, $\Phi$, was best modeled as intercept only (but see below).

Recapture probability, $c$, was best modeled as equal to capture probability, $p$ (hereafter detection probability). Detection probability, $p$, was best explained by site and survey specific parameters – supporting the need for modeling variation in survey specific detection as a function of covariates. Once underlying structure of the models was determined, we included linear and quadratic covariates for the environmental predictors: $T_e$, precipitation, and $d_e$. All predictors were standardized by subtracting...
the mean and dividing by a standard deviation. Regression coefficients were estimated using generalized linear models using the logit link in program MARK.

Results

Thermal performance experiment

The main goal of our thermal performance experiment was to quantify thermal optimum for performance, $T_{opt}$, which in this study is an estimate of the temperature at which an individual experiences peak energy assimilation. Energy assimilation was largely determined by $E_A$, or the number of calories consumed during controlled feeding trials (Table 4.3). This result was attributed to high digestive efficiencies, which salamanders maintained across temperatures (Table 4.3). On average, salamanders exhibited a $90.10 \pm 6.25\%$ digestive efficiency, meaning that the amount of energy lost as $E_F$ and $E_S$ was very small relative to $E_A$. The NLME model estimated that mean $T_{opt}$ for energy assimilation was $19.59^\circ C$ for salamanders from Wind Rock, VA. By fitting individual performance curves, we determined that the range of thermal optima was relatively narrow for this population, $17.44 – 21.84^\circ C$, with a standard deviation of $1.30^\circ C$ (Fig. 4.2). On average, $P_{max}$ for this population was $0.94 \pm 0.04$ kJ/g/day. Unlike $T_{opt}$, $P_{max}$ was highly variable among individuals, and random variation among individual $P_{max}$ estimates accounted for a standard deviation of $0.18$ kJ/g/day. The strongest performing individual had a $P_{max}$ that was double that of the weakest performer ($1.25$ and $0.64$ kJ/g/day, respectively; Fig. 2.2).

Temperature preference experiment
By combining data from the thermal performance experiment and the temperature preference experiment, we tested the hypothesis that individuals prefer temperatures that optimize performance in wet and dry conditions. We found that individual $T_{\text{opt}}$ was unrelated to $T_{\text{pref}}$ ($\chi^2 = 0.30; df = 26; P = 0.58$), indicating that salamanders did not regulate body temperature to optimize energy assimilation. However, $T_{\text{pref}}$ was statistically different among moisture treatments, so that salamanders maintained cooler temperatures, farther from $T_{\text{opt}}$, in the dry treatment compared to the wet treatment ($\chi^2 = 12.18; df = 294; P < 0.001$; Fig. 4.3). On average, salamanders preferred temperatures 8.60°C below $T_{\text{opt}}$ in the dry treatment and 6.74°C below $T_{\text{opt}}$ in the wet treatment. $T_{\text{set}}$, or middle 50% of temperatures selected in the lab, was 7.20 – 13.91°C in the dry treatment and 6.96 – 16.35°C in the wet treatment. There was only one instance where $T_{\text{pref}}$ exceeded $T_{\text{opt}}$, in which the individual’s $T_{\text{pref}}$ was 17.60°C and its $T_{\text{opt}}$ was 17.44°C. Finally, we did not find evidence for an interactive effect of $T_{\text{opt}}$ and moisture treatment on $T_{\text{pref}}$ ($\chi^2 = 0.32; df = 294; P = 0.57$). We assessed model fit using a likelihood ratio test and found that our model fit the data significantly better than an intercept-only (null) model for $T_{\text{pref}}$ ($P = 0.006$).

**Mark-recapture field study – microhabitat selection**

The first goal of our mark-recapture study was to determine whether *P. cinereus* uses behavioral thermoregulation to select optimal microhabitats when active. To address this goal, we first analyzed operative temperatures, $T_e$, to understand the range of environmental temperatures available for behavioral exploitation at each field site on nights when salamanders are surface-active. We found that $T_e$ was interactively
related to month (quadratic term) and thermal quality category ($\chi^2 = 217.905; df = 769; P < 0.001$; Fig. 4.4A). Across all sites, $T_e$ was almost always within or below the $T_{opt}$ range (Fig. 4.4A). At the field site with superior thermal quality, $T_e$ was within $T_{opt}$ 82.31% of the times we observed active salamanders (107/130 observations). For the poor thermal quality sites (Horsenettle Trail and Virginia Walks Trail), $T_e$ was within $T_{opt}$ 89.76% of the times we observed active salamanders (114/127 observations), suggesting that salamanders regulate aboveground activity times to avoid suboptimal temperatures that often occur at these sites. However, mean $T_e$ was closer to the upper limit of the $T_{opt}$ range at the poor-quality sites compared to the superior quality site (mean $T_e = 19.21$ and $18.62^\circ C$, respectively), and exceeded $T_{opt}$ in one case. In contrast, mean $T_e$ ($17.35^\circ C$) was slightly below the $T_{opt}$ range at sites with moderate thermal quality (Wind Rock and John’s Creek Trail), with $T_e$ falling below $T_{opt}$ 60.23% of the time, and within the $T_{opt}$ range 39.77% of the time. Patterns of $T_b$ largely reflected those of $T_e$, indicating a lack of microhabitat selection on nights when salamanders are surface-active (Fig. 4.4B).

