DISSERTATION

LINKING ORGANISMAL PHYSIOLOGY AND THE LANDSCAPE TO PREDICT

VULNERABILITY TO CLIMATE CHANGE

Submitted by

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Graduate Degree Program in Ecology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2023

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ABSTRACT

LINKING ORGANISMAL PHYSIOLOGY AND THE LANDSCAPE TO PREDICT
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Global temperatures continue to increase at unprecedented rates, both in mean and in variance. Thus, a major challenge for scientists of the 21st century is to predict whether species will persist through these changes. One way to partly assess vulnerability to climate change is to investigate the relationships between the environment and traits that are either particularly sensitive to temperature or may confer resilience against thermal changes. In ectotherms, external temperatures dictate their physiology, thus thermal physiological traits may be key to understanding ectothermic persistence. Although population variation is integral to the evolvability of thermal physiological traits, most studies using these traits to infer vulnerability extrapolate data from one or few populations to represent the species. Furthermore, many studies also use coarse metrics of environmental temperatures which may not fully capture the variation experienced by the organism. Here, using a cold-water frog system, I demonstrate the relationships between thermal physiological traits and local environmental temperatures among populations. In my first chapter, I provide a brief overview of ectothermic physiology, environmental thermal landscapes, and the ecology of the two species of tailed frogs that I investigated. In my second chapter, I show that populations of tailed frogs vary in their critical thermal limit (CTmax) plasticity, which impacts species-level assessments of vulnerability. I also demonstrate the methodological impacts of ignoring acute responses to temperature when estimating plasticity in this trait. For my third chapter, I demonstrate relationships between CTmax and local thermal environments, including temporal and spatial variability in

temperature, among populations of tailed frogs. These results show that tailed frogs have limited opportunity for behavioural avoidance of warm temperatures, and that populations of one tailed frog species show a positive relationship between CTmax and maximum stream temperature while populations of the other species does not. In my fourth chapter, I test the critical assumption that CTmax is related to fitness, specifically mortality in ecologically relevant temperatures. My results show that populations with higher estimates of CTmax experience less mortality from thermal stress in temperatures experienced in nature, demonstrating the link between CTmax and fitness. Lastly, in my fifth chapter, I return to the plasticity in CTmax results and demonstrate the relationship between this trait and local thermal environments, showing that populations experiencing greater temperature fluctuations have greater estimates of plasticity in CTmax.

Overall, these results underscore the importance of sampling widely among populations when inferring vulnerability to climate changes from physiological traits. The population variation in CTmax and its plasticity that I uncovered demonstrate the differing trends in vulnerability to climate change for the two species investigated. This work also highlights the importance of quantifying local thermalscapes and highlight how similar environments can differentially shape physiological tolerance and patterns of vulnerability among populations, in turn impacting vulnerability to future warming.

ACKNOWLEDGEMENTS

I would like to thank my committee: Cameron Ghalambor, Kim Hoke, Yoichiro Kanno, and Erin Landguth for all their support and efforts throughout my degree. When I was putting together my committee, I felt like I was creating my own team of superheroes. Now I know I was. I have learned so much from each of you and am a better scientist because of it. Thank you all for your feedback, support, mentorship, and magic. Thank you for challenging me pushing me to think outside the box. I look forward to continuing to work with you and am so fortunate to have been given the chance to learn from you.

I would like to express my deepest gratitude to my incredible advisor, Chris Funk. Thank you for providing me with the opportunity to join your lab and embark on this research. I greatly appreciate all the support you've provided me, including feedback and encouragement on my science as well as the support beyond the walls of Biology. For example, thank you for joining me in Oregon and Montana to collect data (and leading the OR spatial temperature collection), for teaching me how to change a truck tire just in case, for bringing me snacks and well-wishes when **** hit the fan. Thank you for answering my stressed phone calls when something went wrong (and for helping me fix it) and my excited phone calls when something went right! Thank you for sharing your knowledge and enthusiasm for science with me, for nerding out about stats and results, for brainstorming future groundbreaking work. Thank you for challenging me and pushing me out of my comfort zone, for providing me a safe space to fail, and reminding me [sometimes too often] that I belong here. Thank you for being a dedicated mentor and a positive role model. Thank you for believing in me.

My PhD would not have been the same without the many coffee breaks, late night Thai food visits, and definitely only a few Trailhead outings with my wonderful friends. I have so many cherished memories with you all and cannot wait to see what you all achieve!

Beyond my [phenomenal] genes, my family has provided me with much love and support that have been critical to my journey. I could not have done this without you all. Many special thanks to my cousins, Maxime and Maya, and to my Tata (Aunt) Christine, and Uncle Rahul for listening to me talk *ad nauseum* about frogs and supporting me throughout my degree (even visiting me from England!). Tata, thank you for organizing the many family zooms that provided me with a platform for frog pontification. A huge thank you to Barry – I am so lucky to have you as part of my family and am so grateful for your support and guidance. Thank you for the many snacks, chocolate bars, trips to the airport, and stellar advice. My siblings, Michelle and Alex, provide me with constant inspiration and encouragement. It is never a dull moment when the three of us are together. You are both incredibly smart (geniuses, really), generous, fun people. Thank you for always picking up the phone, helping me with math, splitting the last piece of pie, and bringing home almond croissants. So many thanks and truly endless love to my grandparents, my Papy and Nanou. Thank you for teaching me how to tell stories, how to love and cook food, how to leap confidently into adventures in foreign countries, and how to find art and beauty in life. You two inspire me to fearlessly chase my dreams and remind me to take the time to smell the flowers and watch the birds along the way. Finally, I would like to thank my Mom. You are my fiercest supporter and my biggest role model. Thank you for teaching me my love of learning, for encouraging me to be curious about nature and letting me keep tadpoles and salamanders as a child, for picking me up whenever I fall down (figuratively and literally, I am very clumsy), for the countless phone calls, for flying me home when I miss being cold, and for modeling the kind of person I want to be. One day, when I grow up, I hope to be the strong, brave, brilliant person that you are. Thank you.

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CHAPTER 1: INTRODUCTION

Introduction

Climate Variation and Trait Responses

Environmental variation plays a large role in shaping biodiversity (Sunday et al., 2011). Indeed, many examples of trait variation within and between species can be partly explained by environmental variation (Coyne and Orr, 2004; Sultan 2015). When faced with changes to their environments, populations may respond *in situ* through evolution, plasticity, or a combination of both (Chevin & Lande, 2010; Snell-Rood et al., 2018). Evolved responses require adequate standing genetic variation for environmental selection to act upon and may take multiple generations to realize. Conversely, plastic responses occur within a single generation (Snell-Rood et al., 2018), allowing for trait changes in response to environmental cues on a more rapid timescale. Thus, plasticity is often considered the first line of defense for organisms facing rapid changes in their environment. Trait plasticity may have genetic underpinnings and could be considered as adaptations if they lead to increased fitness (Gottard and Nylin,1995). However, plastic responses may not always be beneficial. They can be maladaptive, moving a phenotype further from their fitness optimum (Ghalambor et al., 2007; Merilä & Hendry, 2014) and/or may reduce the speed at which evolved selection occurs by shifting the phenotypic distribution closer to the optimum (Huey et al., 2003; Ghalambor et al., 2015). These two major mechanisms (evolution and plasticity) linking trait variation and environmental variation can explain contemporary patterns of variation but are also key to predicting future responses to environmental change.

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Environments are changing at unprecedented rates due to climate change. The consequences of these changes are already being realized through declines in species' abundances and local extirpations (Butchart et al., 2010; Bellard et al., 2012). As such, predicting species' responses to climate change is increasingly important. Doing so requires the investigation into the potential evolved and plastic responses in traits that may confer resilience against these changes (Dawson et al., 2011; Nicotra et al., 2015; Beever et al., 2016; Thurman et al., 2020). As climate change is causing increases in temperature means and (Jackson et al., 2009), traits associated with temperature, such as thermal physiological traits, are potentially key resilience traits.

Ectothermic Thermal Physiology

Ectotherms are particularly sensitive to environmental change as their physiology is dependent on the external thermal environment. External temperatures dictate physiological performance of ectotherms through thermodynamic and biochemical relationships (Gillooly et al., 2001; Angilletta, 2009). The overall relationship between temperature and physiology is often left-skewed, such that performance increases more slowly toward the optimum temperature (where performance is maximized) than the rate at which it decreases after peaking – demonstrated by thermal performance curves (Figure 1; Huey and Stevenson 1979). Thermal limits bound physiological performance such that function ceases when temperature falls below the lower thermal limit or exceeds the upper thermal limit. Multiple components of this curve can respond to environmental variation through plasticity (i.e., acclimation) or evolution, leading to changes in the width and/or amplitude of the curve (Huey, 1982; Angilletta et al., 2003). As such, critical limits (Sunday et al., 2011; Shah et al., 2017), thermal optima (Casteñeda et al., 2004; Logan et al., 2014; Gilbert and Miles, 2017), and thermal breadth (Sunday et al., 2011;

Lancaster et al., 2015; Shah et al., 2017; Rohr et al., 2018) have all been demonstrated to vary with environmental temperatures.

The Thermal Landscape

Different components of the temporal dimension of thermal variation can influence thermal physiological traits. For example, extreme temperatures (e.g., the warmest temperature experienced) have been shown to be related to thermal limits (e.g., Sunday et al., 2019) while thermal variability (e.g., seasonality) has been documented to explain variation in thermal breadths (width of the thermal performance curve) (e.g., Shah et al., 2017). Thermal temporal variability indeed affects many aspects of population performance and growth (Bernhardt et al., 2018, 2020; Slein et al., 2023) and is expected to select for traits conferring wider thermal performance, through increased thermal breadth and/or plasticity (the "climate variability hypothesis, CVH) (Janzen, 1967; Ghalambor et al., 2006; Sheldon et al., 2018). However, short-term temperature variability, such as diurnal fluctuations, may mask the effects of longer-term variability like seasonality (Temple & Johnston, 1998; Padilla et al., 2019). Therefore, consideration of the temporal thermal dimension across multiple scales is important for uncovering its role in shaping thermal physiological traits.

Local environmental temperatures can also vary along the spatial dimension at the scale of the organism, driven by variation in microhabitat features (Sears et al., 2011; Woods et al., 2015; Pincebourde et al., 2016; Garcia et al., 2019; Neel et al., 2021). Fine-scale spatial temperature variation provides organisms the opportunity to behaviorally thermoregulate, which in turn can shield them from temporal extremes in environmental temperatures (Bogert, 1949; Huey et al., 2003; Beever et al., 2017; Muñoz & Losos, 2018; Bodensteiner et al., 2021; Muñoz, 2021). However, the efficacy of behavioral thermoregulation is dependent not just on the

presence of spatial thermal variability, but on the accessibility of it. The bioenergetics of the organism and the configuration of spatial temperatures dictate the benefits of behavioral thermoregulation (Row & Blouin-Demers, 2006; Sears & Angilletta, 2015; Sears et al., 2016). Thus, consideration of the range of spatial temperatures available to the organism, its distribution within a habitat, and the ability for an organism to access it are key to adequately assessing the role of spatial thermal variability in shaping thermal physiological traits.

Physiological Vulnerability to Climate Change

The consequences of increasing temperatures and temperature fluctuations on ectotherms is predicted to be far-reaching (Huey & Kingsolver, 2019; Litchman & Thomas, 2022). However, relationships between thermal physiological traits and the environment provide the opportunity to estimate a metric of physiological vulnerability (i.e., vulnerability to warming temperatures due to their effects on physiological traits) (e.g., Deutsch et al., 2008; Huey et al., 2009; Sunday et al., 2011, 2014; Morley et al., 2019; Pinsky et al., 2019). By incorporating current and projected environmental temperatures, scientists are able to determine approximately when in the future species will experience the consequences associated with temperatures exceeding preferred or critical maximum temperatures (Deutsch et al., 2008). Plastic responses can also be incorporated into these assessments as added temporal buffers (Seebacher et al., 2012; Miller et al., 2013; Morley et al., 2019), decreasing physiological vulnerability with increased magnitude of plastic responses. Broad, macrophysiological studies have uncovered patterns in relative physiological vulnerability among species and taxonomic groups, highlighting both freshwater and terrestrial ectotherms as at risk from warming (Sunday et al., 2014, 2019; Gunderson & Stillman, 2015; Comte & Olden, 2017; Rohr et al., 2018; Morley et al., 2019; Pinsky et al., 2019).

Despite large advances in understanding physiological vulnerabilities across the landscape, there remain a few major gaps that limit our ability to assess vulnerability. First, most studies examining the environmental drivers of thermal physiology use coarse, regional temperatures from thermal regime data. These studies may fail to capture relevant temporal variability at the scale of the organism (Bernhardt et al., 2018; Garcia et al., 2019). Furthermore, these studies also rarely incorporate the complete thermal landscape, investigating both temporal and spatial metrics of local thermal variability within a study, despite the known interacting effects they have in shaping thermal physiology (Sunday et al., 2014; Muñoz & Bodensteiner, 2018; Bodensteiner et al., 2021). Second, many studies assume the rigidity of thermal performance curves among populations (e.g., evolutionarily inert, invariable), due to genetic or physiological constraints (Addo-Bediako et al., 2000; Huey et al., 2012; Araújo et al., 2013; Grigg & Buckley, 2013; Muñoz et al., 2014; Gangloff & Telemeco, 2018; Rohr et al., 2018). This rigidity extends to acute responses to temperature, which reflect passive, biochemical reactions that ultimately shape the thermal performance curve. Although often overlooked, these acute responses may vary among populations and may respond to environmental variation (Hochachka & Somero, 2002; Somero, 2004; Schulte et al., 2011; Schulte, 2015). Lastly, many studies of physiological vulnerability investigate one or few populations and extrapolate vulnerability to the entire species. However, population variation in physiological traits and the potential for evolution in these traits, including thermal limits, have been widely observed (Geerts et al., 2014; Cuenca Cambronero et al., 2018; Rolandi et al., 2018; Herrando-Pérez et al., 2019; Nati et al., 2021; Morgan et al., 2022). Excluding population-level variation may thus bias estimate of physiological vulnerability for a species. Therefore, my overarching goal is to fill this

gap in our understanding by testing the extent to which local thermal variability shapes physiological traits and ultimately vulnerability both between and within species.

Study System

I investigated these relationships within an ecologically-unique frog family: Ascaphidae. This family consists of the species *Ascaphus montanus* and *A. truei* and represents the sister clade to the common ancestor of all extant frog species (Ford & Cannatella, 1993; Pyron & Wiens, 2011; Zhang et al., 2013). *A. montanus* and *A. truei* occupy cold, fast-flowing streams in forested landscapes of the Klamath Mountains, Coast Ranges, and Cascade Mountains (*Ascaphus truei*), and northern Rocky Mountains (*A. montanus)* of the United States and Canada (Figure 2). Their distribution along elevation gradients causes naturally occurring thermal gradients, which are ideal for testing hypotheses related to physiology and environment.

Similar to many frog species in the USA and Canada, tailed frogs metamorphose from aquatic tadpoles to semi-terrestrial adults, exposing them both aquatic and terrestrial environments throughout their lifetime (Duellman & Trueb, 1986). However, unlike these other frog species, the tadpole stages of tailed frogs can take up to five years of development before metamorphosing into juveniles (Gaige, 1920; Brown, 1975). Thus, the larval stages of these species must cope with aquatic thermal variability for at least one entire annual cycle. As tadpoles, these species graze on the epilithon of benthic stream rocks using their suctorial mouths and play a critical role in ecosystem function, accounting for a significant portion of algal biomass consumption in these streams (Mallory & Richardson, 2005).

Tailed frogs may be particularly sensitive to changes in their environments. These species have low estimates of thermal tolerance within these species (Brown, 1975; Bury, 2008) and

high rates of desiccation (Claussen, 1973). They have also been shown to be sensitive to habitat disturbance (Wahbe & Bunnell, 2001, 2003; Hossack & Honeycutt, 2017), though may have the capacity to recolonize extremely disturbed habitats (Spear & Storfer, 2010; Spear et al., 2012). Critically, tailed frogs are already declining due to warming stream temperatures and environmental disturbances (Hossack et al., 2023). Investigating this system offers a unique opportunity to uncover trends in a cold-water frog system that holds an important evolutionary, ecological, and conservation position.

Conceptual Framework

I developed a conceptual framework through which to test the various relationships shaping how environmental variation affects vulnerability through physiological traits (Figure 3). This framework incorporates both the temporal and spatial dimensions of thermal variability that make up the thermal landscape. The thermal landscape in turn shapes variation in thermal physiological traits through three major mechanisms: evolution, plasticity, and acute responses. Ultimately, variation in physiological traits influences vulnerability to climate change.

Here, I present the results of multiple studies investigating various components of this framework to ultimately understand population physiological vulnerability. I focus on critical thermal maximum (CTmax), which estimates warm temperature tolerance (Figure 1). In Chapter 2, I test relationships connecting the thermal landscape to variation in evolved CTmax estimates. In Chapter 3, I test the relationships between plasticity and acute responses to temperature in CTmax, and how those responses shape variation in CTmax and relative population vulnerability. In Chapter 4, I investigate the thermal landscape conditions that drive the variation in plasticity in CTmax that I observed in Chapter 3. In Chapter 5, I test the critical assumption that CTmax is related to vulnerability by linking variation in CTmax among populations to

mortality from thermal stress. I conclude with a final chapter testing the physiological outcome when the top node of the framework, "environmental variation", is completely changed.

Tables & Figures
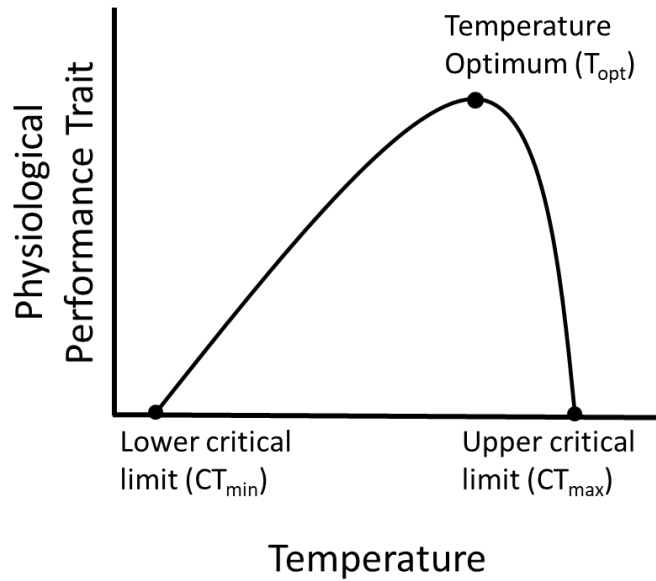
Figure 1. Stylized thermal performance curve demonstrating an increase in physiological performance from a lower thermal limit, a peak in performance at an optimal temperature, and a rapid decrease in performance until the upper thermal limit is reached.
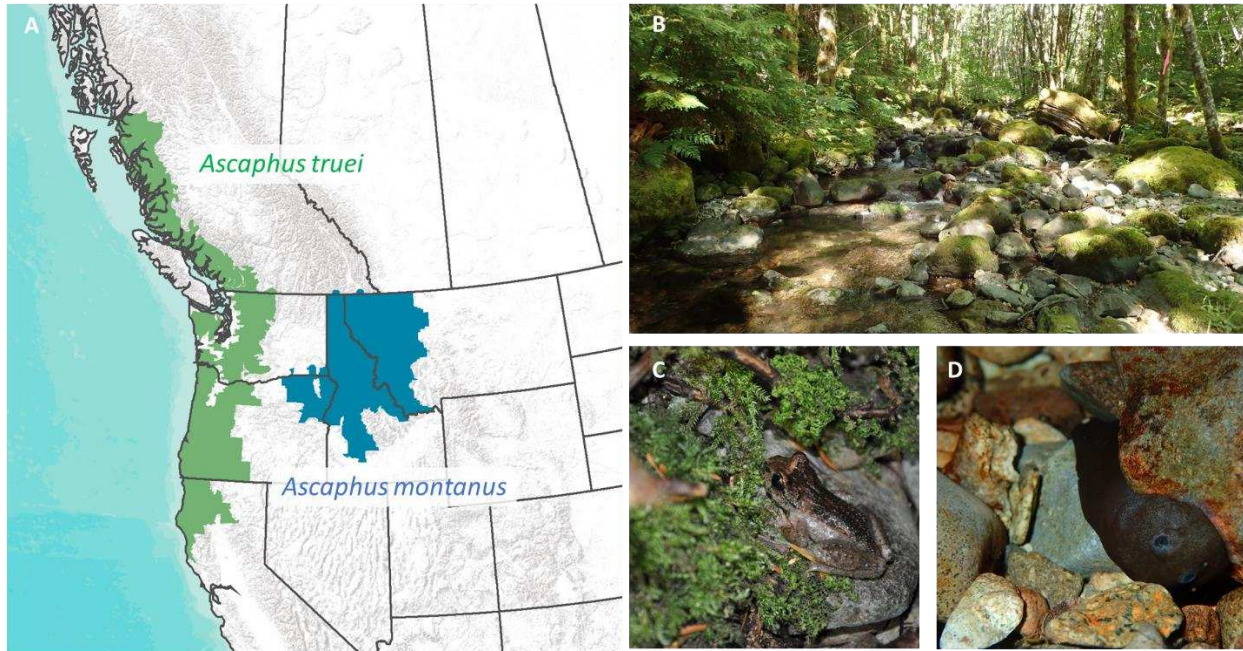
Figure 2. A: Distribution of *Ascaphus montanus* (blue) and *A. truei* (green). B-D: photos from the same stream in Oregon, demonstrating the habitat (B), the adult frog (C), and tadpole (D).
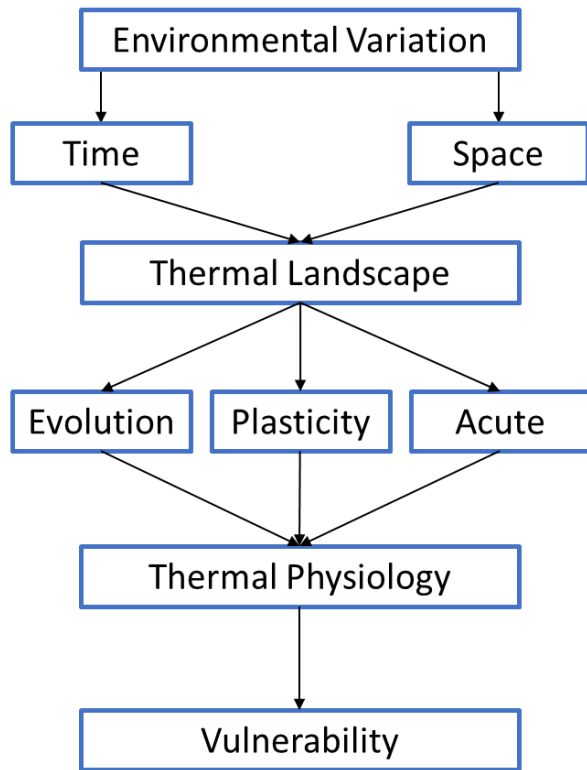
Figure 3. My conceptual framework linking environmental variation and vulnerability to climate change through thermal physiological traits. According to this framework, the thermal landscape is shaped by both temporal and spatial dimensions of temperature variability. The thermal landscape in turn shapes thermal physiological traits through evolution, plasticity, and/or acute physiological responses. These mechanisms dictate variation in thermal physiological traits, which ultimate shape vulnerability to warming temperatures.

