




ARTICLE

Climate Ecology

Acclimation capacity of critical thermal maximum varies among populations: Consequences for estimates of vulnerability

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Abstract

Adaptive plasticity in thermal tolerance traits may buffer organisms against changing temperatures, making such responses of particular interest in the face of global climate change. Although population variation is integral to the evolvability of this trait, many studies inferring proxies of physiological vulnerability from thermal tolerance traits extrapolate data from one or a few populations to represent the species. Estimates of physiological vulnerability can be further complicated by methodological effects associated with experimental design. We evaluated how populations varied in their acclimation capacity (i.e., the magnitude of plasticity) for critical thermal maximum (CTmax) in two species of tailed frogs (Ascaphidae), cold-stream specialists. We used the estimates of acclimation capacity to infer physiological vulnerability to future warming. We performed CTmax experiments on tadpoles from 14 populations using a fully factorial experimental design of two holding temperatures (8 and 15°C) and two experimental starting temperatures (8 and 15°C). This design allowed us to investigate the acute effects of transferring organisms from one holding temperature to a different experimental starting temperature, as well as fully acclimated responses by using the same holding and starting temperature. We found that most populations exhibited beneficial acclimation, where CTmax was higher in tadpoles held at a warmer temperature, but populations varied markedly in the magnitude of the response and the inferred physiological vulnerability to future warming. We also found that the response of transferring organisms to different starting temperatures varied substantially among populations, although accounting for acute effects did not greatly alter estimates of physiological vulnerability at the species level or for most populations. These results underscore the importance

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of sampling widely among populations when inferring physiological vulnerability, as population variation in acclimation capacity and thermal sensitivity may be critical when assessing vulnerability to future warming.

KEYWORDS

amphibian, *Ascaphus*, climate change, conservation physiology, passive plasticity, plasticity, thermal limits

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; Gunderson & Stillman, 2015; Pinsky et al., 2019; Sunday et al., 2014). Although these proxies have their shortcomings (Clusella-Trullas et al., 2021; Garcia et al., 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 (Clusella-Trullas et al., 2011; Deutsch et al., 2008; Kingsolver et al., 2013; Sunday et al., 2014). 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 and extrapolation from few populations), which have led to questions about the accuracy of the predictions (Clusella-Trullas et al., 2021; Sinclair et al., 2016).

One question that has received considerable interest is the degree to which plasticity in thermal tolerance may ameliorate the consequences of global warming (Calosi et al., 2008; Huey et al., 2012; Seebacher et al., 2015; Stillman, 2003). Plastic responses to temperature changes (i.e., thermal acclimation) can alter thermal tolerance traits, including critical thermal maxima (CT_{max})—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 et al., 2017; Gunderson & Stillman, 2015; Morley et al., 2019; Seebacher et al., 2015). For example, Gunderson et al. (2017) found that plasticity in CT_{max} (i.e., thermal acclimation of CT_{max}) reduced the number

of predicted overheating days, but this benefit was variable among the 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 et al., 2017). By contrast, other analyses suggest that acclimation can provide a significant buffer to future warming by increasing CT_{max} (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 & Morley, 2020; Overgaard et al., 2012; Terblanche et al., 2007), 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 & 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 et al., 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 and up- or downregulation of heat-shock proteins; Angilletta, 2009; Sinclair & Roberts, 2005) 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 (Cochrane et al., 2015; Herrando-Pérez et al., 2019; Sears et al., 2011; 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 (Duffy et al., 2021; Feder et al., 2000; Galván et al., 2022; Huey & Kingsolver, 1989), and populations are known to vary in their acclimation capacity (Barley et al., 2021; Gervais et al., 2021; Peck et al., 2014; Seebacher et al., 2012; Tonione et al., 2020). Thus, we would expect acute and acclimation responses in traits such as critical thermal limits (e.g., CT_{max}) to vary within and among populations (Bubliy et al., 2002; Cossins & Bowler, 1987; Somero, 2004). The inclusion of intraspecific variation in vulnerability proxies typically increases the predictive power of models (Barley et al., 2021; Herrando-Pérez et al., 2019) 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 CT_{max} on estimates of vulnerability for two species of tailed frogs (family: Ascaphidae). We chose to investigate CT_{max} as it occurs in the stressful temperature range of an organism, when heat stress accumulates and normal functions (e.g., development and growth) are halted (Ørsted et al., 2022). Therefore, the acclimation capacity of CT_{max} may be critical for organisms to reduce the consequences of heat stress in warming environments.