Thermal quality indices were very close to 0 across all sites during summer field surveys, indicating high thermal quality on nights when active salamanders were observed (Fig. 2.4C). The interaction between month (quadratic term) and thermal quality category was a significant predictor of $\tilde{d}_e$ ($\chi^2 = 16.14; df = 759; P = 0.003$; Fig. 2.4A). However, most variation in $\tilde{d}_e$ was attributed to the survey month ($\chi^2 = 270.09; df = 759; P < 0.001$), rather than thermal quality category ($\chi^2 = 1.39; df = 759; P = 0.50$). This mismatch between thermal quality category and thermal quality on nights when salamanders are active suggests that salamanders regulate activity.
time to avoid suboptimal temperatures. Salamanders had the lowest thermoregulatory accuracy, $\bar{d}_p$, at sites with moderate thermal quality, likely because the environmental temperature rarely fell within $T_{opt}$ (Figs. 4.4A and 4.4D). Salamanders exhibited a higher accuracy of temperature regulation at sites with poor and superior quality (Fig. 4.4D).

Sample sizes were greatly reduced from 780 to 351 observations when analyzing thermoregulatory effectiveness, $E$, because it was undefined for each observation where $T_e$ fell within the range of $T_{opt}$. With this reduced dataset, we found that $E$ was related to survey month ($\chi^2 = 45.59; df = 335; P < 0.001$), but unrelated to thermal quality of the site ($\chi^2 = 4.69; df = 335; P = 0.09$). Additionally, the interaction term between month and thermal quality category was non-significant ($\chi^2 = 5.59; df = 335; P = 0.23$). Because $E$ was undefined for most of the surveys at the poor and superior quality sites, we tested the relationship between precipitation and $E$ for the moderate quality sites only. We found that $E$ was strongly, negatively related to total rainfall ($\beta = -0.13; \chi^2 = 132.16; df = 308; P < 0.001$).

**Mark-recapture field study – surface activity**

The second goal of our mark-recapture study was to determine whether *P. cinereus* uses behavioral thermoregulation to regulate surface activity based on environmental conditions. Therefore, we performed mark-recapture analyses to determine whether salamanders adjust surface activity, estimated by detection probability, in response to environmental temperature or moisture at our study sites. Quadratic relationships outperformed linear relationships for all environmental predictors, and temperature
was the best predictor of salamander detection, followed by thermal quality index and precipitation (Table A1.3). Detection probability was low across all sites, but mean detection was highest at Wind Rock (0.121, 95% CI [0.103, 0.142]) followed by John’s Creek Trail (0.093, 95% CI [0.077, 0.111]), Virginia Walks Trail (0.077, 95% CI [0.053, 0.111]), Cherokee Flats (0.060, 95% CI [0.046, 0.080]), and then Horsenettle Trail (0.048, 95% CI [0.032, 0.072]). All environmental relationships with detection followed concave down quadratic relationships, indicating a detection optimum. Optimum detection occurred at 17.9°C, 29.8 mm precipitation, and 0.35 thermal quality index (Fig. 4.6). Regression coefficients for all environmental quadratic parameters had confidence intervals that did not overlap zero, suggesting clear relationships with detection probability (Table 4.4). Annual survival probabilities varied across each of the five sites, but confidence intervals were wide, indicating low power (Table 4.5). When looking at survival from the best model (model 3), survival rates were 0.659 ± 0.093 SE 95% CI [0.462, 0.814].

**Discussion**

This study used an integrative approach to assess thermoregulatory behavior, and its performance consequences, in a widespread terrestrial amphibian. We found that *P. cinereus* did not conform to the thermal coadaptation hypothesis in a laboratory setting, where individuals preferred body temperatures 6-9°C below $T_{opt}$, on average. Salamanders preferred temperatures farther from $T_{opt}$ in dry, relative to wet, conditions, suggesting a tradeoff between performance and hydration. Field observations, on the other hand, supported the thermal coadaptation hypothesis –
summer surface activity peaked when environmental temperatures matched $T_{\text{opt}}$. We also found that salamanders from poor thermal quality sites were more accurate thermoregulators than those from moderate thermal quality sites. Specifically, salamanders from warmer sites almost always avoided temperatures above $T_{\text{opt}}$, whereas salamanders from cooler sites rarely avoided temperatures below $T_{\text{opt}}$. Further, we present preliminary evidence that rain decreases thermoregulatory effectiveness, suggesting that opportunistic foraging may incur long-term performance costs.