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CHAPTER 2: ACCLIMATION CAPACITY OF CRITICAL THERMAL MAXIMUM VARIES AMONG POPULATIONS: CONSEQUENCES FOR ESTIMATES OF VULNERABILITY

Introduction

For ectothermic organisms whose performance is tightly linked to environmental temperatures (Gillooly et al. 2001), limits of physiological tolerance to temperatures (i.e., thermal tolerance) are frequently used to estimate proxies for vulnerability to warming (e.g., Addo-Bediako et al., 2000; Calosi et al., 2008; Sunday et al., 2014; Gunderson & Stillman, 2015; Pinsky et al., 2019). Although these proxies have their short-comings (Clusella-Trullas et al. 2021; Garcia, Allen, and Clusella-Trullas 2019), they continue to be valuable in a comparative framework and contribute to the understanding of relative population/species potential vulnerability to future warming (e.g., Deutsch et al., 2008; Sunday et al., 2014). For example, macrophysiological studies of ectotherms have revealed substantial geographic variation in risk to future warming by relating thermal tolerance to the magnitude of expected temperature change (Deutsch et al. 2008; Clusella-Trullas, Blackburn, and Chown 2011; Sunday et al. 2014; Kingsolver, Diamond, and Buckley 2013). However, such measures of thermal physiological vulnerability to warming (hereafter, simply referred to as "vulnerability") are often based on several underlying assumptions and limitations (e.g., accuracy of experimental design, extrapolation from few populations), which have led to questions about the accuracy of the predictions (Sinclair et al., 2016; Clusella-Trullas et al., 2021)

One question that has received considerable interest is the degree to which plasticity in thermal tolerance may ameliorate the consequences of global warming (Stillman 2003; Calosi,

Bilton, and Spicer 2008; Huey et al. 2012; Seebacher, White, and Franklin 2015). Plastic responses to temperature changes (i.e., thermal acclimation) can alter thermal tolerance traits, including critical thermal maxima (CTmax) – the highest temperature an organism can withstand before loss of function (Angilletta 2009). However, it is unclear whether the magnitude of change in tolerance, referred to as acclimation capacity, can suffice to provide a long-term coping mechanism to warming temperatures for most ectotherms (Gunderson and Stillman 2015; Gunderson, Dillon, and Stillman 2017; Morley et al. 2019; Seebacher, White, and Franklin 2015). For example, Gunderson et al. (2017) found that plasticity in CTmax (i.e., thermal acclimation of CTmax) reduced the number of predicted overheating days, but this benefit was variable among taxonomic groups of the 103 ectothermic species investigated. Furthermore, the magnitude of plasticity (i.e., acclimation capacity) was generally an insufficient buffer against long-term warming (Gunderson, Dillon, and Stillman 2017). By contrast, other analyses suggest that acclimation can provide a significant buffer to future warming by increasing CTmax (e.g., Rohr et al., 2018) and/or reducing the sensitivity of physiological rates to temperature (e.g., Seebacher et al. 2015). Given variability in these findings, additional work is needed to understand the capacity for acclimation to buffer organisms against future warming.

A second concern revolves around the experimental approaches used to determine thermal tolerance as they can also influence trait responses (Bates and Morley 2020; Terblanche et al. 2007; Overgaard, Kristensen, and Sørensen 2012) and such methodological issues are particularly important to measures of how much plasticity these traits exhibit (Havird et al. 2020). To estimate acclimation capacity, many experiments require transferring organisms to an experimental starting temperature that is warmer or cooler than the temperature at which they were held (Terblanche and Hoffmann 2020). This rapid exposure to a new temperature may

impact estimates of trait responses due to temperature effects on physiological traits that are not being explicitly quantified. Physiological responses to temperature occur both passively and actively (Havird et al. 2020). Passive responses to temperature can represent acute phenotypic changes that are products of molecular thermodynamic relationships (i.e., Arrhenius-like or Q10 effects; Arrhenius, 1915) and are not regulated by the organism (Kingsolver 2009; Schulte, Healy, and Fangue 2011). Acute responses typically occur quickly (e.g., enzymatic reactions, Schulte et al., 2011). By contrast, active responses to temperature represent plastic/acclimation responses that are due to active regulation by the organism (e.g., changes in membrane lipid permeability, up- or down-regulation of heatshock proteins, Sinclair and Roberts, 2005; Angilletta, 2009) and occur over longer timescales. How much acute responses alter estimates of thermal plasticity has been debated, but new methods that account for acute effects when calculating acclimation responses allow for comparisons of different approaches (Einum et al. 2019). Distinguishing between passive and active (hereafter, acute and acclimation, respectively) responses to thermal exposure may be important for estimating the buffering capacity of thermal tolerance plasticity (Havird et al. 2020).

A third question about the use of thermal tolerance traits in estimating vulnerability that has received less attention, is how representative estimates from a single population or locality are for an entire species (Sears, Raskin, and Angilletta 2011; Cochrane et al. 2015; Herrando-Pérez et al. 2019; Valladares et al. 2014). Many of the existing estimates of acclimation capacity are made from a single or few population(s). For example, in the dataset compiled in Morley et al., (2019), of the 319 species investigated, 282 of the estimates came from a single population, whereas only 37 estimates were from two or more populations. Yet, like any trait, thermal tolerance is expected to vary intraspecifically (Huey and Kingsolver 1989; Feder, Bennett, and

Huey 2000; Galván, Schwartz, and Garland 2022; Duffy et al. 2021) and populations are known to vary in their acclimation capacity (Seebacher et al. 2012; Peck et al. 2014; Tonione et al. 2020; Gervais et al. 2021; Barley et al. 2021). Thus, we would expect acute and acclimation responses in traits such as critical thermal limits (e.g. CTmax) to vary within and among populations (Cossins and Bowler 1987; Somero 2004; Bubliy et al. 2002) The inclusion of intraspecific variation in vulnerability proxies typically increases the predictive power of models (Herrando-Pérez et al. 2019; Barley et al. 2021) and can identify populations with increased risk to future warming. Yet, few attempts have been made to use standardized methods to document how much standing variation exists among populations in either their critical thermal limits or their acclimation capacity and how such variation might alter predictions about future vulnerability.

The aims of this study were to quantify and test the impacts of population variation in acclimation capacity of CTmax on estimates of vulnerability for two species of tailed frogs (family: Ascaphidae). We chose to investigate CTmax as it occurs in the stressful temperature range of an organism, when heat stress accumulates and normal functions (e.g., development, growth) are halted (Ørsted, Jørgensen, and Overgaard 2022). Therefore, acclimation capacity of CTmax may be critical for organisms to reduce the consequences of heat stress in warming environments.

To quantify variation within and between species, we measured CTmax of tadpoles from 14 populations of *Ascaphus montanus* and *A. truei* using a fully factorial design of two holding temperatures and two experimental starting temperatures. We hypothesized that CTmax estimates would be influenced by both acclimation and acute effects of temperature. Here, acute effects are elicited by starting temperature transfers, while acclimation effects are related to holding temperatures. We expected holding temperature to be positively related to CTmax,

demonstrating beneficial acclimation (Angilletta 2009; Gunderson et al., 2017). If acute

responses are primarily dictated by the effects of temperature on biochemical rates, then we

predicted that the direction of the temperature transfer would dictate the effect on CTmax (i.e.,

transfer to a cold temperature would decrease CTmax; transfer to a warm temperature would

increase CTmax). Alternatively, if a rapid exposure to a warmer or colder temperature elicits an

acute stress response (e.g., rapid enzymatic denaturation), then we expected CTmax to decrease

regardless of the direction of temperature transfer. We calculated acclimation capacity using two

methods (Figure 4) to test the consequences of ignoring acute effects of temperature for these

estimates. Lastly, we inferred a proxy for population-level vulnerability using estimates of

acclimation capacity and local temperature projections, expecting population variation and

experimental approaches to significantly influence overall conclusions for the species.

Methods

Study System & Collection

Ascaphus truei and A. montanus occupy cold, fast-flowing streams in forested landscapes

of the Klamath Mountains, Coast Ranges, and Cascade Mountains (*Ascaphus truei*), and

northern Rocky Mountains (*A. montanus*) of the United States and Canada. The larval stages of

these species may take 1—4 years to metamorphose into juvenile frogs (Hayes and Quinn,

2015), during which time they are exposed to daily, seasonal, and annual temperature

fluctuations in streams., which may vary among streams but remain consistent over time within a

stream (Arismendi et al. 2013; Maheu, Poff, and St-Hilaire 2016). These species have relatively

low thermal tolerances (Bury 2008), making them particularly sensitive to increasing

temperatures. The tadpoles of these species play a critical role in stream ecosystems by

consuming benthic algae (Mallory and Richardson 2005), therefore, variation among populations in thermal tolerance or acclimation capacity might contribute to both species and ecosystem resilience.

We collected tadpoles (developmental stages 26.5 to 45; Gosner, 1960) from populations in Oregon (*A. truei*) and Montana (*A. montanus*) in 2017 (July to August) and 2018 (June to July), targeting populations from varying elevations to capture the range of thermal variation experienced by the species. For *A. truei*, we sampled two populations from the Clackamas River basin (Cripple and Shellrock Creeks), two populations from the North Umpqua River basin (Bulldog and Steelhead Creeks), and six populations from the McKenzie River basin (Augusta, Flunky, Lamb, Upper and Lower Lookout and Ore Creeks) for ten populations in total (Figure 4). For *A. montanus*, we sampled two populations from the Clark Fork River basin and from the Lost Horse Creek River basin (four total populations) (Figure 5). We collected approximately 48 tadpoles per population (Appendix 1: Table S1) by placing aquarium nets downstream from overturned rocks, such that the tadpoles would flow into the nets when disturbed. Tadpoles were transported to lab facilities (*A. truei*: H.J. Andrews Experimental Forest; *A. montanus*: Fort Missoula, University of Montana) using the protocol described in Essner et al. (2012).

Acclimation & CTmax Experiments

Tadpole collection, holding, and CTmax experiments were performed one population at a time, when possible given sampling (SI Table 1). For each population, ~24 tadpoles were randomly assigned to one of two holding temperature treatments: 8°C or 15°C for three days. Tadpoles were held in 142L coolers, with only one population in a cooler at a time. We held tadpoles for three days in their temperature treatments to minimize the effects of feeding on CTmax, without inducing lethargy or starvation. We used water collected from their natal

streams and maintained temperatures using a recirculating water chilling unit. High oxygenation

was maintained in each holding tank using standard aquarium air pumps and bubblers. From

each holding temperature treatment, half of the tadpoles (N = 12) were then randomly transferred

to and tested at a starting temperature of 8°C or 15°C, enabling estimates of acute responses to

temperature. We chose 8°C as it is a commonly-experienced *Ascaphus* habitat stream

temperature in the summer months (during our field collections), and 15°C because it is close to

the upper limit of experienced stream temperatures.

CTmax experiments were performed via temperature ramping (Overgaard, Kristensen,

and Sørensen 2012). We placed individual tadpoles into mesh containers that were immersed in a

water bath. As tailed frog tadpoles are typically attached to rocky substrate, we added a small

stone to each container. We maintained approximately 80% oxygen saturation in the water with

air pumps to avoid compounding the effect of temperature with hypoxia in the tadpoles. After

allowing the tadpoles to become familiar with the chambers for two minutes, water temperature

was ramped at a rate of 0.3°C min^{-1} using a temperature controller attached to a titanium heating

rod (500W). Tadpoles were not encouraged to move and were relatively at rest during

experiments. We considered CTmax to be the point at which a tadpole no longer responded to

tactile stimulus with muscular movement (Peck et al. 2009). Because *Ascaphus* tadpoles can

often remain latched onto rocks post-mortem (Gradwell 1971), we removed the rocks from the

mesh containers once tadpoles seemed to respond more slowly to tactile stimulus. Once a tadpole

reached CTmax, it was placed in a tank with cool water (~8°C) to recover. Tadpoles were

considered recovered when they responded to a tactile stimulus and were able to swim. We only

analyzed data from tadpoles that recovered after the experiment, and tested each individual once

to avoid potential cumulative effects from multiple experiments.

Following experiments, we euthanized recovered tadpoles (as requested by permitting agencies and for use in other studies) using a 20% benzocaine solution and photographed them laterally beside a ruler for length measurements. We fixed each individual in 10% formalin for at least one day before transferring them to vials containing 70% ethanol. Tadpole length measurements were made using ImageJ software (Rasband, 2009) on the photographs, measuring each tadpole twice from the tip of the snout to the tip of the tail, and then averaging the two measurements. We used tadpole length as a covariate in our models rather than developmental stage as the two measurements were highly correlated (Pearson's correlation coefficient 0.81, p<0.0001) and to account for potential size effects on CTmax (Lindmark et al. 2018; Brown et al. 2004; Angilletta, Steury, and Sears 2004; Peralta-Maraver and Rezende 2021) regardless of developmental rates, which may vary along elevation gradients (Riha and Berven 1991; Arendt 1997; Conover and Present 1990).

Assessing Temperature Effects & Acclimation Capacity

We tested for the effects of holding temperature (i.e., acclimated effects) and starting temperature (i.e., acute effects) on CTmax using mixed effects models. With CTmax as our response variable, we first tested a model with holding temperature, starting temperature, and species as predictors, length as a covariate, and population as a random intercept. We included a three-way interaction of holding temperature, starting temperature, and species to test for the interdependence of these predictors. Because the three-way interaction was significant (Appendix 1: Table S2), suggesting that holding temperature effects and starting temperature effects were dependent on species, we subsequently used species-specific models. For each species, the model included CTmax as the response variable, holding temperature, starting temperature, and population as fixed effects with a three-way interaction. The three-way

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interaction term allowed us to test whether the effects of holding temperature on the relationships between CTmax and starting temperature were dependent on population (and vice versa). We accounted for body size by including length as a covariate. Using these models, we calculated the estimated marginal means, hereafter referred to as estimated marginal CTmax, for each population and treatment combination, which were finally used for the calculation of acclimation capacity below. All analyses were performed in R version 3.6.1 (R Core Team 2019); data plots were made using the ggplot2 (Wickham, 2016) package in R. Statistical significance was evaluated using $\alpha=0.05$ for all analyses.

We calculated acclimation capacity using two approaches (Figure 4), both of which using population as our sampling unit. First, we used the Acclimation Response Ratio (ARR; Claussen, 1977), which does not account for acute effects of temperature (Figure 4i). The ARR is calculated as the slope of the line describing the trait response when held at two temperature treatments and tested at those temperatures. We also calculated acclimation capacity of CTmax using an approach that accounts for the acute effects of temperature, described by Einum et al. (2019; Figure 4ii). This approach subtracts the average of the acute effects slopes (start temperature effects) for each holding temperature treatment from the ARR. Acclimation capacity is then estimated as the absolute value of this difference. We used a paired t-test to investigate differences in acclimation capacity due to the approach used to estimate it.

Assessing Vulnerability to Climate Change

To characterize the current variation in stream temperatures, we deployed two temperature data loggers (Hobo Water Temperature Pro v2 and 64K Pendant Water Temperature Data Loggers, Onset Computer Corporation, Bourne, MA USA) within the sampled stream reach and logged temperatures every 4 hours for a mean period of 12 months (Appendix 1: Table S1).

Temperature loggers were housed PVC tubes with holes drilled to allow for water flow, and secured to an in-stream metal rebar pole such that the bottom of the PVC pipe was slightly above the stream substrate. Preference for location of the rebar was a pool with flow to increase the chances that the logger remained in water all year. The PVC housing was also secured to a nearby tree using a steel wire cord. We screened the temperature logger time series data for errors by visually inspecting time series plots and standard deviation time series plots (Dunham et al. 2005).

To test if acclimation capacity could buffer to projected warming, we calculated buffering capacity (our proxy for vulnerability), adapted from the approach used in Morley et al. (2019), for each population and estimation approach (Figure 6). We used our *in-situ* temperature logger data to calculate the average of the ten warmest recorded temperatures to represent current maximum environmental temperature (stream temperature intercept), and used NorWeST temperature projections (Isaak et al. 2016) to estimate the rate of temperature increase predicted for each population (stream temperature slope). We used CTmax averages for each population (held and tested at 8°C) as our current CTmax estimate (intercept) and modeled CTmax changes through time based one (1) no acclimation capacity, (2) acclimation capacity estimated without accounting for acute effects, and (3) acclimation capacity estimated by accounting for acute effects. The time (in years) when modeled CTmax estimates intersected with the stream temperature estimates were used to estimate the number of years that each acclimation scenario (scenarios 2 and 3) added to baseline CTmax intersection estimates (scenario 1). These differences, representing an added temporal buffer to warming temperature via plasticity, are subsequently referred as buffering capacity (measured in years). We performed a paired t-test to

test whether the approach for estimating acclimation capacity yielded differences in buffering capacity estimates.

Results

We sampled 665 individuals from 14 populations across the two species' ranges. From these, one individual died during acclimation and nine individuals did not recover from the CTmax acclimation experiments (presumably from surpassing their CTmax) and were removed from analyses. We also removed four individuals for which we were missing length measurements. Therefore, the data presented represent 651 individuals from four *A. montanus* populations and ten *A. truei* populations (see SI Table 1 for treatment sample sizes).

Acclimation & Acute Effects

Within both species investigated, the effects of holding temperature (acclimated effect) and starting temperature (acute effect) were dependent on each other and the population sampled (i.e., significant three-way interaction; *A. montanus* p= 0.067, *A. truei* p<0.001; Table 1). Thus, for both species, the magnitude of CTmax responses to starting and holding temperature varied among populations. We also found a significant main effect of population (p<0.001) and holding temperature (p=0.008) in *A. montanus*, and a significant main effect of population (p<0.001) and starting temperature (p=0.042) in *A. truei.*

We found that being held in the warm temperature treatment (15°C) generally resulted in an increase in CTmax regardless of starting temperature (Figure 7A). Within the cold-start temperature treatments (8°C), only three populations experienced a decrease in CTmax when held in the warm temperature (two *A. montanus*, one *A. truei*). Within the warm-start treatments,

only one *A. truei* population experienced a decrease in CTmax when held in the warm temperature.

Broadly, the effects of a transfer to a warm experimental temperature from a cold holding temperature (T_1-held acute effect) caused a decrease in CTmax in all four *A. montanus* populations and five *A. truei* populations – the other five *A. truei* populations exhibited an increase in CTmax (Figure 7B). When held in a warm temperature and transferred to a colder temperature (T_2-held acute effect), CTmax was lower at the cold starting temperature for two *A. montanus* populations and six *A. truei* populations. The other two *A. montanus* populations and four *A. truei* populations exhibited higher CTmax values when tested at 8°C (Figure 7B).

Acclimation Capacity & Vulnerability

We found evidence for acclimation capacity in CTmax (i.e., magnitude greater than 0) in populations of both *Ascaphus* species, regardless of the consideration of acute effects. When ignoring acute effects, estimates of acclimation capacity showed a positive effect of acclimation (i.e., higher CTmax after being held in a warmer temperature) in all but two populations. However, when including acute effects of temperature, all estimates of acclimation capacity were positive (Figure 8). Although the magnitude of acclimation capacity differed between approaches for some populations (Figure 8), the means were not different within *A. montanus* (ignoring acute effects mean 0.041, SD 0.033; accounting for acute effects mean 0.044, SD 0.018; Paired t-test, t=0.269, df=3, p=0.805) or *A. truei* (ignoring acute effects mean 0.065, SD 0.039; accounting for acute effects mean 0.056, SD 0.027; Paired t-test, t=1.314, df=9, p=0.221).

The mean number of years gained through acclimation until stream temperatures exceed CTmax (buffering capacity – our proxy for vulnerability) did not differ between the two

approaches in *A. montanus* (ignoring acute effects mean 14.63 years, SD 11.76; accounting for acute effects mean 15.59 years, SD 9.70; paired T test, t= 0.332, df=3, p=0.761) or *A. truei* (ignoring acute effects mean 24.89 years, SD 16.81 accounting for acute effects mean 21.05 years, SD 11.32; paired T test, t= 1.280, df=9, p=0.232). However, the estimates from each approach were not always congruent among populations (Figure 9). Patterns of this buffering capacity metric largely mirrored patterns of acclimation capacity, as streams did not vary substantially in their projected rates of warming (0.040—0.047 °C year^{-1}).

Discussion

We used a cold-tolerant frog study system (*Ascaphus montanus* and *A. truei*) to investigate the roles of population variation and experimental design in estimating acclimation capacity of critical thermal maximum (CTmax) to infer vulnerability (buffering capacity). We found support for our hypotheses that CTmax is influenced by both acute temperature effects, elicited when moving tadpoles from their holding temperature to a different experimental starting temperature, and acclimated temperature effects. The magnitude of these effects was dependent on population (as indicated by the significant three-way interaction term in the models), demonstrating that both acute temperature responses and acclimated temperature responses vary within these species. Accounting for acute temperature effects when estimating acclimation capacity did not alter conclusions about acclimation or buffering capacity at the species-level, but resulted in different population-level assessments of vulnerability to warming temperatures. The potential for overestimating coping capacity when ignoring acute temperature effects on estimates of vulnerability proxies may become increasingly important as the consequences of climate change continue to be realized and environmental temperatures approach tolerance limits.