To quantify variation within and between species, we measured the CT_{max} 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 CT_{max} 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 CT_{max}, 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 CT_{max} (i.e., transfer to a cold temperature would decrease CT_{max}; transfer to a warm temperature would increase CT_{max}). Alternatively, if a rapid exposure to a warmer or colder temperature elicits an acute stress response (e.g., rapid enzymatic denaturation), then we expected CT_{max} to decrease regardless of the direction of temperature transfer. We calculated acclimation capacity using two methods (Figure 1) 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 and collection

A. truei and *A. montanus* occupy cold, fast-flowing streams in forested landscapes of the Klamath Mountains, Coast Ranges, and Cascade Mountains (*A. truei*), and Northern Rocky Mountains (*A. montanus*) of the United States and Canada. The fecundity of these species is low compared with other frogs, as females may lay clutches of ~57 eggs (*A. truei*) and ~65 eggs (*A. montanus*) every other year (Hayes & Quinn, 2015; Karraker et al., 2006), suggesting a relatively larger impact of larval (i.e., tadpole) survival on population ecology (Biek et al., 2002). Tadpoles of these species may take 1–4 years to metamorphose into juvenile frogs (Hayes & 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 et al., 2016). The tadpoles play a critical role in stream ecosystems, as they account for much of the benthic algae consumption in these streams (Mallory & Richardson, 2005).

Previous research has demonstrated population variation in tadpole CT_{max} for both *Ascaphus* species (Cicchino, Shah, et al., 2023) and generally low thermal tolerances compared with other frogs (Bury, 2008; Cicchino, Shah, et al., 2023), suggesting sensitivity to increasing temperatures. Many studies have linked thermal physiological traits to population ecology and persistence through stressful temperatures (Bernhardt, 2019;