Terrestrial lungless salamanders (genus *Plethodon*) depend on cool, wet microhabitats for gas exchange (Petranka 1998). As such, *Plethodon* species must balance the immediate risks of desiccation in dry environments with thermal performance outcomes (Feder 1983). Separate studies have quantified thermal sensitivity and temperature preference in *P. cinereus*. Merchant (1970) concluded that the optimal temperature for energy assimilation is 10°C, whereas Feder and Pough (1978) found that *P. cinereus* prefers temperatures between 16 and 21°C. It should be noted that Merchant approximated $T_{\text{opt}}$ based on discrete experimental temperatures, rather than fitting a curve to the data. Regardless, these studies suggest that *P. cinereus* prefers temperatures above the optimal temperature for performance, which is not explained by any theory under the current thermal coadaptation framework (reviewed in Gvoždík 2015). However, it is not possible to reconcile these contradictory findings because the studies occurred at different times and used individuals from different populations. Further, neither study tested the effects of moisture on behavioral thermoregulation.
Our laboratory and field studies showed mixed support for the thermal coadaptation hypothesis. In a laboratory setting, *P. cinereus* selected temperatures far below $T_{\text{opt}}$. A similar thermal mismatch has been observed in European newts, and may be explained by variation in evolutionary rates among thermal traits (Gvoždík 2015). In newts, the lower boundary of $T_{\text{pref}}$ range evolved faster than the mean $T_{\text{pref}}$, the upper boundary of the $T_{\text{pref}}$ range, and $T_{\text{opt}}$, potentially driving a thermal mismatch far greater than expected by the Asymmetry model of behavioral thermoregulation (Martin and Huey 2008; Gvoždík 2015). In our study, the range of $T_{\text{pref}}$ was wide and highly variable among individuals suggesting that they might not have a strong proclivity for regulating body temperatures through microhabitat selection, which was the main mechanism being tested in the laboratory thermal gradient. The lack of microhabitat selection is supported by our field data, which showed that regulation of surface activity, rather than aboveground microhabitat selection was the primary strategy for thermoregulation.

In this study, environmental temperature was a relatively strong predictor of surface activity in the field, and surface activity peaked at $T_{\text{opt}}$. This result suggests that the regulation of surface activity during the summer months may optimize thermal performance. However, another study found that fall and spring surface activity in *P. cinereus* peaks at much lower temperatures (11°C and 8.5°C, respectively; Muñoz et al. 2016). There are several possible reasons why salamanders may prefer optimal temperatures during summer activity, but not during fall and spring activity. First, $T_{\text{opt}}$ for energy assimilation may undergo seasonal shifts, or acclimatization, so that optimal temperatures match seasonal environmental
temperatures (Angilletta et al. 2006). For example, the thermal sensitivity of muscle metabolic enzymes shifts in accordance with seasonal body temperatures in the American alligator (Alligator mississippiensis), and in turn, facilitates locomotor performance throughout the year (Seebacher 2003). Second, salamanders have a greater risk of experiencing temperatures near the upper limits of their $T_{opt}$ range in the summer, and thus, precise regulation of surface activity is likely more adaptive in the summer than in other seasons (Hadamová and Gvoždík 2014). Finally, energy assimilation may not be the most ecologically relevant performance metric in the spring and fall when activities associated with mating and courtship are prioritized. Male garter snakes (Thamnophis sirtalis parietalis), for example, perform courtship behaviors rather than basking during mating season likely because the reproductive costs of behavioral thermoregulation are far greater than the benefits of maintaining optimal temperatures (Shine et al. 2006). Future studies of Plethodon performance should test the aforementioned explanations by measuring seasonal acclimatization of thermoregulatory behavior and performance, energetic costs associated with seasonal variation in thermoregulatory accuracy, and the tradeoffs between energy assimilation and courtship behavior during mating season.

Environmental moisture and precipitation are important to consider when assessing thermoregulatory behavior in P. cinereus. I found that salamanders were less likely to choose optimal temperatures for performance in dry conditions in the lab and wet conditions in the field. In a laboratory setting, P. cinereus chose colder temperatures that were farther from $T_{opt}$ in dry conditions, relative to wet conditions. This behavior conforms with prior studies that have showed that hydration state
(Connor and Tracy 1992; Tracy and Christian 2005) and evaporative water loss (Peterman and Semlitsch 2014) are strong predictors of microhabitat selection and spatial distribution in amphibians. However, our results suggest that *P. cinereus* may incur energetic consequences as a result of choosing suboptimal temperatures to reduce water loss. A similar tradeoff can be seen in *L. clamitans*, which efficiently hydroregulate to maintain hydration at the cost of choosing suboptimal temperatures for performance (Mitchell and Bergmann 2015). The opposite trend appeared in the field, where thermoregulatory effectiveness was strongly reduced during heavy rainfall. Aboveground activity is highly constrained by moisture in *P. cinereus*, with most foraging occurring during and immediately after rain events (Jaeger 1978). This constraint may result in a tradeoff where salamanders exploit wet conditions despite the risk of experiencing non-optimal body temperatures that reduce thermal performance. However, thermoregulatory effectiveness was undefined during most field surveys, so the relationship between precipitation and thermoregulatory effectiveness should be considered preliminary. Future studies should repeat these measurements in the spring and fall when salamanders likely experience a greater range of thermoregulatory effectiveness, as they are often active at temperatures below $T_{\text{opt}}$ (Muñoz et al. 2016).