The Role and Impact of Population Variation

Estimates of acclimation capacity in thermal tolerance traits are often used as a proxy of vulnerability to climate change (Nicotra et al. 2015; Gunderson and Stillman 2015; Gunderson, Dillon, and Stillman 2017; Morley et al. 2019) and thus should accurately reflect a species' ability to cope with changing temperatures. The inclusion of intraspecific variation in thermal tolerance traits can affect inferred climate impact (Seebacher et al. 2012; Valladares et al. 2014; Bennett et al. 2019; Senior et al. 2019; Herrando-Pérez et al. 2019) and conclusions about broad physiological trends (Herrando-Pérez et al. 2020), yet population variation in thermal tolerance plasticity is unknown for many taxa. We found that estimated acclimation capacity varied over three-fold among populations (*A. montanus*: accounting for acute effects = 3.5X, ignoring acute effects = 6X; *A. truei*: accounting for acute effects = 4.5X, ignoring acute effects = 11X) and inferred buffering capacity varied by decades. Therefore, limited sampling of populations from this study could lead to skewed results that suggest a high acclimation ability, leading to overly optimistic buffering capacity estimates if generalized for the species. Our results add to the growing evidence that among population variation is important to consider for vulnerability assessments as generalizations from a few populations can be misrepresentative (Herrando-Pérez et al. 2019).

Acute responses to temperature varied markedly among populations and holding temperature treatments and did not solely reflect expectations based on thermodynamic relationships. Regarding our predictions of the underlying relationships dictating the acute temperature responses (thermodynamic versus stress responses), we found that acute effects of temperature were similar in magnitude across acclimation treatments, but were not consistent with expectations of directionality. First, for the cold-acclimated treatments, transfer to a warmer

temperature resulted in a mean decrease in CTmax estimates, consistent with a stress response and associated decreased function or performance (Galloway and Kieffer 2003; O'Steen and Bennett 2003). Six of the fourteen populations, however, exhibited a greater CTmax with a higher start temperature, consistent with the prediction that thermodynamic relationships shape acute effects. Second, in the warm-acclimated treatments, transfer to a colder start temperature resulted in a mean decrease in CTmax, consistent with both thermodynamic principles and stress responses, as well as previous studies (Terblanche et al. 2007; Kittner and Riisgård 2005), although four populations exhibited a slightly higher CTmax after transfer from warm to cold. Taken together, these results suggest that acute effects of temperature may not be dictated by a singular process (thermodynamic relationships or thermal stress) but rather a combination of multiple processes. Furthermore, acute temperature responses may be partly shaped by other factors, such as baseline heat-shock protein abundance and initial cell membrane structure (Angilletta, 2009). These other factors may contribute to both the magnitude and directionality of responses to temperature transfer and to the population variation in acute responses that we observed.

Population variation in acclimation capacity was the product of variation in acclimated responses to temperature and acute responses to temperature, which may be related to factors not investigated in this study. Acclimation responses may be related to local environmental conditions among species and populations of ectotherms (e.g., Narum et al., 2013; Sørensen et al., 2016; Shah et al., 2017; Rohr et al., 2018). As the populations in this study were sampled along elevation gradients, local thermal regimes may vary and contribute to the variation observed at this scale (e.g., Freidenburg & Skelly, 2004). Further studies are required to test the effects of local environment on acclimation capacity and to investigate whether acute and

acclimated responses are similarly shaped by the environment in this system. Acute and acclimated responses may also be influenced by the duration and magnitude of exposure to thermal stress (Einum and Burton 2022; Jørgensen et al. 2021; Rezende, Bozinovic, and Santos 2020). Holding tadpoles for three days was sufficient to elicit an acclimated response in this system, although the effects of holding duration or temperature on CTmax responses among populations in this system remains to be tested.

Effects of Experimental Design and Estimation Approach

Accounting for acute temperature effects in this study did not change mean estimates of acclimation or buffering capacities, although acute effects did have an outsized impact on inferences for some populations. After accounting for acute temperature effects on CTmax, buffering capacity estimates slightly decreased for most populations (Figure 9). Physiological proxies represent one aspect of vulnerability (Clusella-Trullas et al. 2021; Dawson et al. 2011; Nicotra et al. 2015; Beever et al. 2016), and thus the differences in estimates due to the different approaches may be negligible for most of these tailed frog populations. However, a few populations from this study exemplified the potential consequences of not accounting for acute temperature effects when making inferences of vulnerability. When acute temperature effects are responsible for much of the trait value change after holding, estimates of acclimation and buffering capacity will be overestimated (Einum et al. 2019). Overestimated buffering capacities may artificially lower inferences of vulnerability for populations facing warming temperatures. For example, for the Lamb Butte population, buffering capacity (years of added buffer) decreased by about 21 years (36%) when accounting for acute temperature effects. Alternatively, when acute effects of temperature reduce CTmax and acclimation effects compensate for this reduction (R.B. Huey and Berrigan 1996), acclimation capacity can only be uncovered when

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accounting for acute temperature effects. This was the case for four populations, such that ignoring acute effects of temperature when estimating acclimation capacity would provide a more conservative estimate of buffering capacity, but underestimate the population's capacity to cope with warmer temperatures.

Our acute temperature response results point to a limitation of the approach used to estimate acclimation capacity outlined by Einum et al. (2019). In their approach, acute responses are assumed to be driven solely by thermodynamic relationships, and thus follow expected patterns of directionality and equal magnitude. Our results demonstrated similar magnitudes, but differing directionality of responses among holding temperature treatments. In situations where acute temperature responses are not equal among holding temperature treatments, it may not be appropriate to subtract the averaged acute effects slope when estimating acclimation capacity at a warmer or colder temperature.

Ultimately, researchers must weigh the costs of a particular experimental design with the potential benefits given their questions and system. When acute temperature effects are generally low, as observed here, studies quantifying acclimation capacity may not need to account for these temperature responses. However, for questions involving conservation implications for individual populations, the quantification of acute temperature effects may be necessary to increase the accuracy of vulnerability proxy estimates. The experimental design needed to quantify acute and acclimated temperature effects may be a challenge for many systems due to the sample sizes required from each population (here, N=48). An alternative experimental design, such as a repeated-measures design, would reduce the sample size requirements and allow for estimation of population variance in acclimation capacity, an added insight into the evolvability of acclimation capacity (Terblanche and Hoffmann 2020). However, with the

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treatments used here, each individual would be subject to four CTmax experiments, potentially introducing a strong experiment-order and stress effects. Other experimental designs, such as transferring organisms from different acclimation treatments to a common temperature before beginning an experiment also have associated biases (Terblanche and Hoffman, 2020) and do not necessarily allow for the quantification and consideration of acute effects of temperature on acclimation capacity estimates. Various aspects of experimental design beyond temperature treatments can influence physiological trait responses and each experimental design has its own benefits and limitations (Terblanche and Hoffmann 2020; Havird et al. 2020).

Climate Impact Insights for Ascaphus

The estimated acclimation capacities of CTmax for tailed frogs are among the lowest estimates for amphibians, regardless of the estimation approach used (see Gunderson and Stillman, 2015). Our estimates, however, are higher than reported in a previous study investigating acclimated responses of CTmax in adult tailed frogs. Claussen (1973) found a lack of an acclimated response between adults held at 10°C and 20°C, although they did report a higher acclimated response than we observed (0.2°C CTmax per °C acclimation) when comparing frogs held at 0°C and 10°C. These differences may be a product of different experimental methodologies (e.g., different ramping rates and holding lengths), but may be due to ontogenetic differences in acclimation capacity and/or increased trait flexibility at the cold end of the species' tolerance. The low acclimation capacity estimates calculated in our study provide a maximum buffer of approximately 50 years to the species' estimated warming tolerance, suggesting that acclimation of CTmax will not provide tailed frog populations a long-term coping strategy for warming temperatures. Although the present-day stream maxima are much lower than tadpole CTmax estimates, physiological consequences of increasing temperatures will

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certainly begin to be experienced before organisms reach their CTmax (Pörtner and Farrell 2008; Thomas et al. 2017; Huey and Kingsolver 2019). Therefore, studies investigating the capacity for these populations to evolve greater CTmax and acclimation capacity, as well the impacts of warming on physiological traits that occur prior to the upper critical limit, would be beneficial for further understanding climate change impacts.

Tables & Figures

Table 1. Mixed model ANOVA (type III) CTmax results for *Ascaphus truei* (top) and *A. montanus* (bottom).

Ascaphus truei				
Factor	**Sum Sq**	**df**	**F value**	**p-value**
(Intercept)	8000.637	1	59109.720	<0.001
Start Temperature	0.563	1	4.158	0.042
Population	12.441	9	10.213	<0.001
Holding Temperature	0.137	1	1.010	0.315
Tadpole Size	3.924	1	28.989	<0.001
Start Temperature: Population	4.517	9	3.708	<0.001
Start Temperature: Acclimation Temperature	1.828	1	13.502	<0.001
Population: Holding Temperature	3.438	9	2.823	0.003
Start Temperature: Population: Holding Temperature	4.330	9	3.555	<0.001

Ascaphus montanus				
Factor	**Sum Sq**	**df**	**F value**	**p-value**
(Intercept)	1750.290	1	12507.633	<0.001
Start Temperature	0.001	1	0.009	0.923
Population	7.363	3	17.540	<0.001
Holding Temperature	1.012	1	7.231	0.008

Tadpole Size	0.104	1	0.746	0.389
Start Temperature: Population	0.377	3	0.899	0.443
Start Temperature: Holding Temperature	0.004	1	0.028	0.868
Population: Holding Temperature	1.058	3	2.521	0.060
Start Temperature: Population: Holding Temperature	1.020	3	2.430	0.067
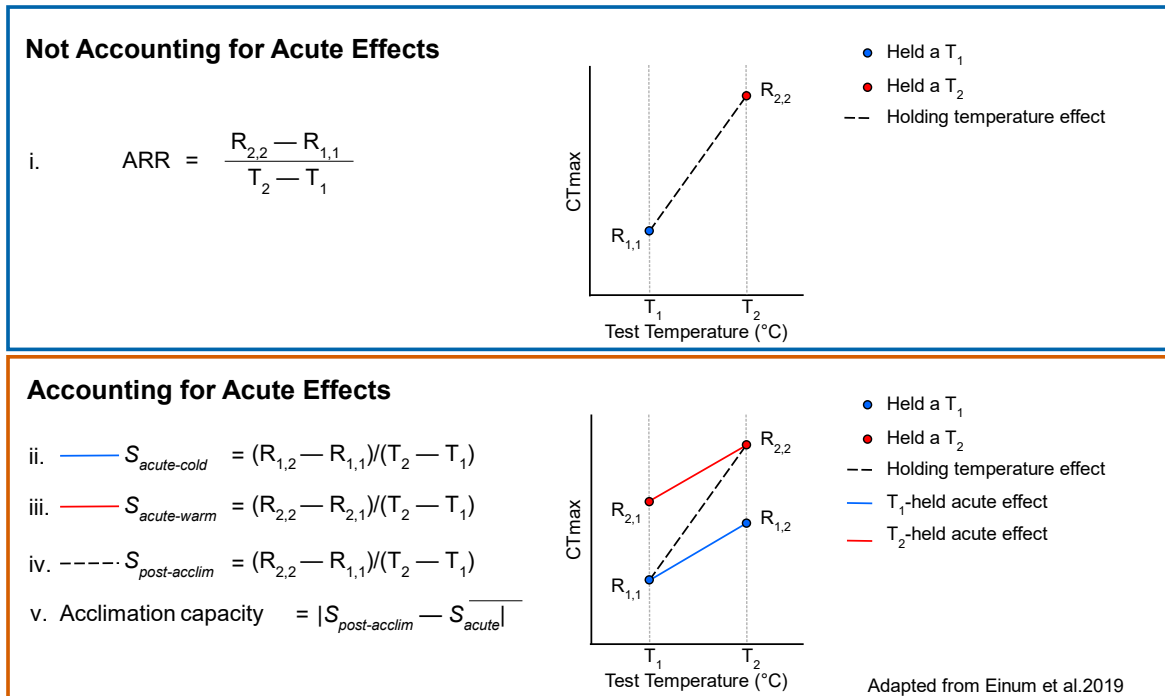
Figure 4. Hypothetical example outlining the two approaches tested in this study. For these examples, the two holding temperatures and the two test temperatures are the same: T1 and T2. "R" denotes the trait value (response, here CTmax) where the first subscript represents the holding temperature, and the second subscript represents the start temperature. (Top) This approach calculates the slope of the line for trait values held and tested at the same temperatures (i.e., test temperature equals holding temperature). We depict this response over test temperature for comparison with the other approach. (Bottom) This approach accounts for acute effects of temperature by subtracting the mean of the two acute effect slopes from the holding temperature effect slope. The absolute value is then taken. Here, the resulting acclimation capacity when ignoring acute temperature effects is greater than when acute temperature effects are accounted for.
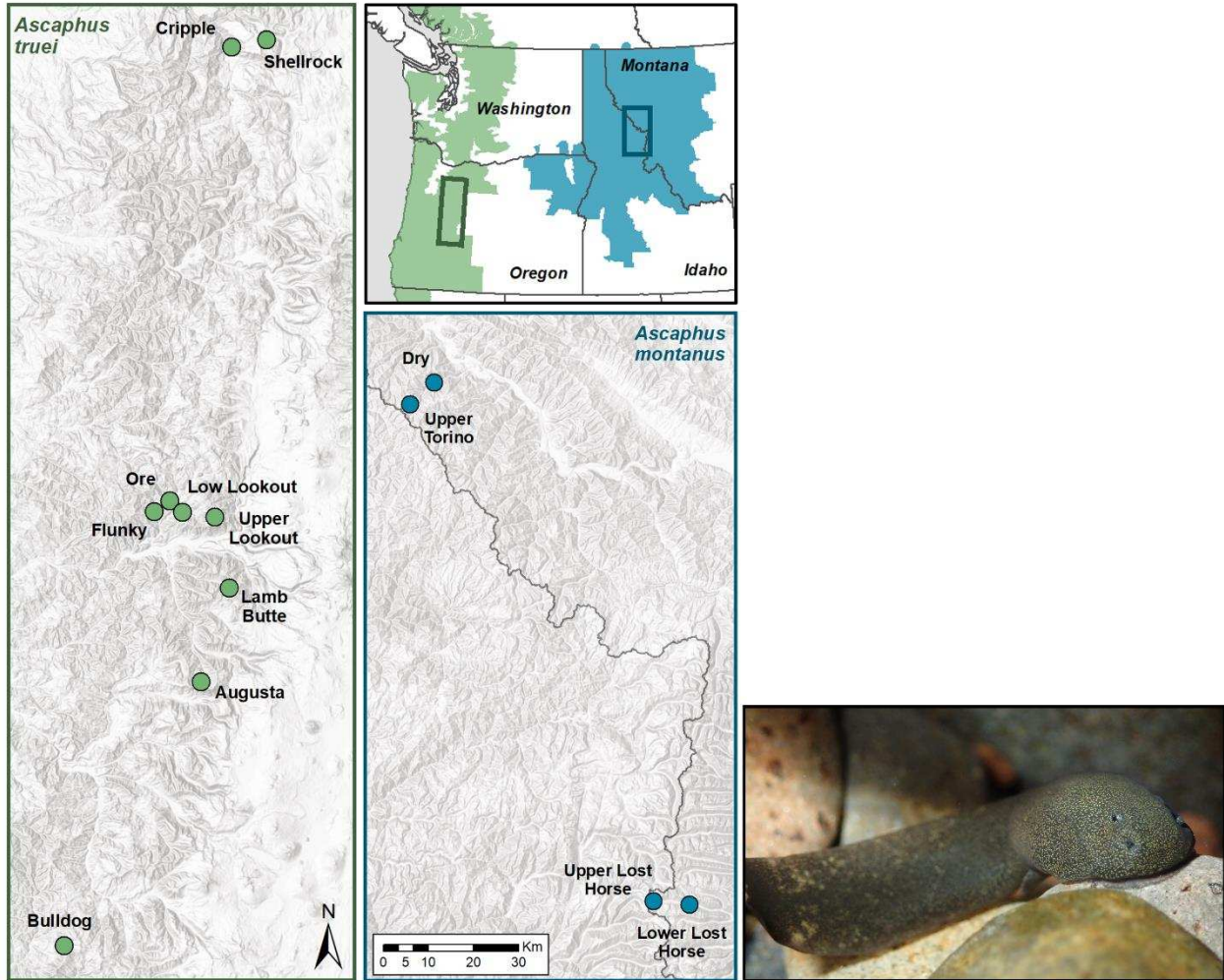
Figure 5. Map of sampling sites: ten populations of *Ascaphus truei* were sampled from Oregon and four populations of *A. montanus* were sampled from Montana. *Ascaphus* tadpoles (pictured right) occupy cold, fast-flowing streams where they use their suctorial mouths to attach themselves to benthic substrate.
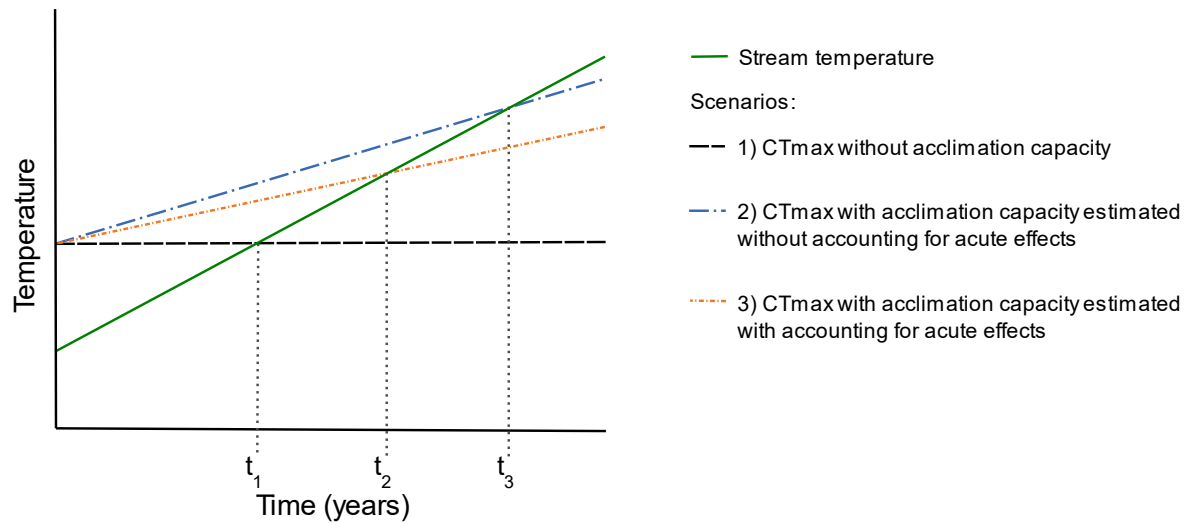
Figure 6. A hypothetical example demonstrating how we estimated buffering capacity, our proxy for physiological vulnerability to warming (calculations adapted from Morley et al. 2019). For each population, we modelled stream temperature changes over time using maximum temperatures calculated from our in-stream data loggers as the intercept and the rate of increase projected for each stream according to Isaak et al. 2016 (green solid line). We modelled CTmax changes over time using population medians as the intercept and a slope that was determined from 1) no acclimation capacity (grey dashed line), 2) acclimation capacity estimated using an approach that accounts for acute effects (orange dotted line), and 2) acclimation capacity estimated while ignoring acute effects (blue dotted and dashed line). Using the time (in years) that each modelled CTmax intercepted modelled stream temperature (t_1, t_2, t_3), we calculated buffering capacity as the change in years that acclimation capacity provided populations before environmental temperatures exceeded CTmax modelled without acclimation capacity (i.e., t_2-t_1 or t_3-t_1). In this example, buffering capacity from estimating acclimation capacity without accounting for acute effects (t_3-t_1) is greater than when acclimation capacity estimates consider acute effects (t_2-t_1).
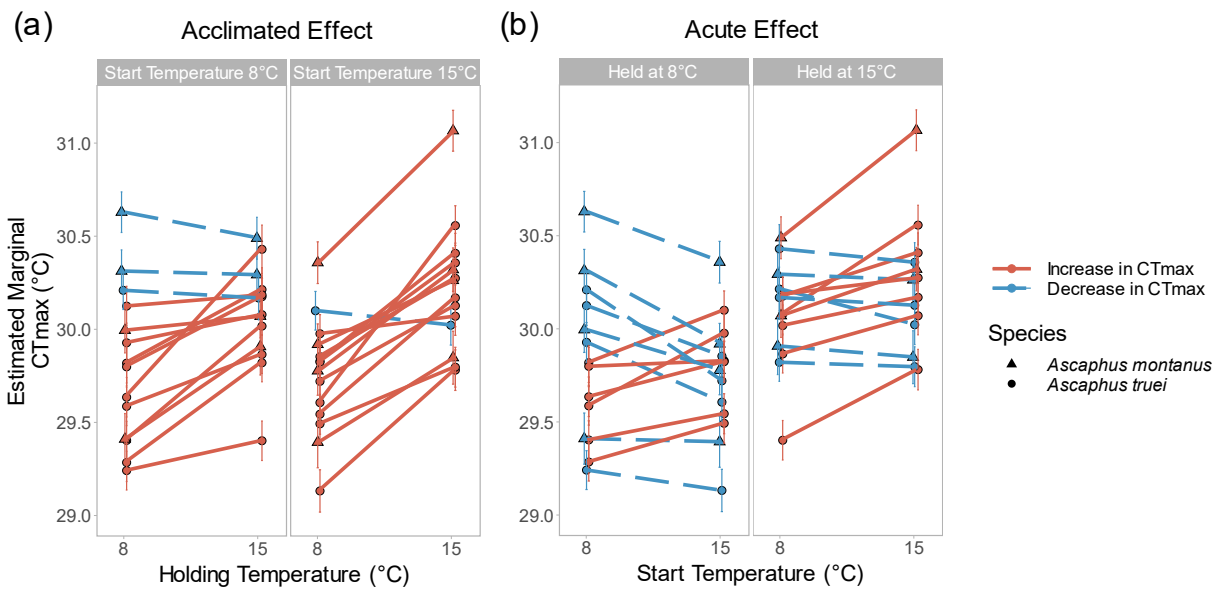
Figure 7. A: With few exceptions, a warmer holding temperature generally led to an increase in CTmax estimates for both *Ascaphus* species, regardless of the experimental starting temperature. **B**: Acute temperature responses of CTmax varied in magnitude and directionality among populations of both species, suggesting multiple processes (thermodynamic and stress) dictating acute responses.
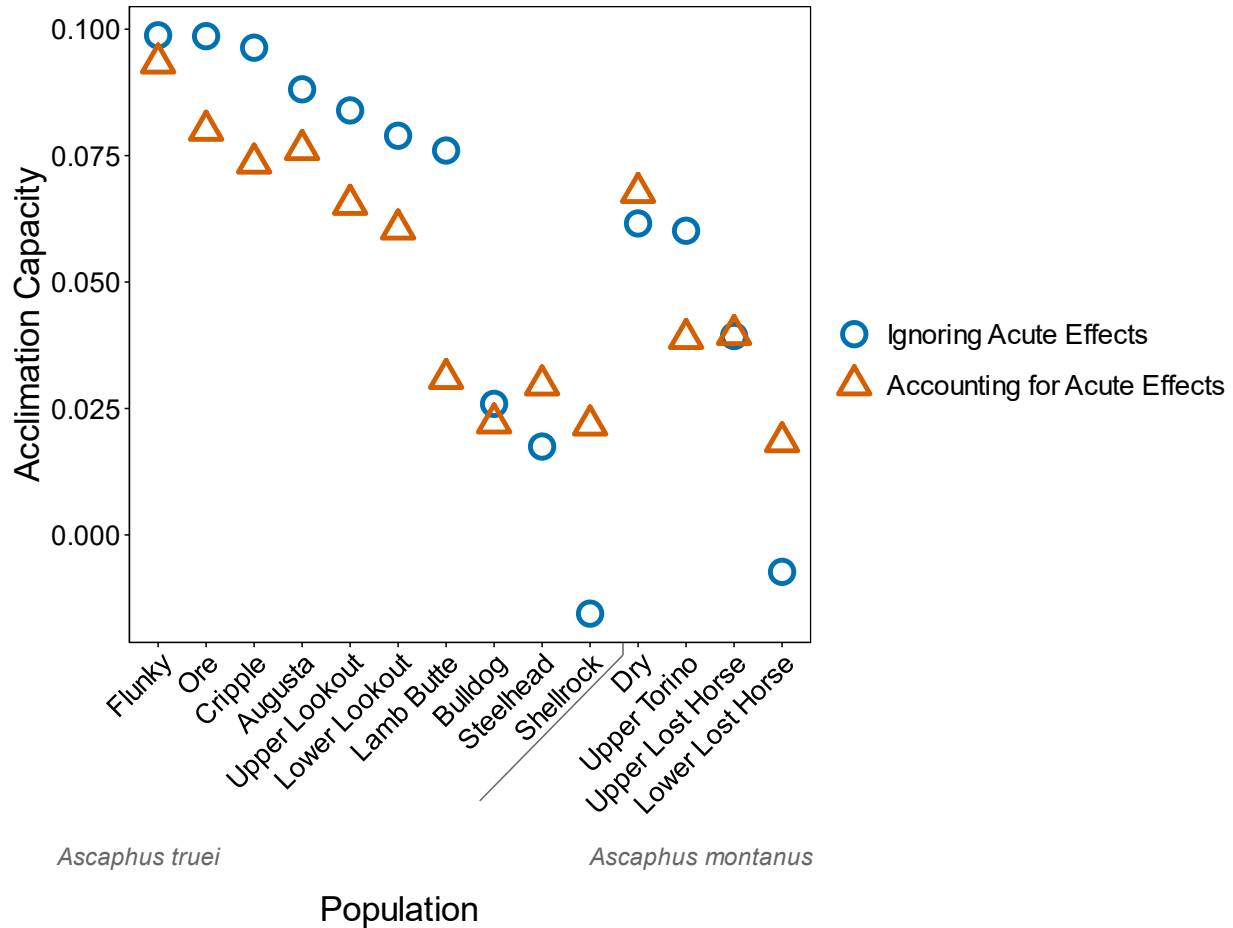
Figure 8. Acclimation capacity (ΔCTmax per degree change in holding temperature; Figure 2) for each population as estimated by ignoring acute effects and accounting for acute effects. Without accounting for acute effects, all but two populations (Lower Lost Horse, Shellrock) exhibited acclimation capacity of CTmax. However, by accounting for acute effects of temperature, all populations were estimated to have acclimation capacity of CTmax (i.e., magnitude of the response greater than 0). Negative values represent populations where the response to being held in a warmer temperature reduced CTmax estimates. Although approach estimates did not differ statistically, they are not congruent within all populations.
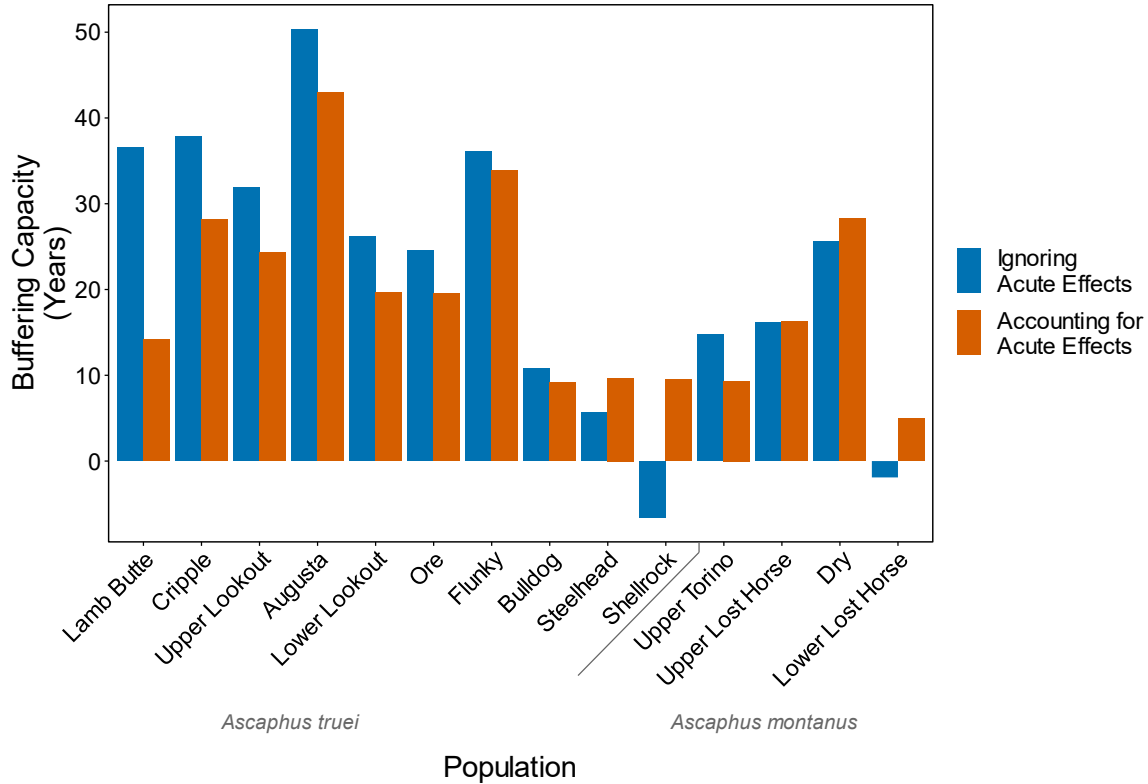
Figure 9. Buffering capacity (years of added buffer; Figure 6) as estimated using both approaches for calculating acclimation capacity (Figure 4). Although the two approaches did not statistically differ from each other in their buffering metric estimates, there is substantial population variation in the magnitude of the buffer depending on the approach used. Ignoring acute effects of temperature for some populations and extrapolating buffering capacity for the whole species with a biased sample of populations could misrepresent the true capacity for coping to warming temperatures.