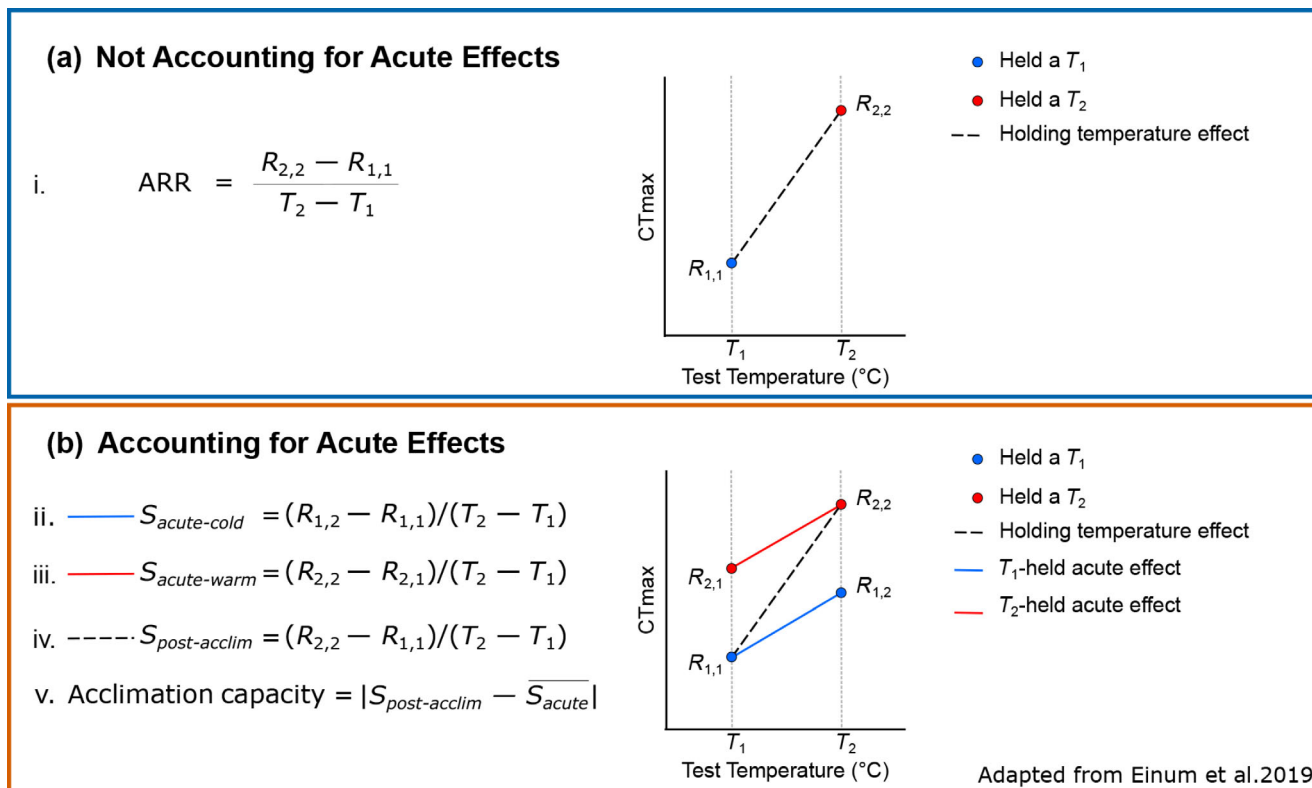


FIGURE 1 Hypothetical example outlining the two approaches tested in this study. For these examples, the two holding temperatures and the two test temperatures are the same: T_1 and T_2 . R denotes the trait value (response, here CTmax) where the first subscript represents the holding temperature, and the second subscript represents the start temperature. (a) 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. (b) 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. ARR, acclimation response ratio.

Bernhardt et al., 2018; Huey & Kingsolver, 2019; Wagner et al., 2023). However, this system in particular has known population-level impacts of CTmax on mortality in stressful temperatures, demonstrating that even a 1° increase in CTmax can have a large impact on estimated mortality (Cicchino, Ghalambor, & Funk, 2023). Therefore, population variation in tadpole acclimation capacity of CTmax may contribute to species resilience against warming temperatures, with impacts on the stream ecosystems they inhabit.

We collected tadpoles (at developmental stages 26.5–45; Gosner, 1960) from populations in Oregon (*A. truei*) and Montana (*A. montanus*) in 2017 (July–August) and 2018 (June–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 10 populations in total (Figure 1). 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 2). We collected approximately 48 tadpoles per population (Appendix S1: 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 and CTmax experiments

Tadpole collection, holding, and CTmax experiments were performed one population at a time, when possible given sampling (Appendix S1: Table S1). For each population, ~24 tadpoles were randomly assigned to one of