Thermoregulatory accuracy, but not effectiveness, varied among sites with differing thermal quality. Salamanders from poor thermal quality sites, which were warmer on average than the other sites, limited surface activity to nights that were within their range of thermal optima. In contrast, salamanders from moderate thermal quality sites, which were colder on average than other sites, were regularly active on
nights when the temperature was below thermal optima. This result supports the Asymmetry model of thermoregulation, as salamanders would experience greater performance costs from heat exposure than cold exposure, in the absence of local adaptation (Angilletta et al. 2006; Martin and Huey 2008). Similar variation can be seen in reptiles, where thermoregulatory behavior is greater in seasons and habitats with lower thermal quality (Row and Blouin-Demers 2006; Edwards and Blouin-Demers 2007; Vickers et al. 2011).

Behavioral thermoregulation may be the most immediate means of avoiding unfavorable temperatures for *P. cinereus* at our field sites, as thermal optimum for performance does not match temperatures experienced across the elevation gradient (See appendices 2 and 3). Our results suggest that regulation of surface activity is an effective means of homogenizing temperatures experienced across sites with differing thermal quality, which reduces the need for microhabitat selection. While this strategy is immediately beneficial, it may reduce physiological adaptation to warming temperatures associated with climate change (Buckley et al. 2015).

In the absence of thermal adaptation to warmer temperatures, we might expect *P. cinereus* to experience a reduction in activity time, and therefore a loss of foraging and courtship opportunities (Caruso et al. 2014). This reduction in activity time will be strongly influenced by changes in precipitation and humidity (Peterman and Gade 2017). We might expect salamander activity time to be further reduced in drier climates, which will compound the loss of foraging and courtship opportunities (Caruso et al. 2014). In a situation where the environment becomes warmer and wetter, however, we might expect salamanders to become opportunistically active.
while suffering exposure to temperatures near or above their range of thermal optima. This climatic shift would likely cause a dramatic decrease in energy assimilation. However, this scenario is complicated by potential plasticity in resistance to water loss, which has been demonstrated in closely related *Plethodon* species (Riddel and Sears 2015). Salamanders may increase resistance to water loss, meaning they experience less cutaneous water loss, when acclimated to warmer temperatures. As such, energetic models intending to predict *P. cinereus*’ response to climate change should incorporate variation in water loss as well as the role of temperature and moisture in regulating surface activity.
Table 4.1. Glossary of the indices and abbreviations used in Chapter 4. Glossary does not include model symbols, which are explained in the text, and used less frequently throughout the chapter.

<table>
<thead>
<tr>
<th>Index</th>
<th>Definition</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\bar{d}_b$</td>
<td>Accuracy of thermoregulation, mean absolute deviation of field active $T_b$ from $T_{opt}$ for each mark-recapture plot</td>
<td>°C</td>
</tr>
<tr>
<td>$d_e$</td>
<td>Thermal quality of the habitat, calculated as the absolute deviation of $T_e$ from $T_{opt}$</td>
<td>°C</td>
</tr>
<tr>
<td>$\bar{d}_e$</td>
<td>Survey thermal quality, mean absolute deviation of $T_e$ from $T_{opt}$ for each mark-recapture plot during survey events when at least 1 salamander was observed</td>
<td>°C</td>
</tr>
<tr>
<td>$E$</td>
<td>An index of thermoregulatory effectiveness, calculated as $1 - (d_b/d_e)$</td>
<td>-</td>
</tr>
<tr>
<td>$P_{max}$</td>
<td>Maximum performance</td>
<td>kJ/g/d</td>
</tr>
<tr>
<td>$T_b$</td>
<td>Body temperature</td>
<td>°C</td>
</tr>
<tr>
<td>$T_e$</td>
<td>Operative temperature</td>
<td>°C</td>
</tr>
<tr>
<td>$T_{opt}$</td>
<td>Thermal optimum for performance</td>
<td>°C</td>
</tr>
<tr>
<td>$T_{pref}$</td>
<td>Preferred body temperature, calculated as the mean $T_b$ selected in the laboratory</td>
<td>°C</td>
</tr>
<tr>
<td>$T_{set}$</td>
<td>Set-point (or target) temperature range, calculated as the central 50% of $T_b$ selected in the laboratory</td>
<td>°C</td>
</tr>
<tr>
<td>$T_{sub}$</td>
<td>Substrate temperature</td>
<td>°C</td>
</tr>
</tbody>
</table>
Table 4.2. Description of field sites in Pembroke, VA used for salamander mark-recapture study from 2014-2016. Observations include the total number of captures at each field site and the number of individually marked salamanders at each field site throughout the course of the study. Thermal quality is reported as mean±sd for each site throughout the active season of *P. cinereus* and categorized into 3 discrete thermal quality groups: superior thermal quality (*d_e* ≤ 0.5), moderate thermal quality (0.5 > *d_e* > 1.0), and poor thermal quality (*d_e* ≥ 1.0).