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CHAPTER 3: MULTI-SCALE RELATIONSHIPS IN THERMAL LIMITS WITHIN AND BETWEEN TWO COLD-WATER FROG SPECIALISTS UNCOVER DIFFERENT TRENDS IN PHYSIOLOGICAL VULNERABILITY

Introduction

As environments across the globe continue to warm, the persistence of ectotherms will be partly dependent on their physiological thermal tolerance (Addo-Bediako et al., 2000; Pinsky et al., 2019). Physiological thermal limits play an important role in organismal thermal tolerance as they represent the upper and lower bounds of performance and activity across a range of body temperatures (Angilletta, 2009) and in turn help shape patterns of species distributions (e.g., Bozinovic et al., 2011; Sunday et al., 2012; Amundrud and Srivastava, 2020). Quantifying thermal limits is therefore critical for understanding a species' capacity to withstand exposures to future temperature changes (Deutsch et al., 2008; Rezende et al., 2011; Sinclair et al., 2016). Macrophysiological patterns of thermal limits have provided insights into species' relative vulnerabilities to climate change across broad geographic scales (Gaston et al., 2009; Pinsky et al., 2019; Sunday et al., 2019). For example, studies have found that upper thermal limits (UTL: maximum temperature tolerated) are relatively invariant across latitude among terrestrial ectotherms, suggesting that species occupying environments with temperature maxima close to their UTLs, such as tropical species, may be most sensitive to warming (Araújo et al., 2013; Deutsch et al., 2008; Sunday et al., 2011). While most comparative studies to date have focused on species differences, patterns of thermal limits among populations within a species can provide insight into relative vulnerability while also quantifying variation – a key component of evolvability to future temperatures (Bennett et al., 2019; Gervais et al., 2021; Nati et al., 2021).

Predicting population and species responses to future warming requires an understanding of the environmental and organismal drivers of variation in thermal limits (Clusella-Trullas et al., 2011). Several hypotheses have been proposed to explain variation (or lack thereof) in the lower thermal limits (LTL: minimum temperature tolerated) or upper thermal limits, including physiological constraints (Araújo et al., 2013; Gangloff & Telemeco, 2018), paleoclimatic pressures (Bennett et al., 2021; Ibargüengoytía et al., 2021), and phylogenetic stasis (Hoffmann et al., 2013; Muñoz et al., 2014). However, selection pressures associated with contemporary environmental temperatures have emerged among these hypotheses as a strong predictor for both the upper and lower thermal limits of species (Bennett et al., 2021). For example, environmental temperature variation is correlated with many patterns in thermal limits observed along latitudinal and elevation gradients (Addo-Bediako et al., 2000; Drummond et al., 2020; Shah et al., 2017; Sunday et al., 2011) as temperature is related to both of those geographic variables (Addo-Bediako et al., 2000; Buckley & Huey, 2016). Indeed, Sunday et al., (2019) showed that extreme environmental temperatures (i.e., maximum and minimum temperatures) experienced within environmental realms (marine, terrestrial, freshwater, intertidal) were associated with global latitudinal patterns of thermal limits among ectothermic species.

While environmental temperature extremes are undoubtedly a powerful predictor of thermal limits (Bennett et al., 2021), they only represent one aspect of the thermalscape (i.e., thermal landscape; e.g., Isaak et al., 2017; Snyder et al., 2020). Temperature extremes are often calculated using temporal temperature variation at one point in space. Local microhabitat features, however, can produce spatial variation in temperatures, substantially impacting temperatures experienced by an organism (Garcia et al., 2019; Neel et al., 2021; Pincebourde et al., 2016; Sears et al., 2011; Woods et al., 2015). This fine-scale temperature variation allows

organisms the opportunity to exploit spatial variation through behavioral thermoregulation, which in turn can shield them from temporal extremes in environmental temperatures (Beever et al., 2017; Bodensteiner et al., 2021; Bogert, 1949; Huey et al., 2003; Muñoz, 2021; Muñoz & Losos, 2018). For example, among sites with variation in temperature maxima, UTLs among *Anolis* species were invariant, partly as a result of behavioral thermoregulation that allowed lizards to maintain consistent body temperatures (Muñoz et al., 2014; Muñoz & Bodensteiner, 2018). Similarly, in aquatic environments the ability to take advantage of such microclimatic variation has been linked to the avoidance of unfavorable temperatures (Berman & Quinn, 1991; Ritter et al., 2020). Thus, substantial fine-scale spatial temperature variation is needed for organisms to have the opportunity to track favorable microclimates, and a lack of fine-scale temperature would suggest a lack of opportunity for behavioral thermoregulation. Yet, most studies examining the drivers of thermal limits typically only measure the relation with regional or local temperatures from thermal regime data, and fail to consider how fine-scale variation can provide a buffer from these thermal extremes (e.g., Huey et al., 2012).

Many species from montane environments, where natural temperature gradients often track elevation gradients, have already begun shifting their distributions upslope in response to warming temperatures (Chen et al., 2011; Comte & Grenouillet, 2013; Freeman et al., 2018; Tingley et al., 2012). The magnitude of these shifts is partly dependent on organismal thermal physiology (Troia & Giam, 2019), which may be shaped by the environment. Freshwater montane ecosystems provide interesting opportunities to investigate environmental drivers of thermal limits both among and within species due to their natural temperature gradients and complex thermal landscapes (Arismendi et al., 2013; Tonolla et al., 2010; Vatland et al., 2015). Montane streams flowing from high to low elevation often follow expected temperature

gradients (colder at higher elevations), providing similar thermal patterns among streams at similar elevations (Isaak et al., 2017). However, stream temperatures can vary within – or even deviate from – the expected gradient due to variation in environmental, geologic, and hydrological conditions (Schultz et al., 2017; Vatland et al., 2015). Among streams, water source (e.g., spring, seep, snowmelt-fed), mountain aspect, and stream connectivity interact to shape thermal landscapes (Steel et al., 2017). Within streams, variation in canopy cover, surface-subsurface energy fluxes, and a host of other processes can produce thermal variability at very fine (≤1m) scales (Caissie, 2006; Dent et al., 2008; Johnson, 2004; Torgersen et al., 1999). As such, ectotherms occupying stream habitats can face varying and complex thermal conditions across the landscape (Hossack et al., 2013).

Here, we investigated organismal thermal limits (LTLs and UTLs) as they relate to local thermalscapes in two tailed frog species (family: Ascaphidae). The only two species in this family, *Ascaphus truei* and *A. montanus*, occupy montane streams and riparian areas along similar elevation ranges (*A. truei*: ~0-2100m; *A. montanus* ~790-2500m) (Hayes & Quinn, 2015; Macedo, 2019). However, *A. truei* is distributed along coastal environments (Klamath Mountains, Coast Ranges, and Cascade Mountains) while the range of *A. montanus* is restricted to more continental landscapes (northern Rocky Mountains of the United States and Canada). Tadpoles of these species spend 1-5 years in their natal streams before metamorphosis, and thus are exposed to at least one annual stream temperature cycle. They primarily graze diatoms from the stream substrate using their modified sucker mouth and have limited vagility, having been observed moving a mean distance of 1.1m/day in old growth habitats (Wahbe & Bunnell, 2001). Adults are highly aquatic and extremely philopatric, remaining close to their natal streams and

surrounding riparian areas. Accordingly, the aquatic thermalscape may play a large role in shaping their thermal limits, but it has been rarely comprehensively considered for these species.

To address this gap and uncover the role of local thermalscapes in shaping population thermal limits, we experimentally determined thermal limits of tadpoles from multiple populations of both tailed frog species, and quantified thermal regimes and fine-scale (≤1m) spatial variation within these streams. We estimated thermal limits using critical thermal minimum (CTmin) and critical thermal maximum (CTmax), which use the loss of response to stimuli as the endpoint of performance. We expected *A. montanus* to have lower CTmin and higher CTmax than *A. truei* populations, reflecting the greater range of temperatures experienced in their continental range in contrast to the narrower range of temperatures experienced by coastal tailed frogs. We also predicted that populations of both species would exhibit positive relationships between thermal limits (CTmin and CTmax) and stream temperature extremes (minima and maxima, respectively). In addition, we expected to observe within-stream spatial heterogeneity in temperatures, which provides the opportunity for behavioral thermoregulation. We therefore predicted that the species experiencing greater fine-scale spatial heterogeneity would have a weaker relationship between CTmax and stream temperature maxima, reflecting the capacity for behavioral thermoregulation to mediate the strength of selection from thermal regimes.

Materials and Methods

Field Sampling

Sampling occurred June to August in each of 2016, 2017, and 2018. We sampled *Ascaphus truei* (coastal tailed frog) tadpoles from 15 stream reaches in Oregon and *A. montanus*

(Rocky Mountain tailed frog) tadpoles from 14 stream reaches in Montana (Figure 10). Sampled stream reaches are hereafter referred to as separate populations due to the substantial distance between reaches and limited species vagility. We targeted populations across elevation gradients to capture varying local thermal regimes. We collected tadpoles (developmental stages 26.5 to 40; Gosner, 1960) from each site by lifting stream substrate directly upstream of a hand-net. We held the sampled tadpoles in 2L insulated containers (maximum 12 tadpoles per container) with stream water during sampling, ensuring the water remained cold with frequent water changes. To transport tadpoles to our lab facilities (*A. truei*: H.J. Andrews Experimental Forest; *A. montanus*: Fort Missoula, University of Montana), we added bubblers to the insulated containers, which were stored in larger coolers packed with ice (Essner et al., 2012). All sampling, transport, holding, and experimental protocols were approved by Colorado State University IACUC (16-6667AA) and University of Montana IACUC (024-17WLDBS-042117).

Temporal and Spatial Stream Temperature Measurements

To characterize stream thermal regimes experienced by each population, which were used to estimate local temperature extremes, we deployed two temperature loggers (HOBO pendant UA-001-64, Onset Corporation) at each end of sampled stream reaches (~100m) and recorded temperatures every four hours. We chose to record temperatures at this time interval as temperatures in Pacific Northwest streams tend to be relatively stable over time (Arismendi et al., 2013; Maheu et al., 2016) and have a higher heat capacitance than air, leading to a slower rate of warming and cooling than air temperatures (Arismendi et al., 2014; Mohseni & Stefan, 1999). Four hours thus represents an interval in which extreme temperatures should be captured, while also minimizing the required frequency of returning to sites to download data. Temperature loggers were housed in PVC pipe with holes to allow water flow. The pipes were secured to an

in-stream rebar pole using zip ties such that the logger was just above the stream substrate (where tadpoles occupy). Pipes were also secured to a nearby tree using a thick steel cable to prevent them from being washed downstream by stream currents. We visually inspected the time-series temperature logger data for error and screened the data using standard deviation time series plots (Dunham et al., 2005).

From these data (Appendix 2: Table S3 for date ranges used), we calculated two temperature metrics for each end of the thermal regime: 1) the absolute minimum daily temperature and absolute maximum daily temperature, and 2) the average of the ten consecutive coldest and the average of the ten consecutive warmest days. These two metrics were highly correlated in Oregon Because of high correlations between maximum temperature metrics in Oregon (minimum temperature: Pearson's r= 0.94, df=13, p=2.58e^{-10}; maximum temperature: Pearson's r= 0.99, df=13, p=4.01e^{-14}) and Montana (minimum temperature: Pearson's r= 0.49, df=12, p=0.08; maximum temperature: Pearson's r= 0.99, df=12, p=1.12e^{-11}). Therefore, further analyses used the absolute minimum and maximum daily temperatures, hereafter referred to as minimum and maximum stream temperature.

We quantified fine-scale (within stream reaches) spatial variability in temperature for 12 stream reaches (i.e., presumed populations) of each species between 3 Aug 2019 and 13 Aug 2019 (Appendix 2: Table S3), which is typically warmest month of the year. As our goal was to capture the spatial variability at a scale relevant to tailed frog tadpoles (Garcia et al., 2019), we focused our temperature measurements within stream reaches (~100m). We measured temperatures at a minimum of 100 points using a field temperature probe (ODO, YSI Incorporated, Yellow Springs, Ohio) that was placed in the water at the interface of the stream substrate, reflecting the microhabitat of *Ascaphus* tadpoles. Temperature measurements were

made along transects of pre-identified *Ascaphus* spp. habitat along the stream reach. Along the stream transects, we measured the temperature of the stream at intervals determined by stream width (<3m wide: measurements every 0.5m; >3m wide: measurements every 1m). For each measurement, we randomly offset the measurements from the interval along the stream transect using a random number table. For streams <3m wide, the offset did not exceed 25cm. For streams >3m wide, the offset did not exceed 50cm. For example, if a narrow (<3m) stream at transect 1, interval 3 (1.5m from stream bank) had an offset of -21, the measurement would be taken at 1.29m from the stream bank. For transect points that were obstructed (e.g., by a large rock), we noted the obstruction and measured the point directly upstream. We opportunistically sampled visible seeps or confluences to capture any potential thermal anomalies present outside of designated transects to ensure full sampling of existing spatial temperature variation available to tadpoles.

Thermal Limit Experiments

Tadpoles collected from each population were held and tested separately. Tadpoles were held in 142L insulated containers (maximum 24 tadpoles per container) filled with water from their natal streams at 8°C (a commonly experienced temperature) for three days using a 1/10 HP recirculating water chilling unit (Coralife, Franklin, WI, USA). We maintained high oxygenation of the holding water using standard aquarium air pumps and bubblers. During this three-day holding period, food was withheld to ensure a similar post-absorptive state during experiments and standardize the condition of individuals prior to the experiments. We checked tadpoles daily for typical behavior and cleaned tanks between each use.

CTmin and CTmax were measured using temperature ramping experiments at a starting temperature of 8°C and a ramping rate of ~0.3°C/minute. The experiment tank contained natal

stream water, an aquarium pump to circulate water, and air bubblers. Tadpoles were transferred to mesh containers in the experiment tank and allowed to acclimate for two minutes before ramping in either direction began. Temperatures were monitored using a real-time thermometer with a platinum temperature probe (HH804U RTD Thermometer, Omega Engineering, Inc.) and a second thermocouple secured to a Proportional Integral Derivative (PID) temperature controller. To ramp water temperature up for CTmax experiments, we used the PID temperature controller with a solid-state relay attached to a titanium heating rod. To ramp water temperature down for LTL experiments, we added ice at pre-determined intervals to the experiment tank (i.e., not the containers holding the tadpoles), adjusting the amount of ice needed to reach our desired cooling rate (e.g., Christian et al., 2008). Critical thermal limits were defined as the temperature at which tadpoles no longer responded to a tactile stimulus. Upon reaching their thermal limits, tadpoles were returned to holding temperatures and allowed to recover for up to an hour. Following experiments, tadpoles were euthanized using a 20% benzocaine solution and photographed laterally with a ruler. We used ImageJ software (Rasband, 2009) to measure the length (tip of the snout to the tip of the tail) of each tadpole. The average of two length measurements was used for analyses.

We performed CTmin experiments on nine tadpoles from each of six populations of *A. montanus* and three populations of *A. truei* (total N=81). Of the 81 tadpoles sampled, all were active and responsive at the lowest temperature we could reach without causing the water to freeze (0-0.3°C; Appendix 2: Figure S1). We therefore discontinued CTmin experiments and did not test the relationships between CTmin and local thermalscapes.