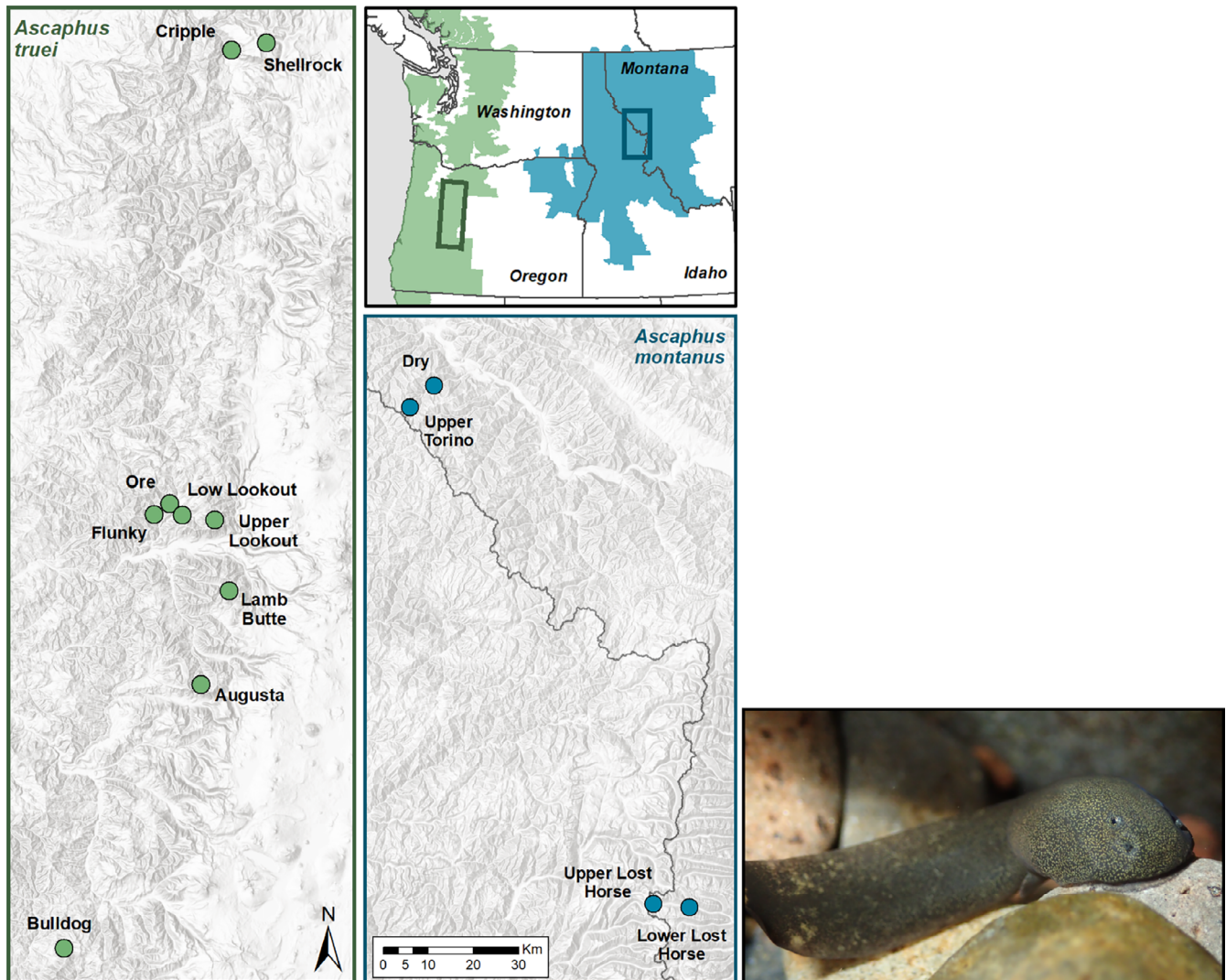


FIGURE 2 Map of sampling sites: 10 populations of *Ascaphus truei* were sampled from Oregon and 4 populations of *A. montanus* were sampled from Montana. *Ascaphus* tadpoles (pictured right) occupy cold, fast-flowing streams where they use their suckorial mouths to attach themselves to benthic substrate. Photo credit: AS Cicchino.

two holding temperature treatments: 8 or 15°C for three days. Tadpoles were held in 142-L 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 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 et al., 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 2 min, water temperature was ramped at a rate of 0.3°C min⁻¹ using a temperature controller attached to a titanium heating rod (500 W). 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 postmortem (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 (Angilletta et al., 2004; Brown et al., 2004; Lindmark et al., 2018; Peralta-Maraver & Rezende, 2021) regardless of developmental rates, which may vary along elevation gradients (Arendt, 1997; Conover & Present, 1990; Riha & Berven, 1991).

Assessing temperature effects and 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 S1: 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 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 1), both of which use 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 1, equation i). 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 1, equation ii). 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 h for a mean period of 12 months (Appendix S1: Table S1). Temperature loggers were housed in 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 SD time series plots (Dunham et al., 2005).

To test whether 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 3). We used our in situ temperature logger data to calculate the average of the 10 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 on (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 was 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 4 *A. montanus* populations and 10 *A. truei* populations (see Appendix S1: Table S1 for treatment sample sizes).

Acclimation and 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;