<table>
<thead>
<tr>
<th>Site Name</th>
<th>Elevation (m asl)</th>
<th>Captures</th>
<th>Individuals</th>
<th>Thermal quality, <em>d_e</em> (°C)</th>
<th><em>d_e</em> category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horsenettle Trail</td>
<td>650</td>
<td>123</td>
<td>100</td>
<td>1.13 ± 2.15</td>
<td>Poor</td>
</tr>
<tr>
<td>Cherokee Flats</td>
<td>750</td>
<td>245</td>
<td>194</td>
<td>0.34 ± 1.0</td>
<td>Superior</td>
</tr>
<tr>
<td>Virginia Walk Trail</td>
<td>950</td>
<td>130</td>
<td>105</td>
<td>1.07 ± 2.22</td>
<td>Poor</td>
</tr>
<tr>
<td>John’s Creek Trail</td>
<td>1,150</td>
<td>404</td>
<td>291</td>
<td>0.53 ± 1.68</td>
<td>Moderate</td>
</tr>
<tr>
<td>Wind Rock</td>
<td>1,250</td>
<td>488</td>
<td>322</td>
<td>0.67 ± 1.80</td>
<td>Moderate</td>
</tr>
</tbody>
</table>
Table 4.3. Results of thermal performance trials, as determined by bomb calorimetry.

All values are reported as mean±sd (n = 38). $E_A$ is the energy acquired through ingestion, $E_F$ is the energy lost as feces, and $E_S$ is the energy lost as shed skin.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$E_A$ (kJ/g/day)</th>
<th>$E_F + E_S$ (kJ/g/day)</th>
<th>Energy assimilation (kJ/g/day)</th>
<th>Digestive efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.14 ± 0.11</td>
<td>&lt; 0.001 ± 0.0004</td>
<td>0.12 ± 0.10</td>
<td>83.99 ± 16.94</td>
</tr>
<tr>
<td>10</td>
<td>0.66 ± 0.16</td>
<td>0.002 ± 0.001</td>
<td>0.59 ± 0.14</td>
<td>89.36 ± 5.18</td>
</tr>
<tr>
<td>15</td>
<td>0.84 ± 0.31</td>
<td>0.002 ± 0.001</td>
<td>0.76 ± 0.30</td>
<td>90.18 ± 4.04</td>
</tr>
<tr>
<td>20</td>
<td>1.02 ± 0.37</td>
<td>0.004 ± 0.002</td>
<td>0.88 ± 0.30</td>
<td>86.33 ± 5.03</td>
</tr>
<tr>
<td>25</td>
<td>0.84 ± 0.36</td>
<td>0.002 ± 0.001</td>
<td>0.77 ± 0.33</td>
<td>91.12 ± 5.92</td>
</tr>
</tbody>
</table>
Table 4.4. Regression coefficients from three generalized linear models (mean ± SE [95% CI]) used in mark-recapture analyses to estimate surface activity and survival. Estimates for survival, $\Phi$, and temporary emigration $\gamma$, are followed by each site’s detection coefficient. The respective environmental linear and quadratic predictors are referred to as $\beta_{\text{Envr}}$ and $\beta_{\text{Envr}}^2$ for each of the three models. Environmental covariates were standardized prior to analysis and are therefore unit-less.