We performed CTmax experiments on a total of 736 tadpoles from 15 populations of *A. truei* and 14 populations of *A. montanus*. The number of tadpoles sampled from each population

varied from 10 to 97 (*A. truei* median 13; *A. montanus* median 24; see Appendix 2: Table S3). Six tadpoles that did not recover after the experiments and two tadpoles that were missing length measurements were excluded from further analyses. One tadpole had a spurious CTmax estimate (>12X SD) and was also excluded from analyses. Therefore, the analyzed data represent CTmax from 727 tadpoles (370 *A. truei*, 357 *A. montanus*).

Data Analysis

Analyses were performed in R version 4.1.2 (R Core Team, 2021). We tested the assumptions of subsequent parametric tests before performing them. We tested for differences in thermal regimes between continental (*A. montanus*) and coastal (*A. truei*) using t-tests to compare the mean maximum and minimum stream temperatures and F-tests to compare the variances in these metrics. We characterized fine-scale variability within stream reaches by inspecting data density distributions, standard deviations, and temperature ranges for each stream. For streams with fine-scale spatial heterogeneity, defined as >2°C in temperature range, we constructed spatially-explicit thermal maps to characterize the configuration of temperature variability. We used linear mixed effects models to test relationships between tadpole CTmax and local thermalscapes. Having found that stream reaches were generally spatially homogenous in temperature (see Results), we did not include fine-scale thermal heterogeneity in these models.

In our analyses of the effects of temperature extremes on CTmax, we first investigated whether the relationship between CTmax and maximum stream temperature was dependent on the species. This mixed effects model included CTmax as the response variable, maximum stream temperature and its interaction with species as predictors, tadpole length as a covariate to account for any variation due to size, and the population sampled as a random effect to account for variation due to population effects. We found a significant interaction term (Appendix 2:

Table S4), demonstrating that species affected the relationship between CTmax and local

thermalscapes, and therefore tested each species separately. Our species-specific models included

maximum stream temperature as a predictor, tadpole length as a covariate, and the random effect

of population. The residuals were visually inspected for assumptions regarding normality and the

resulting models were validated using leave-one-out cross validation.

Results

Temporal and Spatial Stream Temperatures

Across all streams, temperatures reached near-freezing, though minimum stream

temperatures from Oregon (*A. truei* habitat) were significantly different than those from Montana

(*A. montanus* habitat) (Mann Whitney U test, W=57.5, p=0.04). Despite representing coastal

(Oregon) and continental (Montana) environments, mean maximum stream temperatures from

Oregon did not significantly differ from those sampled in Montana (Oregon mean = 14.67°C ±

2.04; Montana mean = 14.75°C ± 2. 79; t-test, t= 0.08, df=23.69, p=0.93). However, streams

from Montana had a slightly higher range of temperatures and experienced greater maximum

temperatures (Oregon: range = 8.11°C, max = 17.28°C; Montana range = 8.80°C, max =

20.42°C). Maximum stream temperatures were related to elevation in Oregon (Pearson's

correlation = -0.76, p=0.001), but not in Montana (Pearson's correlation = 0.08, p=0.79). Within

species, streams in Oregon exhibited variation in minimum and maximum stream temperatures,

whereas in Montana, only maximum temperatures considerably varied. (Figure 10).

At a fine-scale, sampled streams for both species were spatially homogenous in

temperature (Appendix 2: Figure S2). *A. truei* streams ranged in standard deviation from 0.05 to

0.64 and *A. montanus* streams ranged from 0.04 to 1.35. Only two *Ascaphus montanus* streams

and two *A. truei* streams had temperature ranges greater than 1.6°C (*A. montanus* – Hoodoo: 8.0°C; Lower Lost Horse: 8.3°C; *A. truei* – Augusta: 2.8°C; Hardy: 3.0°C). For these streams, the high range values were driven by one or very few data points, demonstrating a gradient and/or clumped spatial distribution (Appendix 2: Figure S3).

Upper Thermal Limits (CTmax)

A. *truei* tadpoles had a mean CTmax of 29.5°C (SD 0.52) and *A. montanus* tadpoles had a mean UTL of 29.8°C (SD 0.54). The range of CTmax based on individual estimates was slightly larger in *A. truei* (3.1: 27.8°C to 30.9°C) than *A. montanus* (2.5: 28.7°C to 31.2°C), although *A. montanus* tadpoles exhibited the highest UTL estimates (Appendix 2: Figure S4).

Results from the *A. truei* linear mixed effects models show that populations varied in CTmax. The covariate of length had a negative relationship with CTmax; maximum stream temperature did not influence CTmax (Table 2; Figure 11). Results from the linear mixed effects model for *A. montanus* showed that maximum stream temperature had a positive relationship with CTmax after accounting for the effects of tadpole length and population (Table 2; Figure 11). Results from the leave-one-out model cross-valuation indicate that the predictive power of the *A. truei* model (Mean Squared Predicted Error = 0.16) is comparable to that of the *A. montanus* model (Mean Squared Predicted Error = 0.14), reinforcing the lack of a relationship between CTmax and maximum temperature in *A. truei*. To determine if *A. montanus* model results were biased by the three populations with maximum stream temperatures exceeding the range found among *A. truei* populations, we performed the same *A. montanus* linear mixed effects model described above but without those populations included in the dataset. We found that the relationships described with the full dataset were maintained even with this restricted dataset (Appendix 2: Table S5).

Discussion

Future temperature extremes are predicted to increase in frequency (Fischer & Knutti, 2015; Meehl & Tebaldi, 2004), making it imperative to understand the variation among species and populations in their tolerance to thermal challenge. To date, most studies investigating variation in thermal physiology have compared species occupying different environments (e.g., Deutsch et al., 2008; Sunday et al., 2011, 2012, 2019). Our results here demonstrate that closely related species occupying similar habitats may have different thermal physiological relationships with their external environments, leading to different conclusions regarding their vulnerability to warming temperatures. Understanding these physiological trends requires the quantification of local thermalscapes as environmental conditions may deviate from expected paradigms, as we observed in streams in Montana along elevation gradients. Lastly, by quantifying fine-scale temperature variation, we uncovered unexpected homogeneity in temperatures within stream reaches and only a few sampled streams having possible thermal refuges.

Among terrestrial ectotherms, most studies have found greater variation in LTLs than UTLs, as well as strong relationships between LTLs and environmental temperatures (Addo-Bediako et al., 2000; Araújo et al., 2013; Bodensteiner et al., 2021; Hoffmann et al., 2013). However, in aquatic environments, UTLs have been found to track environmental temperatures (e.g., Sunday et al. 2012). For example, Shah et al. (2017) found that temperate aquatic insects did not vary in LTLs along an elevation gradient, despite a decrease in UTLs with elevation. This pattern was explained by the thermal regimes experienced at each elevation, as summer maximum temperatures decreased with increasing elevation while winter minimum temperatures we similar (Shah et al., 2017). We expected to observe similar relationships within tailed frogs, however we only found this relationship for CTmin and CTmax in one of the species. Both tailed

frog species had CTmin that were extremely close to freezing, suggesting the capacity to resist or tolerate freezing temperatures. *A. montanus* adult frogs have been observed responding to stimuli at -3°C and have demonstrated some capacity to recover after freezing (Werner II, 2015). In our experiments, we did not reach freezing temperatures because our behavioral assays relied on a response to tactile stimulation (which cannot be performed on tadpoles in frozen water). Thus, whether tailed frog tadpoles or adults vary in freeze tolerance remains to be tested. However, given that stream temperatures from our sampled populations did not drop below freezing, our experimental results suggest that cold tolerance in these organisms may exceed the cold stress they typically experience in nature.

Patterns of evolution in UTLs of terrestrial ectotherms have generally shown that UTLs are invariant across space and phylogeny (Araújo et al., 2013; Bodensteiner et al., 2021; Sunday et al., 2019). UTLs, however, may still be influenced by environmental extremes (Bennett et al., 2021; Shah et al., 2017; Sunday et al., 2019), even among closely-related species (Senior et al., 2019). Our CTmax estimates for these species are within the range observed previously (Bury, 2008; Claussen, 1973) and, within both species, represent ranges in trait values that have been observed among species (e.g., Herrando-Pérez et al., 2020). We found slight differences in CTmax between the species that were dependent on the thermal environment of the sampled populations. Within species, we found that CTmax varied among populations, but were only related to stream temperatures in *A. montanus*, even when populations were subsampled to represent the range of maximum temperatures observed in *A. truei*. The lack of a relationship among *A. truei* populations may be due to the lower range of variation in stream temperatures, resulting in less variation in thermal selective pressures. Indeed, some studies have reported *A. montanus* tadpoles to be present in stream temperatures warmer than we studied herein (Adams

& Frisell, 2001; Dunham et al., 2007; Huff et al., 2005; Welsh Jr & Hodgson, 2008), though the full range of thermal conditions both species experience across their ranges has yet to be fully described. Regardless of the ultimate cause (e.g., plasticity, adaptation) of this difference in intraspecific relationships between CTmax and stream temperatures, these different relationships lead to different conclusions regarding physiological vulnerability to climate change.

The difference between maximum environmental temperatures and UTLs (i.e., warming tolerance, Deutsch et al., 2008) provides an index of vulnerability, such that a smaller difference is interpreted as a narrower window before environmental temperatures exceed UTLs. The positive relationship that we observed among populations of *A. montanus* suggests that each population is similarly vulnerable to future warming. However, the lack of a positive relationship between CTmax and maximum stream temperature among *A. truei* populations suggests that populations currently experiencing high stream temperatures may be relatively more vulnerable to future warming than those occupying colder streams due to a narrower warming tolerance.

Our results also highlight an important consideration for inferring physiological vulnerability across elevation gradients: local conditions can cause thermal heterogeneity among streams and potentially expose organisms to thermal pressures that diverge from the expected gradient. In environments with these deviations, interpretations of physiological vulnerability cannot rely on elevation as a proxy for temperature. We found that maximum stream temperatures from Oregon (*A. truei* habitat) followed the expected pattern across elevation, however, maximum stream temperatures from Montana (*Ascaphus montanus* habitat) did not decrease with elevation. Many of the sampled high elevation streams in Montana are outlets of lakes, which warm up in the summer and may be driving the observed trend (Isaak et al., 2017). Although this result may not represent regional patterns, it exemplifies the importance of

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investigating local conditions. Therefore, physiological vulnerability among these populations is related to elevation only in *A. truei*, such that low elevation populations are at a relatively higher risk from warming than high elevation populations.

We found minimal fine-scale variation in temperature within stream reaches in both species, indicating limited capacity for realized behavioral thermoregulation for the populations studied. Temperatures were spatially homogeneous within stream reaches for both species, although some reaches had one or few variable temperature readings. Even so, the magnitude of temperature differences and spatial configuration of these outliers, along with organismal ecology, suggest that they do not serve as thermal refuges. In the four sites where temperatures spatially ranged over 1.6°C, the variation could be characterized as a gradient or clumped distribution (Appendix 2: Figure S3). According to EPA standards (EPA, 2003), cold-water refuges should be at least 2°C lower than the surrounding water temperature. Fine-scale temperature variation with gradient spatial distributions in streams, such as those in our study, may therefore be too small (i.e., < 2°C) to provide adequate thermal refuge for inhabitants. Clumped distributions of thermal refuges can lead to inaccuracy in behavioral thermoregulation (Sears et al., 2016), and may be inaccessible to organisms with limited movement, such as tailed frogs (Altig & Brodie, 1972; Feminella & Hawkins, 1994; Wahbe et al., 2004; Wahbe & Bunnell, 2001). Although we may have missed sampling a thermal refuge that was outside of our 100m survey extent, we did not observe a greater abundance of tadpoles in cooler areas of stream reaches, suggesting that these tadpoles are not congregating in outlier temperatures.

The possible refuges we uncovered may not be facilitating behavioral thermoregulation in this system but underscore an important alternative outcome: isolated or clumped thermal refuges may incur other costs and have the potential to form ecological traps (*sensu* Battin,

2004). For example, even in spatially heterogeneous streams, organisms do not always choose to exploit thermal refuges (e.g., Barrett et al., 2022), perhaps due to the costs associated with behaviorally thermoregulating, such as increased predation risk and/or associated bioenergetic costs (e.g., Huey and Slatkin, 1976; Carrascal et al., 2001; Snyder et al., 2022). Studies that incorporate bioenergetic information into spatially-explicit forecasting models with other components of adaptive capacity (plasticity, evolvability, dispersal) will fill a key gap in our understanding of vulnerability to changing environments.

Without fine-scale temperature variation to serve as refuges through behavioral thermoregulation (Logan et al., 2019; Sears et al., 2016; Sears & Angilletta, 2015), organisms are fully exposed to environmental temperatures and thermal tolerance trait divergence among populations/species can occur more quickly (Logan et al., 2019; Muñoz & Bodensteiner, 2018). Thus, although fine-scale spatial variation in temperature may not explain variation in upper thermal limits among the populations studied here, the lack of variation may still have contributed to the evolution of their thermal limits. Moreover, this lack of fine-scale thermal variation indicates an increased vulnerability to warming temperatures for all populations in this study as they lack within-reach thermal refuges. Tailed frogs are already declining due to warming stream temperatures and environmental disturbances (Hossack et al., 2023). This result, along with previous studies demonstrating that these species are sensitive to habitat disturbance (Hossack & Honeycutt, 2017; Wahbe & Bunnell, 2001, 2003) and have low acclimation capacities (Cicchino et al., 2022 *in review*), suggests that their capacity to cope with future environmental change is limited. Whether the significant population variation in thermal tolerance that we uncovered in these species provides the potential for evolutionary responses to future changes in climate remains to be determined.

Tables & Figures

Table 2. Linear mixed effects model estimate and results from ANOVA (type III, Satterthwaite's method) testing the effects of maximum stream temperature on CTmax within each species. Tadpole length was included as a covariate and population as a random effect.

Ascaphus truei				
Fixed Effects	**Estimate (SE, df)**	**SumSq**	**F (df)**	**p-value**
(Intercept)	29.63 (0.08,13.25)	-	-	$2E^{-16}$
Maximum stream temperature	-0.09 (0.06, 14.37)	0.32	2.06 (1,14.37)	0.17
Tadpole Length	-0.13 (0.02, 366.27)	4.01	26.00 (1.00,366.28)	$5.29E^{-7}$
Random Effects	**Variance +/- SD**			
Population	0.08 +/- 0.28			
Residual	0.15 +/- 0.39			

Ascaphus montanus				
Fixed Effects	**Estimate (SE, df)**	**SumSq**	**F (df)**	**p-value**
(Intercept)	29.75 (0.07, 12.09)	-	-	$2E^{-16}$
Maximum stream temperature	0.27 (0.06, 12.81)	2.97	21.52 (1,12.81)	0.0005
Tadpole Length	-0.18 (0.02, 345.08)	7.56	54.81 (1,345.08)	<0.0001
Random Effects	**Variance +/- SD**			
Population	0.06 +/- 0.24			
Residual	0.14 +/- 0.37			
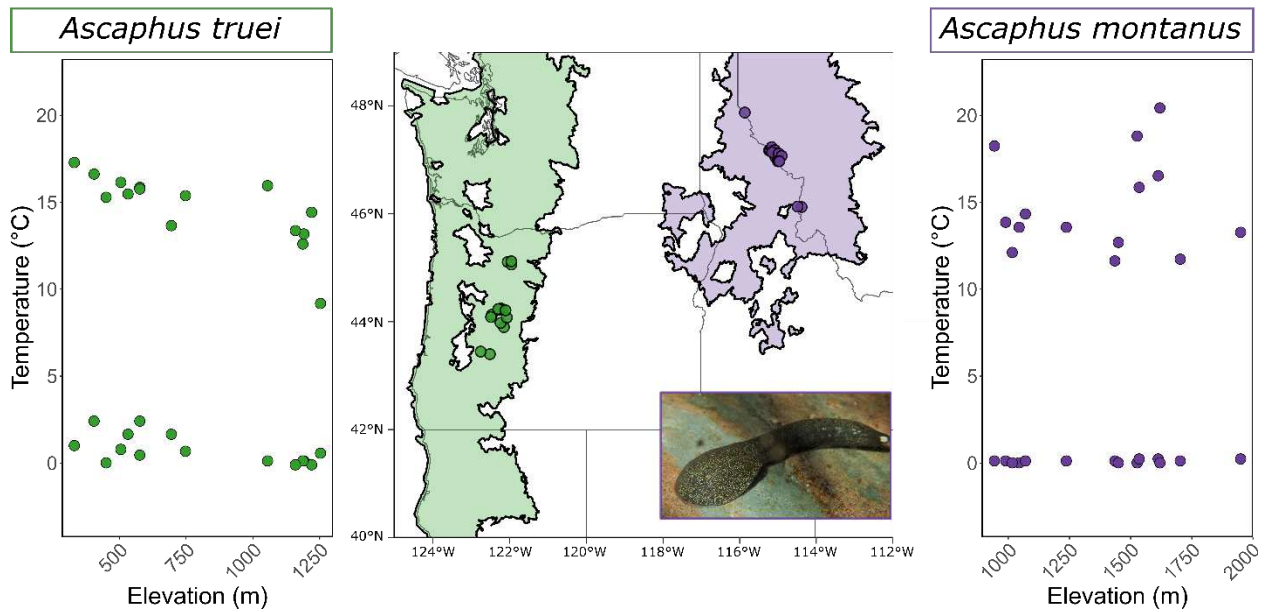
Figure 10. (Center map) Sampling locations for *Ascaphus truei* tadpoles from 15 populations in Oregon (USA species range in green) and *A. montanus* tadpoles from 14 populations in Montana (USA species range in violet). *A. montanus* tadpole shown in bottom right inset. (Outer panels) Maximum stream temperatures varied more than minimum stream temperatures within both species.
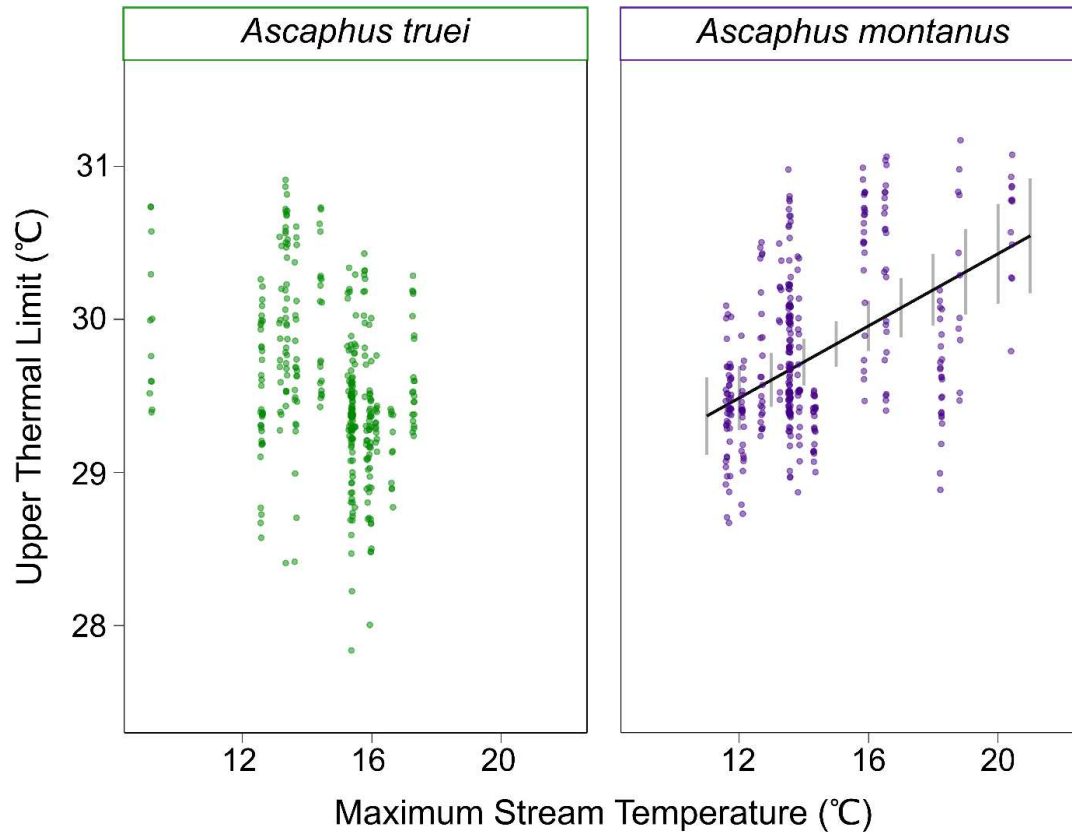
Figure 11. Upper thermal limits, measured as critical thermal maximum (shown here as raw data points), among populations were unrelated to maximum stream temperatures in *Ascaphus truei* (left) but were positively related in *A. montanus* (right). Prediction slope and 95% confidence intervals estimated by the linear mixed effects model for *A. montanus* (right, p= 0.0005).

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CHAPTER 4: LINKING CRITICAL THERMAL MAXIMUM TO MORTALITY FROM THERMAL STRESS IN A COLD-WATER FROG

Introduction

Physiological traits provide insight into the initial response of organisms when their environment changes – a key aspect of quantifying vulnerability to climate change (Addo-Bediako et al., 2000; Chown et al., 2010; Nicotra et al., 2015; Beever et al., 2016; Pinsky et al., 2019). In particular, thermal tolerance traits such as critical thermal maximum (CTmax – maximum temperature tolerated before loss of function) are increasingly used to partly assess organismal risk from warming as they indicate the temperature bounds of performance and mortality (Deutsch et al., 2008; Angilletta, 2009; Walters et al., 2018; Morley et al., 2019; Clusella-Trullas et al., 2021). For example, investigations of ectothermic warming tolerance – the difference between maximum environmental temperature and CTmax (Deutsch et al., 2008; Pinsky et al., 2019) – and related metrics have uncovered patterns of thermal physiological vulnerability (hereafter, vulnerability) at both interspecific and intraspecific scales (Deutsch et al., 2008; Valladares et al., 2014; Bennett et al., 2019; Sunday et al., 2019; Gervais et al., 2021; Nati et al., 2021). Yet, the usefulness of these patterns and metrics in informing predicted responses to future warming events depends on the assumption that CTmax accurately predicts thermal tolerance in ecologically relevant conditions, resulting in uncertainty about its adequacy as a metric of thermal tolerance for inferring vulnerability (Rezende et al., 2014; Kingsolver & Buckley, 2017; Clusella-Trullas et al., 2021; Ørsted et al., 2022).