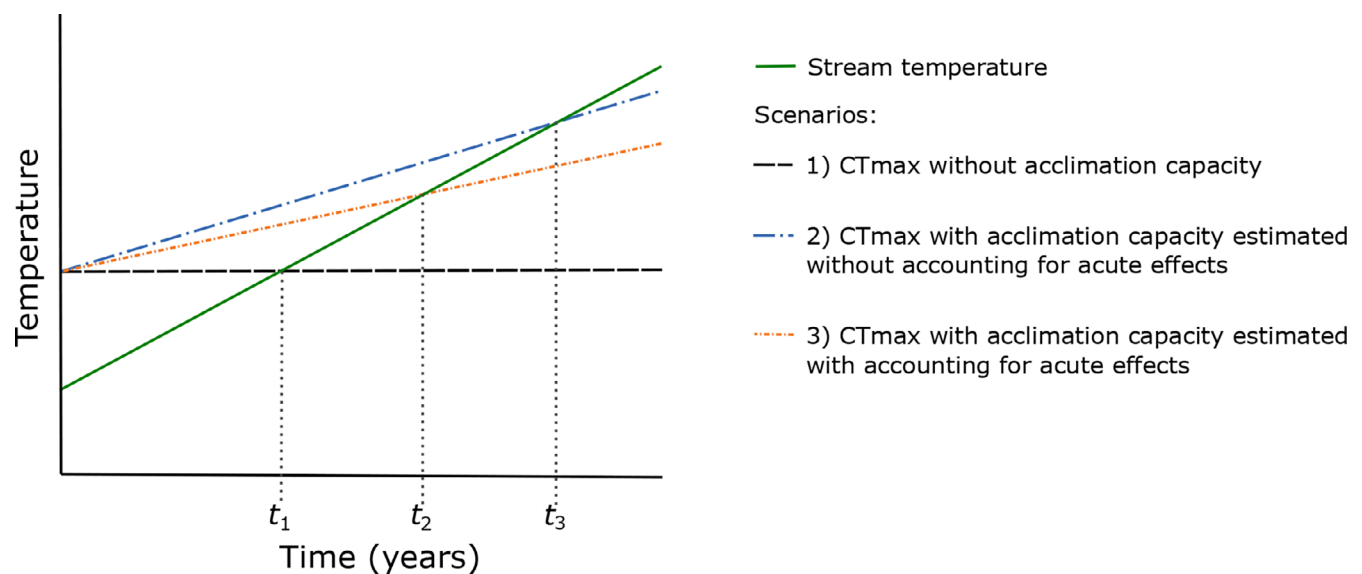


FIGURE 3 A hypothetical example demonstrating how we estimated buffering capacity, our proxy for physiological vulnerability to warming (calculations adapted from Morley et al., 2019). For each population, we modeled stream temperature changes over time using maximum temperatures calculated from our 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 modeled critical thermal maximum (CTmax) changes over time using population medians as the intercept and a slope that was determined from no acclimation capacity (gray dashed line), acclimation capacity estimated using an approach that accounts for acute effects (orange dotted line), and acclimation capacity estimated while ignoring acute effects (blue dotted and dashed line). Using the time (in years) that each modeled CTmax intercepted modeled 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 modeled 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 estimating acclimation capacity considering acute effects ($t_2 - t_1$).

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 4a). 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* and 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 4b). 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 4b).

Acclimation capacity and 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 5). Although the magnitude of acclimation capacity differed between approaches for some populations (Figure 5), 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, $t = 1.314$, $df = 9$, $p = 0.221$).

TABLE 1 Mixed model ANOVA (type III) critical thermal maximum results for *Ascaphus truei* and *A. montanus*.

Factor	SS	df	F	p
<i>Ascaphus truei</i>				
(Intercept)	8000.637	1	59,109.720	<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
<i>Ascaphus montanus</i>				
(Intercept)	1750.290	1	12,507.633	<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