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Temperature (°C)</th>
<th>Precipitation (mm)</th>
<th>Thermal quality (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_\Phi$</td>
<td>$0.661 \pm 0.415 [-0.152, 1.48]$</td>
<td>$0.754 \pm 0.441 [-0.111, 1.62]$</td>
<td>$0.637 \pm 0.410 [-0.166, 1.44]$</td>
</tr>
<tr>
<td>$\beta_\gamma$</td>
<td>$-1.38 \pm 0.947 [-3.24, 0.472]$</td>
<td>$-1.35 \pm 0.923 [-3.16, 0.457]$</td>
<td>$-1.40 \pm 0.963 [-3.29, 0.485]$</td>
</tr>
<tr>
<td>$\beta_{\text{CHK}}$</td>
<td>$-2.74 \pm 0.151 [-3.04, 2.45]$</td>
<td>$-2.80 \pm 0.151 [-3.09, 2.50]$</td>
<td>$-2.85 \pm 0.150 [-3.14, 2.55]$</td>
</tr>
<tr>
<td>$\beta_{\text{HNT}}$</td>
<td>$-2.98 \pm 0.217 [-3.41, 2.56]$</td>
<td>$-2.99 \pm 0.21 [-3.41, 2.57]$</td>
<td>$-3.11 \pm 0.216 [-3.54, 2.70]$</td>
</tr>
<tr>
<td>$\beta_{\text{JCT}}$</td>
<td>$-2.28 \pm 0.103 [-2.48, 2.08]$</td>
<td>$-2.32 \pm 0.107 [-2.53, 2.11]$</td>
<td>$-2.33 \pm 0.102 [-2.53, 2.13]$</td>
</tr>
<tr>
<td>$\beta_{\text{VWT}}$</td>
<td>$-2.48 \pm 0.204 [-2.88, 2.08]$</td>
<td>$-2.75 \pm 0.200 [-3.14, 2.35]$</td>
<td>$-2.66 \pm 0.201 [-3.06, 2.67]$</td>
</tr>
<tr>
<td>$\beta_{\text{WR}}$</td>
<td>$-1.98 \pm 0.09 [-2.16, 1.80]$</td>
<td>$-2.05 \pm 0.090 [-2.23, 1.88]$</td>
<td>$-2.03 \pm 0.091 [-2.21, 1.85]$</td>
</tr>
<tr>
<td>$\beta_{\text{Envr}}$</td>
<td>$-0.028 \pm 0.046 [-0.120, 0.063]$</td>
<td>$0.468 \pm 0.061 [0.348, 0.588]$</td>
<td>$-0.061 \pm 0.057 [-0.174, 0.051]$</td>
</tr>
<tr>
<td>$\beta_{\text{Envr}}^2$</td>
<td>$-0.198 \pm 0.030 [-0.258, 0.139]$</td>
<td>$-0.115 \pm 0.027 [-0.167, 0.063]$</td>
<td>$-0.095 \pm 0.027 [-0.147, -0.043]$</td>
</tr>
</tbody>
</table>
Table 4.5. Apparent annual survival estimates (mean ± SE [95% CI]) for five populations of *P. cinereus*. Results from the intercept only model (model 3) are represented by site “All”. Site specific estimates are based off model 4 where sites varied by thermal quality index.

<table>
<thead>
<tr>
<th>Site</th>
<th>Survival Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>0.659 ± 0.093 [0.462, 0.814]</td>
</tr>
<tr>
<td>CHK</td>
<td>0.567 ± 0.113 [0.347, 0.764]</td>
</tr>
<tr>
<td>HNT</td>
<td>0.819 ± 0.172 [0.319, 0.978]</td>
</tr>
<tr>
<td>JCT</td>
<td>0.639 ± 0.096 [0.438, 0.800]</td>
</tr>
<tr>
<td>VWT</td>
<td>0.805 ± 0.167 [0.338, 0.971]</td>
</tr>
<tr>
<td>WR</td>
<td>0.688 ± 0.111 [0.443, 0.859]</td>
</tr>
</tbody>
</table>
Figures

Fig. 4.1. Sample timeline for energy assimilation trials. The faded section shows a transition to the next experimental temperature. In total, this process was carried out 5 times to measure performance at 5, 10, 15, 20, and 25°C, though the duration of fecal collection varied with temperature.
Fig. 4.2. Individual thermal performance curves fit to energy assimilation data for *P. cinereus* from Wind Rock, Pembroke, VA. Energy assimilation was measured at 5, 10, 15, 20, and 25°C and assumed to be 0 kJ/g/day at 0 and 30°C. Data are jittered along the x-axis to improve their visibility.
Fig. 4.3. Body temperatures selected by *P. cinereus* in wet and dry linear thermal gradients in the laboratory. Points represent the mean for each moisture treatment and error bars represent standard error.
Fig. 4.4. Temporal trends in environmental temperatures and salamander body temperatures during the mark-recapture study (May-August 2014-2016). Site thermal quality is categorized as poor (circles and dotted lines), moderate (crosses and dashed lines), and superior (filled circles and solid lines). The blue shaded areas in A and B represent the range of thermal optima for energy assimilation, determined during thermal physiology experiments in the lab, across all field sites. The gray shading represents 95% CIs for predictions from nonlinear models.
Fig. 4.5. Temporal trends in thermoregulatory effectiveness, $E$, across all mark-recapture sites (A) and the relationship between precipitation and $E$ for sites with moderate thermal quality (B). Thermal quality is categorized as poor (circles and dotted lines), moderate (crosses and dashed lines), and superior (filled circles and solid lines). The gray shading represents 95% CIs for predictions from nonlinear models.
Fig. 4.6. Detection probability estimated modeled as a function of environmental predictors – temperature, precipitation, and thermal quality index. Maximum detection occurred at 17.9°C (A), 29.8 mm precipitation (B), and 0.35 thermal quality (C). Graphs reflect detection at the site with highest detection (WR). Detection probability is used as an estimate of surface activity throughout this study.
Appendix 1: Supplementary methods – model selection and preliminary analyses used to assess thermoregulatory accuracy and effectiveness

Table A1.1: Results of model selection for thermal performance curves. Names are based on the nonlinear functions used to fit each model, which were adapted from prior studies of thermal performance (Angilletta 2006; Estay et al. 2014; Ashrafi et al. 2017; Clay and Gifford 2017). The Beta model best fit our data, and was used to estimate thermal performance for energy assimilation. Reported values include the number of parameters in each model (K), Akaike Information Criteria adjusted for small sample size (AICc), the difference in AICc between each model and the most informative model (Δ AICc), and the log-likelihood (LL) for each model.