Estimates of CTmax are sensitive to the experimental approach used to measure them due to the interacting effects of the magnitude and duration of thermal stress on tolerance

(Terblanche et al., 2007; Kingsolver & Umbanhowar, 2018; Ørsted et al., 2022). CTmax is estimated using a dynamic experimental approach, which exposes organisms to gradually increasing temperatures (i.e., increasing magnitude of thermal stress) until physiological failure is reached (e.g., loss of righting response). The rate of temperature increase is positively related to estimates of CTmax as faster ramping rates shorten the duration of thermal stress experienced and thus allow for higher estimates of CTmax (Terblanche et al., 2007; Chown et al., 2009). Furthermore, experimental ramping rates are often faster than warming rates experienced in nature, suggesting that experimental CTmax may overestimate organismal thermal tolerance in natural conditions (Hoffmann et al., 2013; Huey & Kearney, 2020). However, recent conceptual frameworks suggest that CTmax measured using dynamic approaches quantifies the same additive accumulation of heat stress as metrics estimated using static/non-dynamic approaches, suggesting estimates from both approaches should be correlated if they follow the same failure rate function (Jørgensen et al., 2019, 2021; Ørsted et al., 2022). Thus, empirically connecting dynamic and static metrics of thermal tolerance would help resolve concerns that the sensitivity of CTmax to methodology limits its ecological significance and would bolster CTmax as an adequate proxy for estimating thermal tolerance (Kingsolver & Buckley, 2017; Braschler et al., 2021; Ørsted et al., 2022).

The effectiveness of CTmax to infer vulnerability to climate change may also be limited by the perception that there is insufficient variation within and among taxa. Across broad geographic and taxonomic scales, several studies have found CTmax to be relatively invariant compared to metrics of other physiological traits (Addo-Bediako et al., 2000; Araújo et al., 2013; Grigg & Buckley, 2013; Muñoz et al., 2014). Low levels of variation may be the outcome of physiological constraints (Araújo et al., 2013; Gangloff & Telemeco, 2018) and suggests limited

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ability for populations to evolve higher CTmax as a response to warming temperatures (Grigg &
Buckley, 2013; Hoffmann et al., 2013). However, CTmax has been observed to vary among
populations (Geerts et al., 2014; Herrando-Pérez et al., 2019; Nati et al., 2021) which would
suggest there is the potential for CTmax to evolve in response local temperature variation (Geerts
et al., 2015; Mesas et al., 2021; Morgan et al., 2022). Directly linking variation in CTmax across
populations to measures of fitness like mortality would provide necessary insight the capacity for
different populations to exhibit different vulnerability to future warming scenarios.

Here, we tested the assumption that CTmax is predictive of mortality from thermal stress
among populations of a cold-water frog, *Ascaphus montanus* (Rocky Mountain tailed frog). *A.
montanus* inhabits cold streams in the northern US Rocky Mountains and extreme southern
Rocky Mountains of Canada. This species is already facing population declines that are partly
due warming stream temperatures (Hossack et al., 2023), and low CTmax relative to other
amphibians (Bury, 2008; Bennett et al., 2018; Cicchino et al., 2023) suggests continued risk from
warming. We tested the relationship between measurements of CTmax via the dynamic method
and mortality at different constant temperatures (i.e., static approach) in tadpoles. We expected
these two approaches to be related, such that populations with higher CTmax experienced less
mortality. We also tested the strength of CTmax as a predictor of mortality from thermal stress by
comparing its performance against local thermal conditions, which are commonly related to
physiological traits (Clusella-Trullas et al., 2011; Shah et al., 2017; Sunday et al., 2019;
Drummond et al., 2020).

Methods

We sampled tadpoles to ensure that survival was the sole contributor to fitness during the experiments. For both dynamic and static experiments (detailed below), we used handheld fish nets to collect tadpoles from streams. Tadpoles were held in stream water in insulated containers during sampling with frequent water changes to maintain temperatures. We transported tadpoles to laboratory facilities (Fort Missoula, University of Montana) for experiments via the protocol outlined in (Essner et al., 2012).

Dynamic CTmax Experiments

Full details of CTmax experiments can be found in (Cicchino et al., 2023). Briefly, we sampled 10-24 tadpoles (developmental stages 26.5 to 44 (Gosner, 1960); Appendix 3: Table S6) from each of seven populations of *A. montanus* in Montana. Due to the distance between these streams and limited vagility of the species (Wahbe & Bunnell, 2001), we refer to these sampled sites (n=7) as populations. We held tadpoles for three days at 8°C without food to reduce the effects of natal stream temperature and feeding. For CTmax experiments, tadpoles were moved to an experimental tank and held in individual mesh containers. They were given two minutes to acclimate before temperature ramping at 0.3°Cmin^{-1} began. CTmax was defined as the temperature at which tadpoles lost the ability to respond to tactile stimuli but fully recovered when placed in cooler temperatures. We used population median CTmax for analyses, hereafter referred to as CTmax$_p$, representing previously-characterized CTmax.

Static Thermal Stress Experiments

A. montanus tadpoles overwinter in their natal streams for at least one year (Brown, 1975). To collect tadpoles for static thermal stress experiments, we returned two years later (3 July – 28 July, 2019) to substantially decrease the probability of sampling the same cohort. Since

we permanently removed tadpoles from the population for CTmax$_p$ experiments, we did not resample the same individual. We collected ~60 tadpoles from each population (total n= 420).

Tadpoles from each population were randomly assigned to one of five holding temperature treatments: 5°C, 10°C, 15°C, 20°C, 25°C; ~12 tadpoles per treatment (Appendix 3: Table S6). Temperatures were decided based on thermal regime data (Cicchino et al., in revision TW): 5°C and 10 °C are commonly experienced temperatures; 15°C and 20°C treatments are near maximum stream temperatures; 25°C is greater than current maximum stream temperatures but is ecologically relevant given current rates of warming (Isaak et al. 2017). Tadpoles were held in the temperature treatments for three days and fed *ad libitum* by placing rocks collected from their stream in the holding tanks, from which they graze algae. We maintained oxygenation using flowing water and bubblers. After three days, we counted surviving tadpoles.

Local Stream Temperatures

We characterized stream temperatures experienced by the tadpole at three timescales: 1) immediate, 2) annual, and 3) long-term/ multi-generational. To characterize the immediate and annual stream temperature metrics, we used quality-controlled (Dunham et al., 2005) logged temperature data at 4 hour intervals from each stream (detailed in (Cicchino et al., 2023)). Using Water Year 2018 data, we calculated "immediate thermal experience" by averaging maximum daily temperatures of the three days preceding sampling for the static mortality experiments. We calculated "annual thermal experience" by measuring the maximum temperature experienced in a year for each population. Lastly, we used modeled temperature data of 40-year averages of the mean temperature during the warmest month (August) for each stream (Isaak et al. 2016, 2017) to quantify "long-term thermal experience". These metrics were uncorrelated with each other (Appendix 3: Table S7).

Data Analysis

Analyses were performed in R version 4.1.2 (R Core Team 2021). Statistical significance was evaluated using $\alpha=0.05$. To test differences in mortality among temperature treatments, we used a Fisher's Exact Test for count data. Due to limited variation in mortality in other temperature treatments, we only investigated the relationship between the probability of mortality and $CTmax_p$ in the 25°C treatments. For this and subsequent models, we used a logistic regression with a 2-column matrix of number of deaths and number survived was used as the response variable. To assess the strength of $CTmax_p$ as a predictor of mortality, we compared the performance of this model against ten other models: (1) null; each thermal experience metric modelling separately as (2 – 4) independent predictors, (5 – 7) additive predictors with $CTmax_p$, and (8 – 10) interactive predictors with $CTmax_p$. All models used a binomial distribution. We compared models using Akaike's Information Criterion (Akaike, 1973) adjusted for sample size (AICc), AICc weights, and evidence ratios with the top model.

Results

The probability of tadpole mortality was different among temperature treatments (Fisher's Exact Test, $p<0.001$). There was no mortality in the 5°C, 10°C, or 15°C temperature treatments. In the 20°C treatments, two populations experienced mortality (Lower Cedar Creek: 1 individual; Oregon Gultch Creek: 2 individuals). All populations experienced some degree of mortality in the 25°C treatment (Figure 12), which was not related to the order of population sampling (Appendix 3: Table S8).

$CTmax_p$ ranged from 29.4°C to 30.8°C (variance=0.28). Results from the logistic regression with $CTmax_p$ as the sole predictor of mortality in 25°C demonstrated that a one degree

increase in $CTmax_p$ is associated with a multiplicative decrease in the predicted odds of mortality by 0.07 (Odds Ratio = 0.07, p<0.001; Figure 13; Appendix 3: Table S9 for regression results and detailed interpretation). $CTmax_p$ was the best predictor of mortality from thermal stress when compared to the stream thermal environment at any temporal scale (Appendix 3: Table S10). The top model with $CTmax_p$ as the sole predictor was almost 18 times better supported than the next model ($CTmax_p$ and immediate thermal experience) and 57 times better supported than the top model without $CTmax_p$ (Table 3).

Discussion

Thermal tolerance traits are important for assessing vulnerability to climate change as they contribute species' capacity to cope with environmental change *in situ* and through movement to preferable conditions (Thurman et al., 2020). The strength of vulnerability assessments thus relies on the accuracy of experimental measurements of thermal tolerance, garnering much debate over dynamic versus static approaches (Overgaard et al., 2012; Rezende et al., 2014; Jørgensen et al., 2019; Terblanche & Hoffmann, 2020; Ørsted et al., 2022). Here, we show a clear relationship between CTmax and mortality from thermal stress among populations of *Ascaphus montanus* and demonstrate its strength as a predictor of mortality over environmental metrics. These results support the assumption that CTmax estimated by dynamic approaches is related to mortality from chronic thermal stress and is a relevant metric for vulnerability assessments.

We found that populations with greater CTmax experienced significantly less mortality when held in a stressful temperature for three days. This result aligns with previous studies showing a relationship between dynamically–measured CTmax and thermal tolerance metrics

from static approaches (e.g., sub-critical limits (Braschler et al., 2021), lethal temperature (Dallas & Ketley, 2011; Kingsolver & Umbanhowar, 2018; Jørgensen et al., 2019, 2021)) and highlights that the methodological sensitivity of CTmax does not preclude the trait from assessing thermal tolerance. This result also supports the conceptual framework proposing that dynamic and static experimental approaches ultimately measure similar tolerance outcomes (Ørsted et al., 2022). Under this framework, exposure to thermal stress causes a shift from the "permissive temperature range", where growth and reproduction occur, to the "stressful temperature range" (Jørgensen et al., 2022; Ørsted et al., 2022), where cellular and molecular damages accumulate until heat failure (Pörtner & Farrell, 2008; Pörtner, 2010; Rezende et al., 2014; Huey & Kingsolver, 2019). The transition point between permissive and stressful temperatures is rarely estimated, yet here, the observed mortality in the 20°C and 25°C treatments suggest that it falls between 15-25°C.

Reduced variation in CTmax relative to other physiological traits has suggested that CTmax may be evolutionarily constrained (Araújo et al., 2013; Grigg & Buckley, 2013; Hoffmann et al., 2013). Here, we showed that relatively small differences in CTmax can have a large impact on mortality from thermal stress. We found that the predicted probability of mortality in 25°C after three days for a population with a $CTmax_p$ of 29.5°C was 93%, but a one degree increase in $CTmax_p$ (39.5°C) reduced the predicted probability of mortality by almost half (47%). $CTmax_p$ also outperformed any metric of local thermal environment as the strongest predictor of mortality despite having been collected two years before the mortality experiments. The degree to which variation in CTmax reflects underlying genetic variation and acclimation to an individual's thermal history will be critical to understanding its potential as an evolved response to warming temperatures.

Climate change is exposing species to both low magnitude – long duration thermal stress and high magnitude – short duration thermal stress through chronic warming and extreme temperature fluctuations (Jackson et al., 2009), respectively. The connection we found between mortality from a static experimental approach (low magnitude – long duration thermal stress) and CTmax from a dynamic approach (high magnitude – short duration thermal stress) suggests that either metric can be used to understand how *A. montanus* populations may tolerate these different patterns of warming – a key but often missing characteristic of traits used in vulnerability assessments (Bates & Morley, 2020). Our results also indicate that CTmax can be used to understand the consequences of subcritical thermal stress in *A. montanus*, which will be experienced before CTmax is reached in nature (Isaak et al., 2017). Further combining physiological inferences with those from other components of vulnerability (Nicotra et al., 2015; Beever et al., 2016; Thurman et al., 2020) will strengthen predictions of *A. montanus* persistence through climate change.

Tables & Figures

Table 3. Model comparison results identifying the top predictors of variation in mortality at 25°C. AICc weights demonstrate the probability that a model is the best model. Only included are models with weights > 0. See Appendix 3: Table S10 for full results. Evidence Ratios are presented in reference to the top model.

Model Predictors	AICc	ΔAICc	AICc Weight	Evidence Ratio
$CTmax_p$	30.93	0	0.86	1
$CTmax_p$ + Immediate Thermal Experience	36.70	5.77	0.05	17.9
$CTmax_p$ + Long-term Thermal Experience	37.09	6.16	0.04	21.75
$CTmax_p$ + Annual Thermal Experience	37.80	6.86	0.03	30.92
Immediate Thermal Experience	39.04	8.11	0.01	57.54
Annual Thermal Experience	39.39	8.46	0.01	68.78
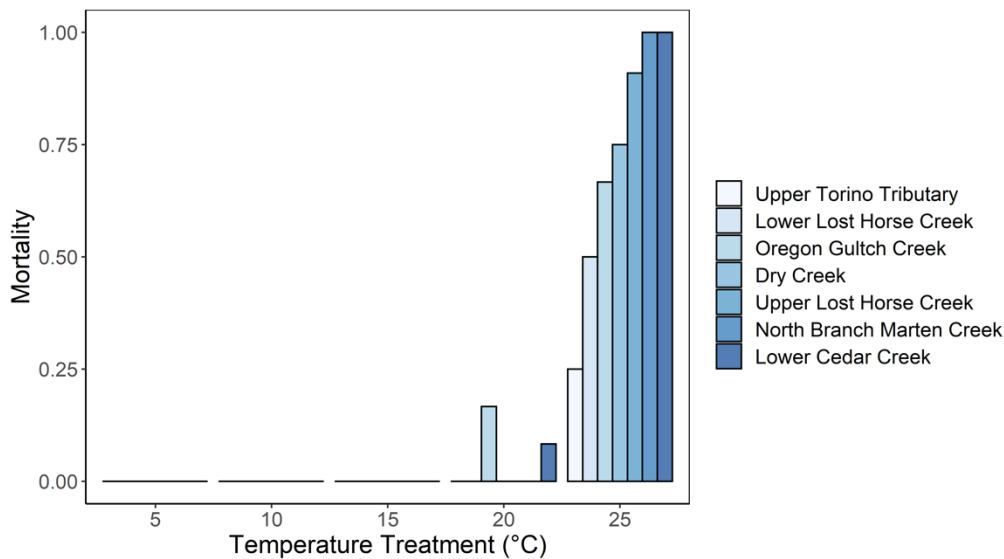
Figure 12. The proportion of tadpoles within populations that experienced mortality after being held for three days at one of five temperatures. Bars and color values represent one population and are ordered by increasing mortality in the 25°C treatments.
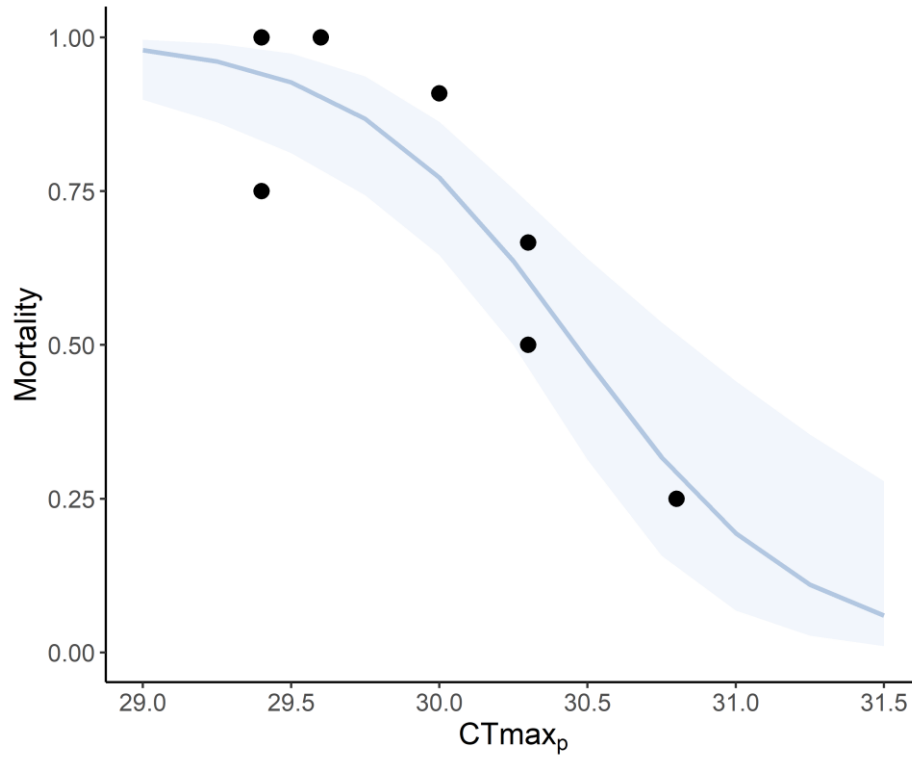
Figure 13. Mortality of *Ascaphus montanus* tadpoles when held in 25°C was related to past estimates of population CTmax (CTmax$_p$). The trend line shows the predicted probabilities and 95% confidence intervals as estimated by the logistic regression model with CTmax$_p$ as the sole predictor of mortality (p<0.0001).

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Chapter 5: Plasticity in CTmax increases with annual temperature range in spatially homogenous habitats of coastal tailed frogs

Introduction

Climate change is causing increases in global temperatures as well as the frequency and severity of extreme climatic events, altering both mean temperatures and temperature variability experienced by organisms (Jackson et al., 2009). The consequences of these changes may be magnified among ectotherms as external temperatures dictate their physiology (Gillooly et al., 2001; Angilletta, 2009). Organisms with the capacity for active, reversible physiological changes as a response to thermal cues (Gabriel et al., 2005), hereafter referred to as plasticity, may be buffered from these consequences (Stillman, 2003; Seebacher et al., 2015; Kingsolver & Buckley, 2017; Fox et al., 2019; Morley et al., 2019). Although plasticity may not be a sufficient coping mechanism against long-term, chronic warming among ectotherms (Gunderson et al., 2017), it may be particularly useful during short term acute climatic events, such as heatwaves (Kefford et al., 2022). As such, quantifying plasticity in thermal tolerance traits, such as critical thermal maximum (CTmax, the upper limit of performance), is an important component of assessing vulnerability to climate change (Thurman et al., 2020). Understanding the environmental drivers of plasticity in CTmax provides necessary *a priori* expectations of buffering ability for species in which plasticity data in CTmax are unknown and are not easily obtained.

The Climate Variability Hypothesis (CVH) is a well-established hypothesis explaining variation in thermal tolerance traits across the landscape (Janzen, 1967; Ghalambor et al., 2006). The CVH postulates that species from temporally thermally variable environments evolve

thermal tolerance traits that widen performance along temperatures, including plasticity in

CTmax (Janzen, 1967; Ghalambor et al., 2006; Calosi et al., 2008, 2010; Sheldon et al., 2018).

However, many studies to date investigating environmental variability as a driver of plasticity in

CTmax have focused on broad, macrophysiological trends among species or taxonomic groups

(e.g., Shah et al., 2017a; Morley et al., 2019). Among those focusing within species (e.g., Barria

& Bacigalupe, 2017; Mottola et al., 2022), it is exceedingly rare that studies have the statistical

power to uncover trends between plasticity in CTmax and thermal variability because they test

too few populations.

One challenge of investigating the role of temporal thermal fluctuations in driving

thermal tolerance plasticity is accounting for the effects of spatial variability in temperature on

physiological traits. Various components of microhabitats, such as the level of canopy cover and

the diversity of abiotic structures, can lead to spatial thermal variability (Woods et al., 2015) and

provide the potential for behavioral thermoregulation (Sears & Angilletta, 2015; Pincebourde et

al., 2016; Sears et al., 2016). This behavior allows organisms to avoid unfavorable temperatures,

lessening the strength of selection for traits that confer increased thermal tolerance, including

plasticity in thermal physiological traits (Bogert, 1949; Huey et al., 2003, 2012; Buckley et al.,

2015; Farallo et al., 2018; Muñoz & Bodensteiner, 2018; Enriquez-Urzelai et al., 2020). For

example, daytime behavioral thermoregulation by *Anolis* lizards has been found to shield them

from maximum temperatures, and explains reduced rates of evolution in upper thermal limits

among *Anolis* species (Muñoz et al., 2014; Muñoz & Losos, 2018). However, effective

behavioral thermoregulation requires vagile organisms that can sample the thermal environment

and a spatial configuration of temperatures that maintains a benefit of behavioral

thermoregulating relative to the costs (Sears & Angilletta, 2015; Pincebourde et al., 2016; Sears

et al., 2016). Therefore, ideal systems for testing hypotheses related to temporal temperature effects on plasticity of thermal limits are those in which organisms inhabit spatially invariable habitats such that they experience the full range of temporal fluctuations in temperature (Kefford et al., 2022).

Here, we tested the relationship between temporal temperature variability and plasticity in CTmax among populations of coastal tailed frogs, *Ascaphus truei*. The larval tadpole stage of *A. truei* inhabits cold fast-flowing headwater streams along the mountainous regions of the Pacific Northwest, USA, and western Canada. Tadpoles occupy these streams for 1-5 years before metamorphosis, experiencing the full annual temperature cycle at least once. Variation in structural microhabitat, such as canopy cover and water source, can cause temperatures within streams to vary across space (Torgersen et al., 1999; Johnson, 2004; Caissie, 2006; Dent et al., 2008; Vatland et al., 2015; Schultz et al., 2017). However, *A. truei* tadpoles are highly limited in movement, moving an average of 1.1m/day (Wahbe & Bunnell, 2001). Therefore, the fast-flowing nature of these streams may homogenize spatial temperatures at a scale relevant to this species, regardless of variation in structural microhabitat. Thus, this system may provide a unique opportunity to test the CVH among populations of a species without the confounding effects of spatial variability in temperature.