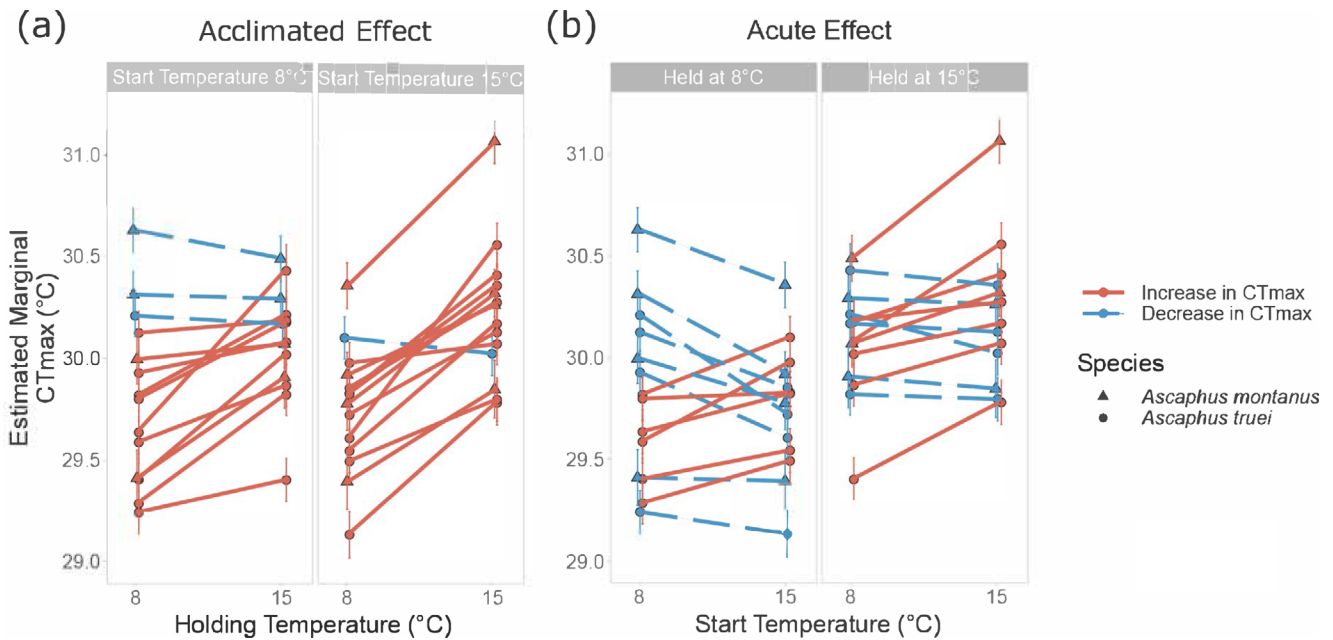


FIGURE 4 (a) With few exceptions, a warmer holding temperature generally led to an increase in critical thermal maximum (CTmax) estimates for both *Ascaphus* species, regardless of the experimental starting temperature. (b) Acute temperature responses of CTmax varied in magnitude and directionality among populations of both species, suggesting multiple processes (thermodynamic and stress) dictating acute responses.

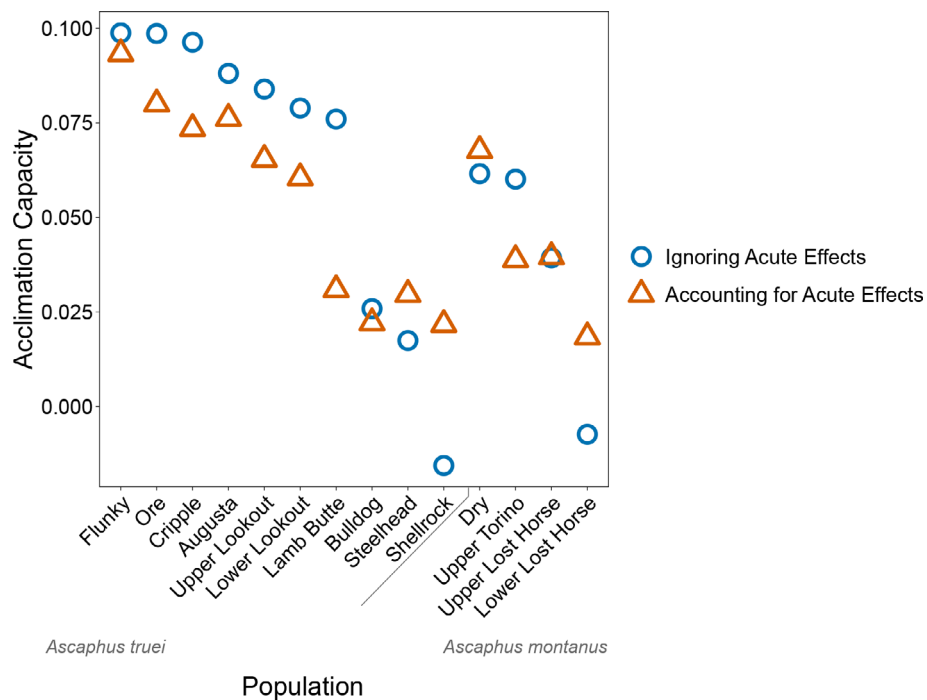


FIGURE 5 Acclimation capacity (Δ CTmax per degree change in holding temperature; Figure 2) for each population as estimated by ignoring acute effects and accounting for acute effects. Without accounting for acute effects, all but two populations (Lower Lost Horse, Shellrock) exhibited acclimation capacity of critical thermal maximum (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.