<table>
<thead>
<tr>
<th>Model Name</th>
<th>K</th>
<th>AICc</th>
<th>Δ AICc</th>
<th>LL</th>
</tr>
</thead>
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<td>10</td>
<td>2394.14</td>
<td>0.00</td>
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<tr>
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<tr>
<td>Candidate</td>
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<tr>
<td>Briere</td>
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<tr>
<td>Quadratic</td>
<td>7</td>
<td>2506.62</td>
<td>112.49</td>
<td>-1246.10</td>
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Table A1.2. Final set of models run in mark-recapture analyses. Models were used to predict detection probability, \( p \), temporary emigration, \( \gamma \), and apparent survival \( \Phi \). K indicates the number of parameters in each model. Environmental covariates include precipitation (prec), temperature (temp), and site thermal quality (TQ).

<table>
<thead>
<tr>
<th>Model #</th>
<th>Model</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( p=\text{site} + \text{temp}; \gamma=\text{intercept}; \Phi=\text{intercept} )</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>( p=\text{site} + \text{temp}; \gamma=\text{intercept}; \Phi=\text{intercept} + \text{TQ} )</td>
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<tr>
<td>3</td>
<td>( p=\text{site} + \text{temp} + \text{temp}^2; \gamma=\text{intercept}; \Phi=\text{intercept} )</td>
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<tr>
<td>4</td>
<td>( p=\text{site} + \text{temp} + \text{temp}^2; \gamma=\text{intercept}; \Phi=\text{intercept} + \text{TQ} )</td>
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</tr>
<tr>
<td>5</td>
<td>( p=\text{site} + \text{prec}; \gamma=\text{intercept}; \Phi=\text{intercept} )</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>( p=\text{site} + \text{prec}; \gamma=\text{intercept}; \Phi=\text{intercept} + \text{TQ} )</td>
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</tr>
<tr>
<td>7</td>
<td>( p=\text{site} + \text{prec} + \text{prec}^2; \gamma=\text{intercept}; \Phi=\text{intercept} )</td>
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</tr>
<tr>
<td>8</td>
<td>( p=\text{site} + \text{prec} + \text{prec}^2; \gamma=\text{intercept}; \Phi=\text{intercept} + \text{TQ} )</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>( p=\text{site} + \text{TQ}; \gamma=\text{intercept}; \Phi=\text{intercept} )</td>
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</tr>
<tr>
<td>10</td>
<td>( p=\text{site} + \text{TQ}; \gamma=\text{intercept}; \Phi=\text{intercept} + \text{TQ} )</td>
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<tr>
<td>11</td>
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<tr>
<td>12</td>
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Table A1.3. Results of model selection from 12 hypothesis models for mark-recapture analyses. The top 2 models are quadratic temperature predictors on detection probability, and indicator of surface activity. Model 4 included site thermal quality as a predictor of survival. Reported values include the difference in AICc between each model and the most informative model ($\Delta$ AICc), the likelihood (L) for each model, and the number of parameters in each model (K).

<table>
<thead>
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</tr>
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<td>6674.0</td>
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Fig. A1.1. Relationship between body temperature and substrate temperature for surface-active *P. cinereus* during mark-recapture surveys. We found a very strong relationship between body and substrate temperatures with a slope close to 1 (\( \beta = 0.99; R^2 = 0.99; P < 0.001; df = 996 \)), indicating that *P. cinereus* thermoconforms to the substrate it is standing on.
Fig. A1.2. Temporal trends in thermal quality, $d_e$, among mark-recapture study sites throughout the active season of *P. cinereus* (April – October). Values were calculated using temperatures recorded by HOBO temperature loggers deployed on the forest floor of each field site. When $d_e$ is equal to 0, conditions are physiologically ideal for the organism. Mean $d_e$ was used to statistically characterize sites into 3 $d_e$ categories: poor (HNT and VWT), moderate (WR and JCT), and superior (CHK).
Appendix 2: Supplementary methods – Comparing thermal performance across the range of *P. cinereus*

I measured energy assimilation for 68 salamanders from 10 collection sites at 10, 15, 20, and 25°C. Collection sites are the same as those described in Chapter 3 (Table 3.1), with some exceptions. Because of the delayed field collection, I was unable to include salamanders from North Carolina in the physiological performance trials, so Richmond was the southernmost site. Additionally, salamanders from a field site in Pembroke, VA (CHK) were not included in this part of the study. For analysis, I included energy assimilation data collected in a previous study (*n* = 38; Wind Rock, Virginia; Chapter 4). Thus, I measured physiological performance for a total of 106 individuals from 11 representative sites.