We targeted our sampling of *A. truei* population along elevation gradients to capture variation in temporal temperatures. For each population sampled, we calculated spatial temperature variability within streams at scales relevant to tadpole tailed frogs (<100m), captured temporal temperature variation using in-stream temperature loggers, and estimated plasticity in CTmax among tadpoles. Using data from spatially homogenous streams (<2°C range in temperatures (EPA, 2003)), we tested the hypothesis that temporal thermal variability is

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related to plasticity in CTmax (i.e., the CVH). In the absence of spatial variability in temperature, we expected to find a strong, positive relationship between the magnitude of plasticity in CTmax and the level of temporal thermal variability among populations, such that populations from highly temporally variable habitats exhibit more plasticity in CTmax. This result would support for the CVH and provide insight into its role in shaping plasticity in CTmax among populations that do not have the capacity to behaviorally avoid unpreferable temperatures.

Methods

Plasticity in CTmax

We collected 480 tadpoles (developmental stages 26.5 to 45; Gosner, 1960) from ten *A. truei* populations in Oregon using hand-nets (~48 tadpoles/population). Tadpoles from each population were evenly and randomly split into one of two holding temperature treatments: 8°C and 15°C. After three days of holding tadpoles without food, CTmax experiments were performed via dynamic temperature ramping (Overgaard et al., 2012) at a rate of 0.3°C min^{-1}. CTmax experiments were either performed from a starting temperature of 8°C or 15°C to account for acute temperature responses (Cicchino et al., 2023b). Oxygen saturation was maintained throughout holding and experiments using water pumps and air bubblers. CTmax was considered the point at which a tadpole no longer responded to tactile stimuli. After CTmax was reached, tadpoles were moved to a tank with ~8°C water to recover (i.e., defined as swimming and responding to tactile stimuli) to ensure that lethal temperatures were not reached. Following recovery, all tadpoles were euthanized using a 20% benzocaine solution, photographed for length measurements, fixed in 10% formalin, and stored in 70% ethanol. We measured tadpole length (tip of nose to tip of tail) using ImageJ software (Rasband, 2009).

Characterizing Stream Temperatures

Spatial variability in temperature was measured for each sampled population by measuring temperatures at ~100 points in space within he sampled stream area (~100m). The full protocol for these measurements can be found in (Cicchino et al., 2023a). Briefly, stream bed temperatures were measured at fixed intervals with random offsets along horizontal transects of the stream (i.e., across stream width) at pre-identified transects along the length of the stream, targeting areas of *Ascaphus* habitat. We also opportunistically sampled stream seeps and confluences that may lead to thermal anomalies. The United States Environmental Proteiction Agency defines thermal refuges as temperatures with a 2°C difference from surrounding temperatures. Using this definition as a guide, we identified spatially homogenous populations as those having <2°C in the range of spatial temperatures measured.

For each sampled population, we deployed two temperature loggers (Hobo 64K Pendant Water Temperature Data Loggers, Onset Computer Corporation, Bourne, MA USA) within the streams in the sampled area. Temperature loggers were housed in PVC pipes with drilled holes to allow for water flow and secured to a nearby tree using plastic-coated metal wire. Pipes were secured to in-stream rebar poles to ensure loggers were held near the streambed, which is the microhabitat that tadpoles occupy. We logged temperatures every four hours for about one year. Temperature data underwent quality control (Dunham et al., 2005), then were used to calculate the following metrics defined in Table 4: Maximum annual temperature range, Range of mean daily temperatures, Absolute maximum temperature, Mean of the ten warmest days. As these metrics were highly correlated (Pearson's correlation coefficient > 0.7, p<0.01), we used the maximum annual temperature range for subsequent analyses.

Statistical Analyses

All statistical analyses were performed using R version 4.2.2 (R Core Team, 2022). We used a mixed effects model to test the relationship between maximum annual temperature range and plasticity in CTmax by including CTmax as the response variable, holding temperature and maximum annual temperature range as both independent and interacting predictors. We also included the experimental starting temperature, and tadpole length as covariates and population as a random intercept to account for variation due to unaccounted population differences. Using this model, a significant interaction term would mean that the relationship between CTmax and holding temperature (i.e., plasticity) was dependent on maximum annual temperature range. A positive interaction term would signify that plasticity increased with maximum annual temperature range and support our prediction. A a negative interaction term would be interpreted as plasticity being lower in populations that experience greater maximum annual temperature ranges. We visually inspected the residuals of the model for assumption of linearity, error variance for assumption of heteroscedasticity, checked for a lack correlations among predictors variables, and compared model performance against the null model with only a random population effect.

Results

Of the ten populations sampled, one was considered spatially variable, Augusta Creek (Figure 14), and was excluded from the model testing the CVH. We found that annual temperature ranges varied greatly among populations of *A. truei* (8.6 – 15.8°C). Results from our mixed effects model demonstrated that the level of plasticity in CTmax was significantly positively related to stream maximum annual temperature range (Table 5; Figure 15). Interestingly, when Augusta Creek, this significant relationship disappeared when Augusta Creek was included in the model (Appendix 3: Table S11, Figure S5).

Discussion

Organisms that inhabit temporally and spatially variable environments are expected to have physiological traits that confer performance at a wide range of temperatures (i.e., the CVH)(Janzen, 1967; Ghalambor et al., 2006; Calosi et al., 2008, 2010; Sheldon et al., 2018). This hypothesis has been widely tested at the macrophysiological scale (e.g., Sunday et al., 2011; Gutiérrez-Pesquera et al., 2016; Shah et al., 2017b, 2017a; Markle & Kozak, 2018) though less attention has been directed to testing the CVH within species (Barria & Bacigalupe, 2017; Mottola et al., 2022). Furthermore, the mediating effects of spatial thermal variability through behavioral thermoregulation are often challenging to account for (Kefford et al., 2022). Here, we took advantage of a system with known variation in plasticity in CTmax among populations (Cicchino et al., 2023b) and limited capacity for behavioral thermoregulation in nature (Cicchino et al., 2023a) to test the CVH within a species. Investigating spatially homogenous populations, our results demonstrated that populations experiencing higher annual maximum temperature ranges exhibited greater plasticity in CTmax. Our results add to the growing evidence of the CVH, contributing important insight into its role in shaping variation in thermal trait plasticity within species.

We found that populations experiencing increased temporal thermal variability exhibited greater plasticity in CTmax. This pattern has been observed among species and across broad geographic scales (Shah et al., 2017a; Morley et al., 2019). However, within species, few studies have investigated this relationship, and of the ones that have, the pattern is yet to be strongly supported (Barria & Bacigalupe, 2017; Mottola et al., 2022). For example, Barria & Bacigalupe (2017) found that two populations of four-eyed frog (*Plurodema thaul*) occupying different environments varied in plasticity in CTmax, among other traits. They conclude that the variation

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may be due to environmental differences, but do not have the power to test that prediction. Thus, these results represent the first test of the relationship between temporal temperature variation and CTmax plasticity among multiple populations of a species.

The correlation we found among stream temperature metrics suggests that plasticity in CTmax may also be related to other aspects of temperature variation. The streams sampled in this study reach similar, near-freezing temperatures during the winter, thus annual temperature variability is largely dictated by maximum temperatures. As such, plasticity in CTmax among these populations alternatively may be driven by maximum temperatures as a strategy to cope with warm temperatures without maintaining high tolerance year-round. To parse the effects of maximum temperature and temperature variability on plasticity in CTmax, studies need to investigate populations that occupy environments where temporal temperature variability is not driven by maximum temperatures (i.e., minimum temperatures must also vary).

Despite experiencing the lowest level of temporal thermal variability, plasticity in CTmax was unexpectedly high in the Augusta Creek population. This outlier may be explained by the high amount of spatial variability in this stream reach relative to the others in this study (double the range in spatial temperatures; Cicchino et al., 2023b). Although spatial variability is often considered as a buffer from selection for increased thermal tolerance (Muñoz et al., 2014; Muñoz & Bodensteiner, 2018; Bodensteiner et al., 2021), its role in shaping thermal physiological traits is dependent on the ecology of the organism. Due to the limited movement of *A. truei* tadpoles, it is unlikely that tadpoles subsample spatial temperatures to choose a preferred location. Rather, *A. truei* tadpoles may randomly be moved short distances downstream when they lose suction on stream substrate before quickly reattaching (Gaige, 1920). The bioenergetic demands associated with being in a warmer locale and moving out of it to colder temperatures may be greater than

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the cost of physiologically coping in place (Snyder et al., 2022), therefore selection may favor plastic thermal limits to increase coping capacity in these environments.

Tables & Figures

Table 4. Definitions of metrics calculated using in-stream temperature logger data.

Metric	Definition
Maximum annual temperature range	The absolute maximum temperature minus the absolute minimum temperature
Range of mean daily temperatures	Annual maximum of the average daily temperatures minus the annual minimum of the average daily temperatures
Absolute maximum temperature	The highest temperature experienced
Mean of the ten warmest days	Average of the highest ten daily maximum temperatures

Table 5. Results from linear mixed effects model testing the effects of maximum annual temperature range on plasticity in CTmax among populations. Tadpole length and experimental starting temperautre were included as covariates and population as a random effect. A significant interaction term between maximum annual temperature range and holding temperature signifies that plasticity in CTmax is dependent on a population's experienced maximum annual temperature range.

Fixed Effects	Estimate	SE	df	p-value
(Intercept)	29.66	0.07	8.83	$2E^{-16}$
Maximum Annual Temperature Range x Holding Temperature	0.12	0.03	420.08	$8E^{-4}$
Holding Temperature	0.36	0.03	420.08	$2E^{-16}$
Maximum Annual Temperature Range	-0.21	0.07	8.06	0.02
Experimental Starting Temperature	0.07	0.03	420.11	0.04
Tadpole Length	-0.10	0.02	426.98	$2.72E^{-6}$
Random Effects	**Variance +/- SD**			
Population	0.04 **+/-** 0.20			
Residual	0.14**+/-** 0.37			
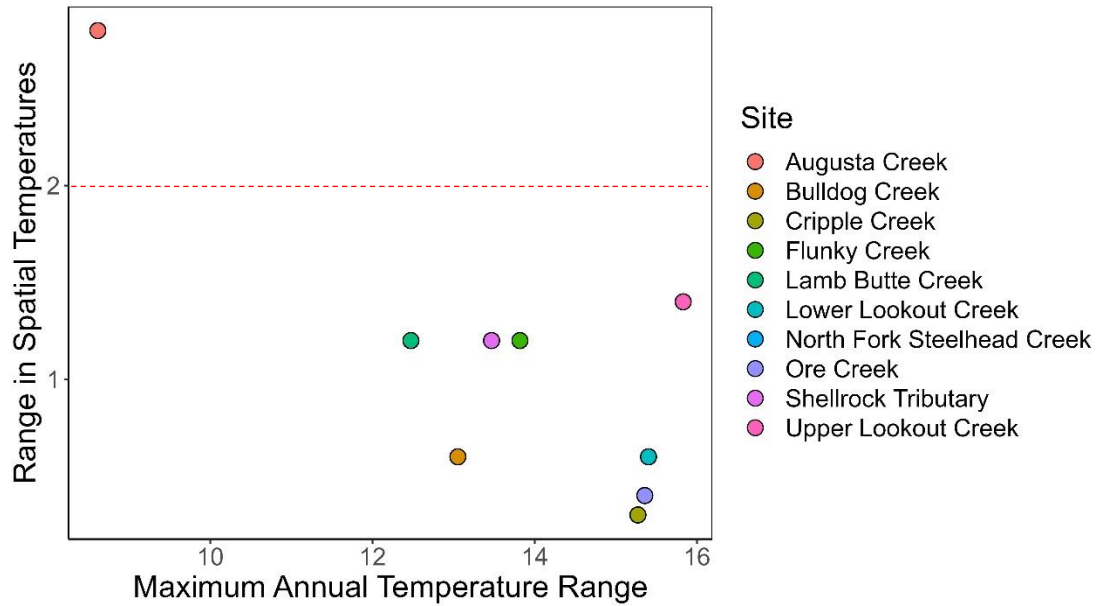
Figure 14. Populations varied in maximum annual temperature range, a metric of temporal temperature variability. Augusta Creek was the only population sampled with spatially variable temperatures (>2°C). The range of spatial temperatures at Augusta Creek (2.8°C) were double the next highest value (1.4°C).
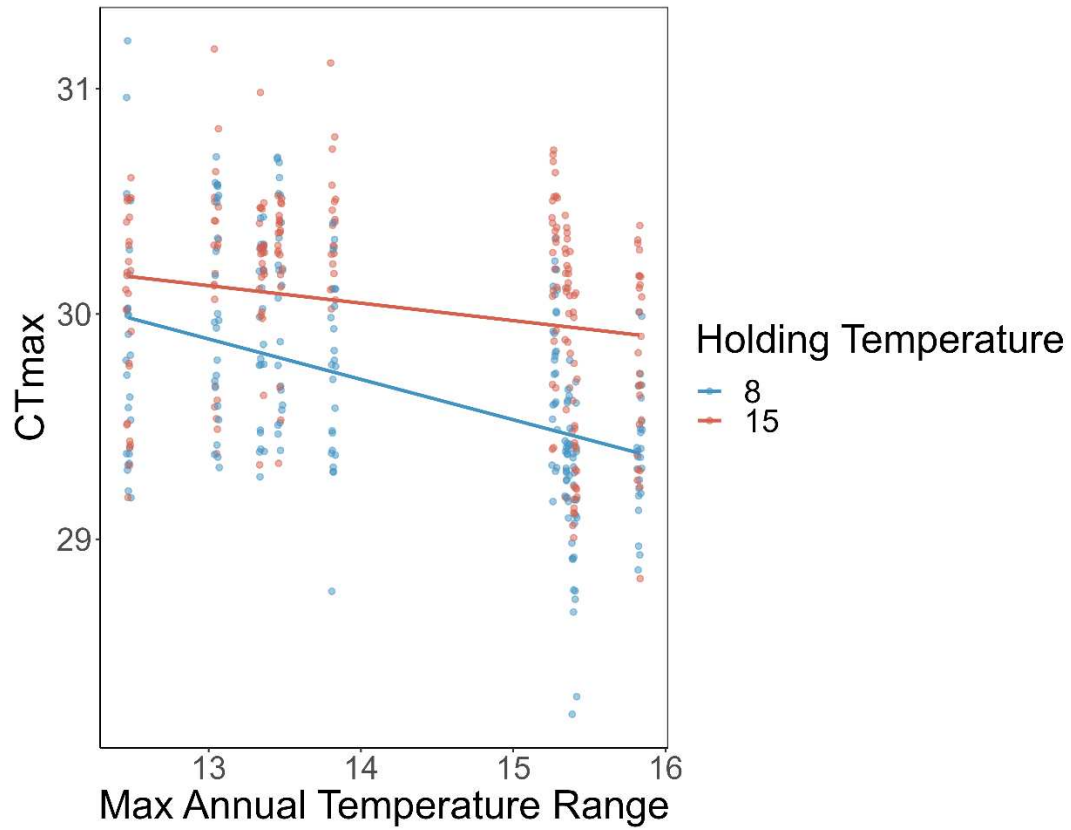
Figure 15. The effect of holding temperature (colors) on CTmax (i.e., plasticity) increased with maximum annual temperature range (p<0.0001). Shown are raw CTmax data points with prediction slopes estimated by the linear mixed effects model.

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APPENDIX 1: SUPPLEMENTAL TABLES AND FIGURES FOR CHAPTER 2

Table S1. Summarized location and sampling information for each population.

Species	Population	Elevation (m)	Latitude, Longitude	Logger Dates	Stream Maximum Temperature (°C)	Date Collected	N					
							Total	Final	Held 8°C; Tested 8°C	Held 15°C; Tested 8°C	Held 8°C; Tested 15°C	Held 15°C; Tested 15°C
Ascaphus montanus	Dry Creek	1044	47.191, -115.099	10/1/2017-9/30/2018	13.12	8/10/2017	35	35	10	7	9	9
	Lower Lost Horse Creek	1527	46.136, -114.402	10/1/2017-9/30/2018	18.09	8/11/2017	48	46	11	11	12	12
	Upper Lost Horse Creek	1950	46.144, -114.505	10/1/2017-9/30/2018	12.51	8/2/2017	44	43	10	12	9	12
	Upper Torino Tributary	1620	47.149, -115.171	10/1/2017-9/30/2018	20.07	8/4/2017	48	48	12	12	12	12
Ascaphus truei	Augusta Creek	1253	43.909, -122.158	07/16/17-05/31/18	8.98	7/15/2017	47	46	12	12	10	12
	Bulldog Creek	1191	43.409, -122.523	10/1/2017-9/30/2018	12.94	7/22/2017	47	47	12	10	13	12
	Cripple Creek	451	45.116, -122.058	08/01/2018-07/31/2019	14.88	7/5/2018	51	51	13	13	13	12
	Flunky Creek	533	44.254, -122.236	10/1/2017-9/30/2018	14.77	7/24/2017	43	43	12	8	11	12
	Lamb Butte	1218	44.087, -122.081	10/1/2017-9/30/2018	11.95	6/27/2018	52	52	13	13	13	13
	Lower Lookout Creek	577	44.232, -122.202	08/01/2018-07/31/2019	15.75	6/21/2018 for treatments held at 8°C ; 6/30/2018 for treatments held at 15°C	49	49	13	13	11	12
	Ore Creek	506	44.234, -122.277	08/01/2018-07/31/2019	19.73	6/19/2018	48	48	13	11	12	12
	Shellrock Creek	1159	45.129, -121.965	10/1/2017-9/30/2018	13.07	7/20/2018	44	44	13	10	10	11
	Steelhead Creek	577	43.398, -122.666	08/01/2018-07/31/2019	15.50	7/11/2018	49	49	12	12	13	12
	Upper Lookout Creek	1074	44.222, -122.117	10/1/2017-9/30/2018	15.35	7/3/2018	50	50	13	13	12	12

Note: All *Ascaphus montanus* populations were sampled from Montana, USA, and all *A. truei* populations were sampled from Oregon, USA. Sample sizes for each population are provided as the total sampled ("N") and the total sample size used for analyses (i.e., after missing data and non-recovered individuals were removed; "Final N"). "Logger Dates" provides in-stream temperature logger date ranges.

Table S2. The results from the linear model investigating starting temperature and holding temperature effects on CTmax, with species included in a three-way interaction with those two variables.

Fixed Effects	Estimate	SE	t-value	df	p-value
(Intercept)	30.471	0.162	187.605	20.758	<0.001
Starting Temperature	-0.241	0.084	-2.875	629.856	0.004
Species	-0.351	0.177	-1.989	14.963	0.065
Holding Temperature	0.054	0.084	0.638	629.951	0.524
Tadpole Size	-0.104	0.017	-6.125	640.766	<0.001
Starting Temperature: Species	0.258	0.097	2.649	629.847	0.008
Starting Temperature: Holding Temperature	0.465	0.118	3.931	629.861	<0.001
Species: Holding Temperature	0.267	0.098	2.728	629.925	0.007
Starting Temperature: Species: Holding Temperature	-0.347	0.138	-2.520	629.842	0.012

Random Effects	Variance	SD
Population (intercept)	0.076	0.275
Residual	0.15	0.387

Note: Tadpole size is included as a covariate. The significance of the three-way interaction led us to investigate these temperature effects within each species.

APPENDIX 2: SUPPLEMENTAL TABLES AND FIGURES FOR CHAPTER 3

Table S3. Sampled streams for each species, including upper thermal limits experiment sample sizes, temperature logger ranges, and the date fine-scale temperature variation was collected. The temperature logger ranges represent the water year (or closest we could achieve) used to quantify thermal regime metrics. This range includes only quality-controlled data, which is why some of the date ranges may vary.

Species	Site	Sample Size (N)	Temperature Logger Range	Fine-scale Temperature Variation Date
Ascaphus montanus	Dry Creek	10	10/01/2017-09/30/2018	8/9/2019
	Hoodoo Creek	25	10/01/2017-09/30/2018	8/8/2019
	[Low] Cedar Creek	24	10/01/2017-09/30/2018	8/8/2019
	[Low] Little Joe Creek	24	10/01/2017-09/30/2018	8/9/2019
	Lower Lost Horse Creek	11	10/01/2017-09/30/2018	8/13/2019
	[Mid] Cedar Creek	24	10/01/2017-09/30/2018	
	[Mid] Torino Tributary	97	10/01/2017-09/30/2018	8/9/2019
	Moore Creek	24	10/01/2017-09/30/2018	
	North Branch Marten Creek	24	10/01/2017-09/30/2018	8/10/2019
	Oregon Gulch	24	10/01/2017-09/30/2018	8/12/2019
	South Fork Trout Creek	24	10/01/2017-09/30/2018	8/4/2019
	Upper Lost Horse Creek	11	10/01/2017-09/30/2018	8/11/2019
	Upper Torino Tributary	12	10/01/2017-09/30/2018	8/12/2019
	Van Ness Creek	24	10/01/2017-09/30/2018	8/3/2019
Ascaphus truei	Augusta Creek	12	07/16/2017-05/31/2018	8/9/2019
	Bear Creek	24	10/01/2016-09/30/2017	
	Bulldog Creek	12	10/01/2017-09/30/2018	8/12/2019
	Cripple Creek	13	08/01/2018-07/31/2019	8/11/2019
	Flunky Creek	12	10/01/2017-09/30/2018	8/7/2019
	Hardy Creek	20	07/08/2017-05/01/2018	8/9/2019
	Kink Creek	25	10/01/2017-09/30/2018	8/11/2019
	Lamb Butte	38	10/01/2017-09/30/2018	8/8/2019
	[Low] Lookout Creek	13	08/01/2018-07/31/2019	8/9/2019
	Marten Creek	12	10/01/2016-09/30/2017	
	South Fork Steelhead Creek	12	08/01/2018-07/31/2019	
	Ore Creek	13	08/01/2018-07/31/2019	8/8/2019
	Shellrock Tributary	37	10/01/2017-09/30/2018	8/11/2019
	Tidbits Creek	97	10/01/2017-09/30/2018	8/8/2019

	[High] Lookout Creek	38	10/01/2017-09/30/2018	8/7/2019

Table S4. Estimated linear mixed effects model parameters and results from ANOVA (type III, Satterthwaite's method) testing the effects of maximum stream temperature and species on CTmax, with tadpole length included as a covariate and population as a random effect.