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 6). 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 (*A. montanus* and *A. truei*) to investigate the roles of population variation and experimental design in estimating acclimation capacity of 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 for vulnerability to climate change (Gunderson et al., 2017; Gunderson & Stillman, 2015; Morley et al., 2019; Nicotra et al., 2015) 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 (Bennett et al., 2019; Herrando-Pérez et al., 2019; Seebacher et al., 2012; Senior et al., 2019; Valladares et al.,

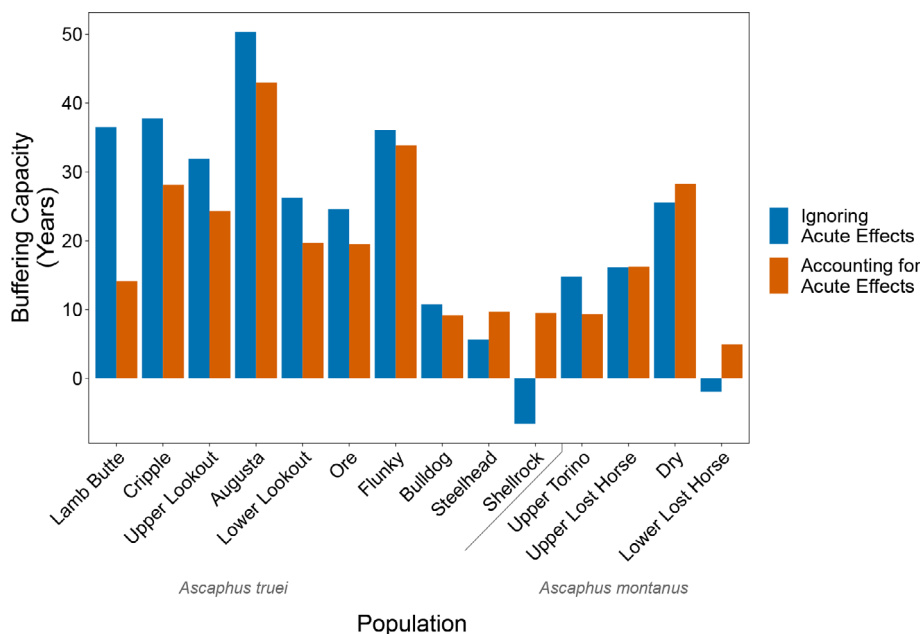


FIGURE 6 Buffering capacity (years of added buffer; Figure 3) as estimated using both approaches for calculating acclimation capacity (Figure 2). Although the two approaches did not statistically differ from each other in their buffering metric estimates, there is substantial population variation in 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.

2014) 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 threefold among populations (*A. montanus*: accounting for acute effects = 3.5×, ignoring acute effects = 6×; *A. truei*: accounting for acute effects = 4.5×, ignoring acute effects = 11×) 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 vs. 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 & Kieffer, 2003; O'Steen & Bennett, 2003). Six of the 14 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 (Kittner & Riisgård, 2005; Terblanche et al., 2007), 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; Rohr et al., 2018; Shah et al., 2017; Sørensen et al., 2016). 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 & Burton, 2022; Jørgensen et al., 2021; Rezende et al., 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 remain to be tested.

Effects of experimental design and estimation approach

Accounting for acute temperature effects in this study did not change the 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 6). Physiological proxies represent one aspect of vulnerability (Beever et al., 2016; Clusella-Trullas et al., 2021; Dawson et al., 2011; Nicotra et al., 2015), 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 (Huey & Berrigan, 1996), acclimation capacity can only be uncovered when 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 & Hoffmann, 2020). However, with the 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 & Hoffmann, 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 (Havird et al., 2020; Terblanche & Hoffmann, 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 & Stillman, 2015). Our estimates, however, are higher than those 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 and 20°C, although they did report a higher acclimated response than we observed (0.2°C CTmax per degree Celsius acclimation) when comparing frogs held at 0 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 certainly begin to be experienced before organisms reach their CTmax (Huey & Kingsolver, 2019; Pörtner & Farrell, 2008; Thomas et al., 2017). Therefore, studies investigating the capacity for these populations to evolve greater CTmax and acclimation capacity, as well as the impacts of warming on physiological traits that occur prior to the upper critical limit, would be beneficial for further understanding climate change impacts.

AUTHOR CONTRIBUTIONS

All authors conceived the ideas and designed the methodology. Amanda S. Cicchino, Brenna R. Forester, and W. Chris Funk collected the data. Amanda S. Cicchino and W. Chris Funk analyzed the data. Amanda S. Cicchino led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Cichino et al., 2023) are available from Dryad: <https://doi.org/10.5061/dryad.fn2z34v1w>. Temperature projections used from Isaak et al. (2016) are freely available at <https://doi.org/10.2737/RDS-2016-0033>.

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