I fit individual performance curves for energy assimilation following the methods described in Chapter 4 to obtain parameter estimates (*T*\textsubscript{opt} and *P*\textsubscript{max}) for each salamander at each site. To determine the effects of evolutionary history (clade) and environmental conditions (local temperature) on physiological performance, I fit ANCOVA models with either *T*\textsubscript{opt} or *P*\textsubscript{max} as the response variable. Explanatory variables included clade (i.e., Northern, Pennsylvanian, or Southern), mean annual temperature, and the interaction term (clade*mean annual temperature). When clade was a significant predictor of physiological performance, I ran a Tukey posthoc test to determine which clades differed from each other. Finally, I tested the relationship between *T*\textsubscript{opt} and *P*\textsubscript{max} by running a linear model with *P*\textsubscript{max} as the response variable and *T*\textsubscript{opt}, clade, and the interaction term (clade*-*T*\textsubscript{opt}) as the explanatory variables.
Appendix 3: Supplementary results – Comparing thermal performance across the range of *P. cinereus*

After extracting parameter estimates from the individual performance curves (Fig. A3.1), I found that both $T_{\text{opt}}$ ($df = 1; \beta = 0.06; F = 7.53; P = 0.007$) and $P_{\text{max}}$ ($df = 1; \beta = 0.04; F = 4.67; P = 0.03$) were positively related to mean annual temperature, but did not differ among clades ($T_{\text{opt}}: df = 2; F = 0.28; P = 0.76$ and $P_{\text{max}}: df = 2; F = 0.33; P = 0.72$; Figs. A3.2 and A3.3). Further, the interaction term was nonsignificant for both parameters ($T_{\text{opt}}: df = 2; F = 0.38; P = 0.68$ and $P_{\text{max}}: df = 2; F = 0.38; P = 0.68$). Overall, the range of $T_{\text{opt}}$ was relatively narrow among individuals, ranging from 19.18 to 22.14°C. In contrast, $P_{\text{max}}$ varied widely from 0.28 in a salamander from Allegany State Park, NY (Pennsylvanian clade) to 2.61 kJ/g/day in a salamander from Richmond, VA (Northern clade). Looking at site averages, salamanders from Cunningham Falls State Park, MD (Northern clade) had the highest mean $P_{\text{max}}$ (1.69 ± 0.40 kJ/g/day) and those from Wind Rock, VA (Southern clade) had the lowest mean $P_{\text{max}}$ (0.97 ± 0.23 kJ/g/day).

$T_{\text{opt}}$ and clade were strong predictors of $P_{\text{max}}$ and accounted for 94.31% of the variation in $P_{\text{max}}$ (Fig. A3.4). I found a significant interactive effect of $T_{\text{opt}}$ and clade on $P_{\text{max}}$, indicating that the slope of the relationship between $T_{\text{opt}}$ and $P_{\text{max}}$ varied among clades ($df = 2; F = 15.21; P < 0.001$). Model coefficients were as follows: $\beta = 0.83$ for the Pennsylvanian clade, $\beta = 0.74$ for the Northern clade, and $\beta = 0.56$ for the Southern clade. Further, $T_{\text{opt}}$ alone was a strong predictor of $P_{\text{max}}$ and accounted
for most of the observed variation ($df = 1; F = 1409.16; P < 0.001$), while clade was
nonsignificant ($df = 2; F = 1.29; P = 0.28$).

Fig. A3.1 – Thermal performance curves for energy assimilation, fit for each
collection site. Line colors represent clade (dark blue = Northern, light blue =
Pennsylvanian, and green = Southern). Data are jittered by 1°C along the x-axis to
improve visibility.
Fig. A3.2. Thermal optimum for energy assimilation ($T_{opt}$) for *P. cinereus* from 11 sites across their geographic range. $T_{opt}$ was positively related to mean environmental temperature (left) but did not significantly differ among clades (right). Line colors represent clade (dark blue = Northern, light blue = Pennsylvanian, and green = Southern). The gray shading around the line represents 95% CIs for predictions from a linear model. The right panel shows mean $T_{opt}$ with standard error bars.
Fig. A3.3. Maximum performance for energy assimilation ($P_{\text{max}}$) for *P. cinereus* from 11 sites across their geographic range. $P_{\text{max}}$ was positively related to mean environmental temperature (left) but did not significantly differ among clades (right). Line colors represent clade (dark blue = Northern, light blue = Pennsylvanian, and green = Southern). The gray shading around the line represents 95% CIs for predictions from a linear model. The right panel shows mean $P_{\text{max}}$ with standard error bars.
Fig. A3.4. Relationship between thermal optimum for performance ($T_{opt}$) and maximum performance for energy assimilation ($P_{max}$) for 3 clades of *P. cinereus* from 11 sites across their geographic range. Line colors represent clade (dark blue = Northern, light blue = Pennsylvanian, and green = Southern). The gray shading around the line represents 95% CIs for predictions from linear models.
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local adaptation on forecasts of species range shifts under climate change.