Fixed Effects	Estimate (SE, df)	SumSq	F (df)	p-value
(Intercept)	29.76 (0.07, 25.18)	-	-	$2E^{-16}$
Maximum Stream Temperature	0.23 (0.05, 26.25)	0.32	2.18 (1, 27.05)	0.15
Species	-0.08 (0.10, 25.57)	0.10	0.72 (1, 25.57)	0.40
Tadpole Length	-0.15 (0.02, 25.57)	1.88	72.59 (1, 721.54)	$2E^{-16}$
Species: Maximum Stream Temperature	-0.33 (0.09, 27.03)	10.67	12.72 (1, 27.03)	0.001
Random Effects	**Variance +/- SD**			
Population	0.07 +/- 2.64			
Residual	0.15 +/- 0.38			

Table S5. Estimated regression parameters and results from ANOVA performed using the *A. montanus* dataset that was restricted to represent the maximum temperature range found in *A. truei* streams.

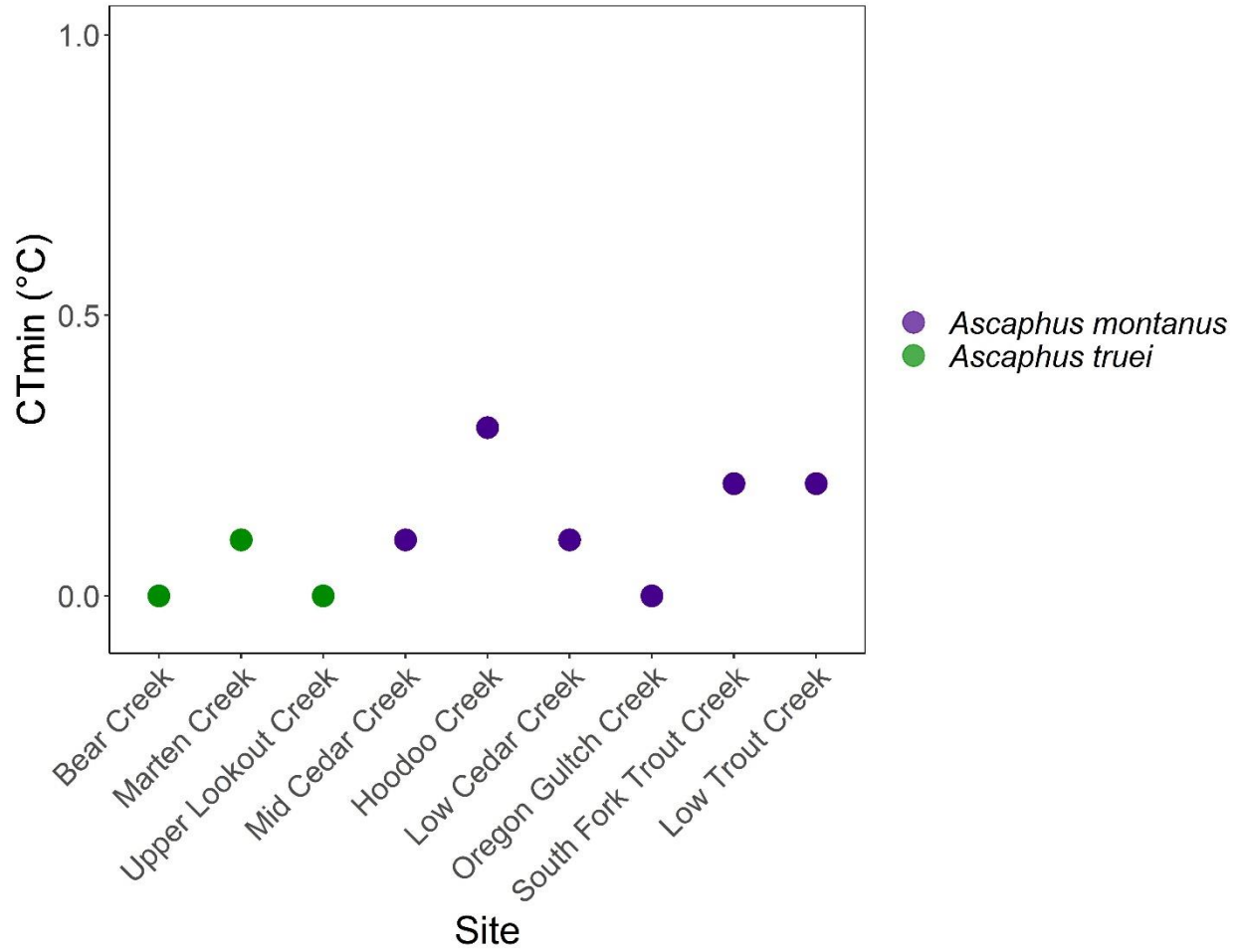| Ascaphus montanus – restricted dataset				
Fixed Effects	**Estimate (SE, df)**	**SumSq**	**F(df)**	**p-value**
(Intercept)	29.71 (0.05, 8.72)	-	-	$2E^{-16}$
Maximum Stream Temperature	0.27 (0.05, 8.50)	4.24	31.70 (1,8.50)	0.0004
Tadpole Length	-0.19 (0.02, 247.66)	7.94	59.36 (1,247.66)	$3.16E^{-13}$
Random Effects	**Variance +/- SD**			
Population	0.03 +/- 0.16			
Residual	0.13 +/- 0.37			

Figure S1. Results from the lower thermal limits experiments measuring critical thermal minimum. Each point represents nine tadpoles. All tadpoles tested were active at the lowest temperatures we could achieve experimentally without freezing (0°C-0.3°C).
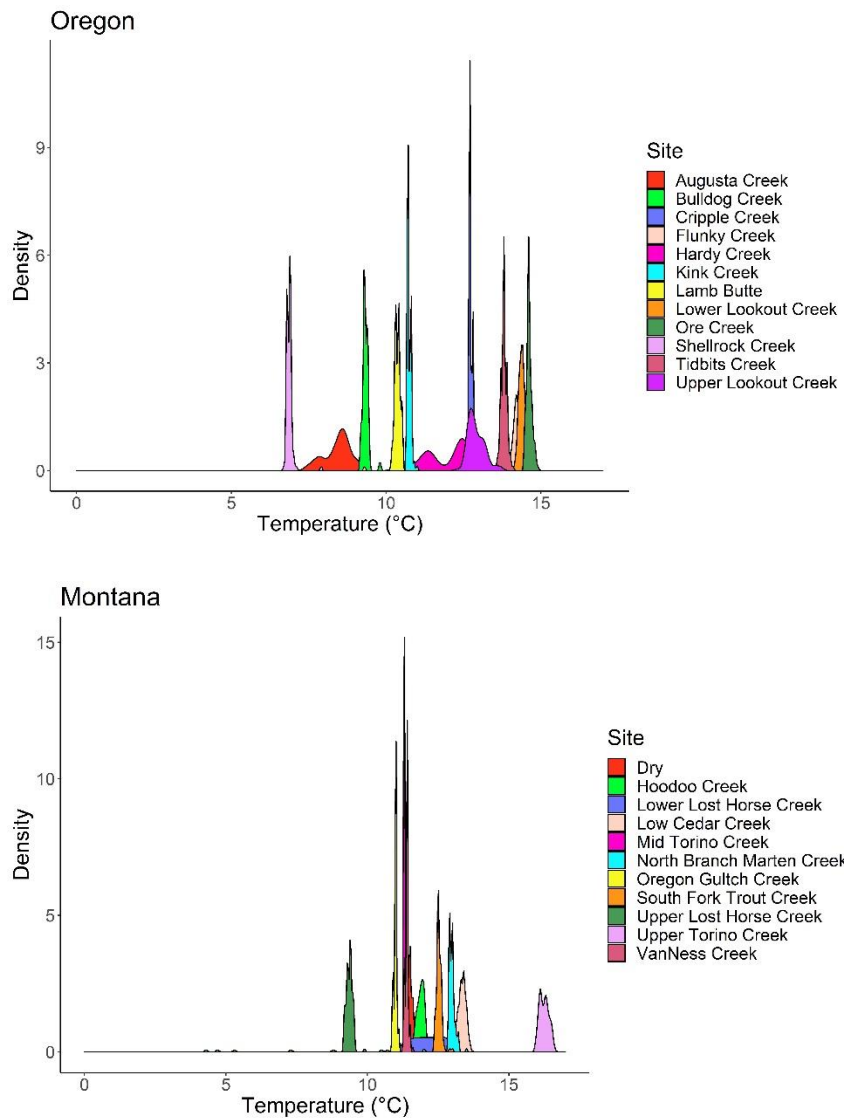
Figure S2. Fine-scale (within stream reach) temperatures are spatially homogenous for both *Ascaphus montanus* (Montana) and *A. truei* (Oregon). Figures show the distributions of temperature recordings (>100 points) for each sampled stream reach in Oregon (top) and Montana (bottom).
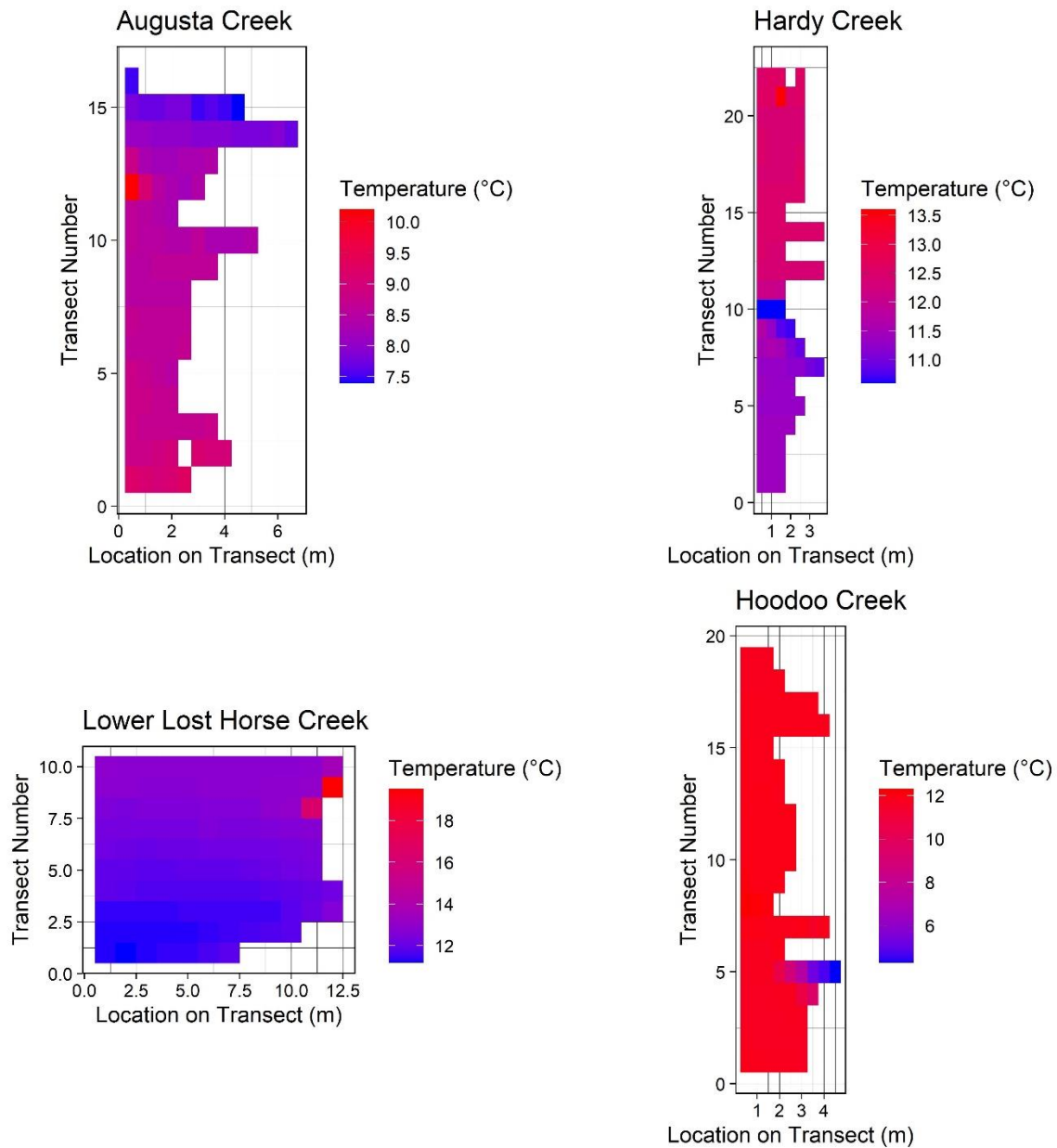
Figure S3. Spatially-explicit temperature maps for the four streams that exhibited a range of temperatures greater than 1.6°C (Oregon: Augusta Creek, Hardy Creek; Montana: Lower Lost Horse Creek, Hoodoo Creek). Note the temperature different scales for each plot. From these figures, we characterized these stream reaches as having a thermal gradient with one or few clumped potential refuges.

Figure S4. Summarized CTmax for each species, with triangles representing the mean values.

APPENDIX 3: SUPPLEMENTAL TABLES AND FIGURES FOR CHAPTER 4
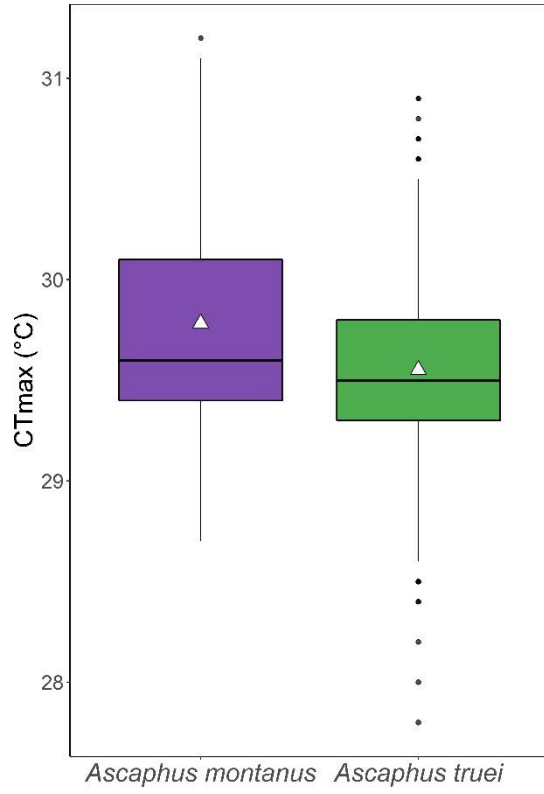
Table S6. Population locality information, tadpole collection dates, and sample sizes for the CTmax and mortality from thermal stress experiments.

Population	Latitude	Longitude	CTmax$_p$		Mortality	
			Date of Collection	Sample Size	Date of Collection	Sample Size
Dry Creek	47.19	-115.10	10-Aug-17	10	13-Jul-19	5°C: 12 10°C: 12 **15°C: 11** **20°C: 13** 25°C: 12
Lower Lost horse Creek	46.14	-114.40	11-Aug-17	11	21-Jul-19	5°C: 12 10°C: 12 15°C: 12 20°C: 12 25°C: 12
Low Cedar Creek	47.13	-115.00	3-Aug-17	24	3-Jul-19	5°C: 12 10°C: 12 15°C: 12 20°C: 12 25°C: 12
North Branch Marten Creek	47.89	-115.88	27-Aug-17	24	28-Jul-19	5°C: 12 10°C: 12 15°C: 12 20°C: 12 25°C: 12
Oregon Gulch	47.06	-115.09	9-Aug-17	24	6-Jul-19	5°C: 12 10°C: 12 15°C: 12 20°C: 12 25°C: 12
Upper Lost Horse Creek	46.14	-114.50	2-Aug-17	11	22-Jul-19	5°C: 12 10°C: 12 15°C: 12 20°C: 12 **25°C: 11**
Upper Torino Tributary	47.15	-115.17	4-Aug-17	12	14-Jul-19	5°C: 12 10°C: 12 15°C: 12 20°C: 12 25°C: 12

Table S7. Results from correlation test among thermal experience metrics presented as Pearson's coefficient (p-value).

	Annual	Long-term
Immediate	0.71 (0.07)	0.54 (0.21)
Annual	-	0.25 (0.59)

Table S8. Results from the logistic regression with the order of tadpole collection as the predictor of mortality in the 25°C treatments. Note that these are in the log scale.

	Estimate	Standard Error	Z-value	P-value
Intercept	0.81	0.54	1.51	0.13
Order of collection	0.04	0.12	0.30	0.76

Table S9. Results from the logistic regression with $CTmax_p$ as the sole predictor of mortality in the 25°C treatments. Note that these are in the log scale.

	Estimate	Standard Error	Z-value	P-value
Intercept	80.61	20.31	3.97	7.20e-05
$CTmax_p$	-2.65	0.67	-3.94	8.28e-05

Note: The odds-ratio for $CTmax_p$ is produced by exponentiating the estimate (=0.07). The odds ratio here is the multiplicative decrease in the odds of mortality per 1°C increase in $CTmax_p$. In Figure 2, we show the predicted probability trend. Predicted probabilities are not constant across $CTmax_p$ and are derived for each modeled point by dividing the odds for each point by the odds +1. For example, if the odds of mortality at a $CTmax_p$ of 30.3 is 1 (6 died: 6 survived), then 1 one degree increase in $CTmax_p$ to 31.3°C is associated with an odds of mortality of 1*0.07=0.07. The probability of mortality at a $CTmax_p$ of 31.3°C is 0.07/1+0.07 = 6.5%.

Table S10. Full model comparison results for models explaining variation in mortality at 25°C. AICc weights demonstrate the probability that a model is the best model. Evidence Ratios are presented in reference to the top model.

Model Predictors	AICc	ΔAICc	AICc Weight	Evidence Ratio
$CTmax_p$	30.93	0.00	0.86	1
$CTmax_p$ + Immediate Thermal Experience	36.70	5.77	0.05	17.9
$CTmax_p$ + Long-term Thermal Experience	37.09	6.16	0.04	21.75
$CTmax_p$ + Annual Thermal Experience	37.80	6.86	0.03	30.92
Immediate Thermal Experience	39.04	8.11	0.01	57.54
Annual Thermal Experience	39.39	8.46	0.01	68.78
Long-term Thermal Experience	43.98	13.05	0.00	680.3
Null	48.34	17.40	0.00	6016.36
$CTmax_p$ + Annual Thermal Experience + $CTmax_p$ x Annual Thermal Experience	49.06	18.13	0.00	8629.27
$CTmax_p$ + Immediate Thermal Experience + $CTmax_p$ x Immediate Thermal Experience	50.58	19.65	0.00	18500.05
$CTmax_p$ + Long-term Thermal Experience + $CTmax_p$ x Long-term Thermal Experience	51.02	20.09	0.00	22988.47

APPENDIX 4: SUPPLEMENTAL TABLES AND FIGURES FOR CHAPTER 5

Table S11. Mixed effect model results after including the one spatially variable population, Augusta Creek, in the analyses.

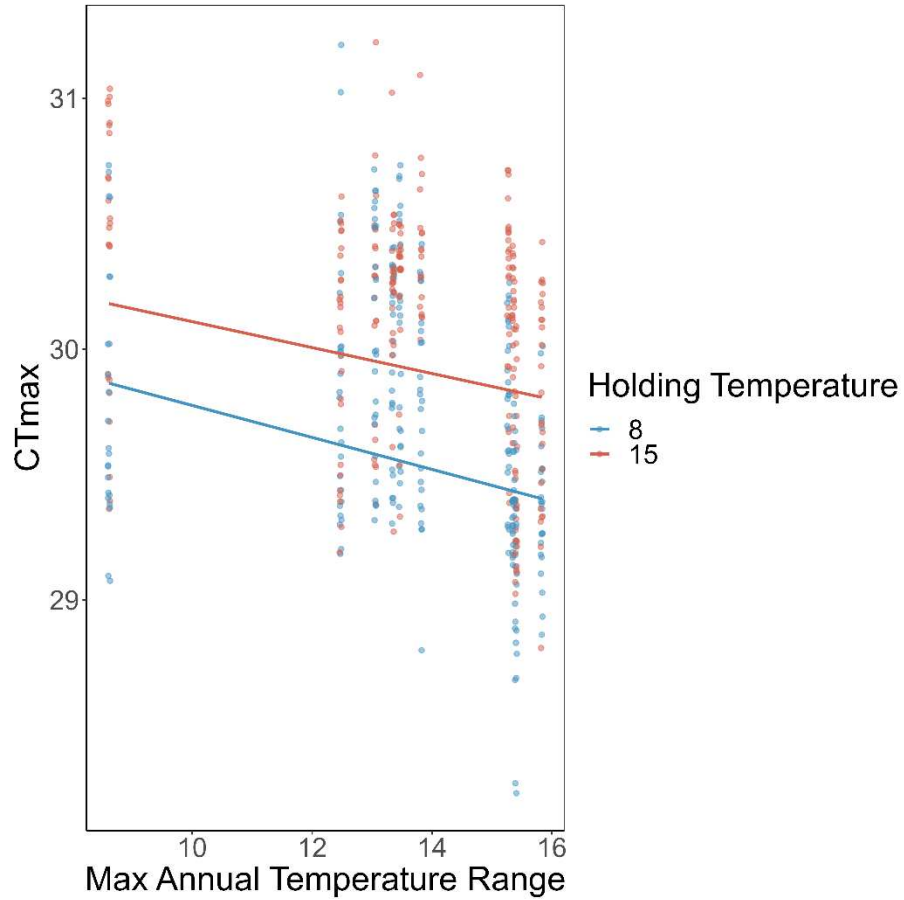| With Augusta Creek				
Fixed Effects	**Estimate**	**SE**	**df**	**p-value**
(Intercept)	29.67	0.73	10.10	2E^{-16}
Maximum Annual Temperature Range x Holding Temperature	0.02	0.03	465.07	0.50
Holding Temperature	0.38	0.04	465.07	2E^{-16}
Maximum Annual Temperature Range	-0.13	0.07	9.26	0.10
Experimental Starting Temperature	0.07	0.03	465.12	0.03
Tadpole Length	-0.12	0.02	472.88	5.64E^{-9}
Random Effects	**Variance +/- SD**			
Population	0.04 **+/-** 0.21			
Residual	0.15 **+/-** 0.39			

Figure S5. When Augusta Creek is included in the analyses testing the effect of stream temporal temperature range in plasticity in CTmax, the relationship previously observed disappears. Here, parallel relationships between holding temperature treatments suggest that the plastic response among populations is equal across temperature ranges.