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Multi-scale relationships in thermal limits within and between two cold-water frog species uncover different trends in physiological vulnerability

Amanda S. Cicchino[1,2] | **Alisha A. Shah**[2,3] | **Brenna R. Forester**[2] | **Jason B. Dunham**[4] | **Cameron K. Ghalambor**[1,2,5] | **W. Chris Funk**[1,2]

[1]Graduate Degree Program in Ecology, Colorado State University, Fort Collins, Colorado, USA

[2]Department of Biology, Colorado State University, Fort Collins, Colorado, USA

[3]W.K. Kellogg Biological Station, Department of Integrative Biology, Ecology, Evolution and Behavior Program, Michigan State University, Hickory Corners, Michigan, USA

[4]U.S. Geological Survey, Forest and Rangeland Ecosystem Science Center, Corvallis, Oregon, USA

[5]Department of Biology, Centre for Biodiversity Dynamics (CBD), Norwegian University of Science and Technology (NTNU), Trondheim, Norway

Correspondence
Amanda S. Cicchino, Department of Biology, Colorado State University, Fort Collins, CO 80521, USA.
Email: cicchino@rams.colostate.edu

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Abstract

1. Critical thermal limits represent an important component of an organism's capacity to cope with future temperature changes. Understanding the drivers of variation in these traits may uncover patterns in physiological vulnerability to climate change. Local temperature extremes have emerged as a major driver of thermal limits, although their effects can be mediated by the exploitation of fine-scale spatial variation in temperature through behavioural thermoregulation.
2. Here, we investigated thermal limits along elevation gradients within and between two cold-water frog species (*Ascaphus* spp.), one with a coastal distribution (*A. truei*) and the other with a continental range (*A. montanus*). We quantified thermal limits for over 700 tadpoles, representing multiple populations from each species. We combined local temporal and fine-scale spatial temperature data to quantify local thermal landscapes (i.e., thermalscapes), including the opportunity for behavioural thermoregulation.
3. Lower thermal limits for either species could not be reached experimentally without the water freezing, suggesting that cold tolerance is <0.3°C. By contrast, upper thermal limits varied among populations, but this variation only reflected local temperature extremes in *A. montanus*, perhaps as a consequence of the greater variation in stream temperatures across its range. Lastly, we found minimal fine-scale spatial variability in temperature, suggesting limited opportunity for behavioural thermoregulation and thus increased vulnerability to warming for all populations.
4. By quantifying local thermalscapes, we uncovered different trends in the relative vulnerability of populations across elevation for each species. In *A. truei*, physiological vulnerability decreased with elevation, whereas in *A. montanus*, all populations were equally physiologically vulnerable. These results highlight how similar environments can differentially shape physiological tolerance and patterns of vulnerability of species, and in turn impact their vulnerability to future warming.

KEYWORDS
behavioural thermoregulation, conservation physiology, CTmax, ecophysiology, thermalscape

1 | INTRODUCTION

As environments continue to warm across the globe, 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 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., Amundrud & Srivastava, 2020; Bozinovic et al., 2011; Sunday et al., 2012). Quantifying thermal limits is therefore critical for understanding a species' capacity to withstand exposure 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 geographical scales (Gaston et al., 2009; Pinsky et al., 2019; Sunday et al., 2019). For example, studies have found that upper thermal limits (UTLs: 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). Most comparative studies to date have focused on species differences, yet 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 lower thermal limits (LTLs: minimum temperature tolerated) or UTLs, including physiological constraints (Araújo et al., 2013; Gangloff & Telemeco, 2018), palaeoclimatic 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 UTLs and LTLs 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 geographical 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 marine, terrestrial, freshwater and intertidal environmental realms were associated with global latitudinal patterns of thermal limits among ectothermic species.

Environmental temperature extremes are undoubtedly a powerful predictor of thermal limits (Bennett et al., 2021), yet 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 behavioural 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 behavioural thermoregulation that allowed lizards to maintain consistent body temperatures (Muñoz et al., 2014; Muñoz & Bodensteiner, 2019). Likewise, in aquatic environments the ability to take advantage of such microclimatic variation has been linked to the avoidance of unfavourable 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 favourable microclimates, and a lack of fine-scale temperature variation would suggest a lack of opportunity for behavioural thermoregulation. Yet, most studies examining the drivers of thermal limits typically only measure relationships 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 as a result of 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 owing to variation in environmental, geological 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 (≤1 m) 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 at similar ranges of elevation (*A. truei*: ~0–2100 m; *A. montanus* ~ 790–2500 m above sea level) (Hayes & Quinn, 2015; Macedo, 2019). However, *A. truei* occurs in 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 1.1 m/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.

In order to address this gap and uncover the role of local thermalscapes in shaping population thermal limits, we determined experimentally the thermal limits of tadpoles from multiple populations of both tailed frog species, and quantified thermal regimes and fine-scale (≤1 m) 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 spatial heterogeneity in temperatures within streams, which provides the opportunity for behavioural 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 behavioural thermoregulation to mediate the strength of selection from thermal regimes.

2 | MATERIALS AND METHODS

2.1 | Field sampling

Sampling occurred June–August in each of 2016, 2017 and 2018. We sampled *A. 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 1). Sampled stream reaches are hereafter referred to as separate populations as a result of 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 26.5–40; Gosner, 1960) from each site by lifting stream substrate directly upstream of a hand-net. We held the sampled tadpoles in 2-L insulated containers (maximum 12 tadpoles per container) with stream water during sampling, ensuring that the water remained cold with frequent water changes. To transport tadpoles to our laboratory 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).

2.2 | Temporal and spatial stream temperature measurements

In order to characterise 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 Corp.) at each end of sampled stream reaches (~100 m) and recorded temperatures every 4 h. 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 minimising 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 (i.e., tadpole habitat). Pipes also were secured to a nearby tree using a thick steel cable to prevent them from being washed downstream. We visually inspected the time-series temperature logger data for error and screened the data using standard deviation (*SD*) time series plots (Dunham et al., 2005).

From these data (see Table S1 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 10 consecutive coldest and the average of the 10 consecutive warmest days. Because of high correlations between maximum temperature metrics in Oregon (minimum temperature: Pearson's $r = 0.94$, df $= 13$, $p = 2.58e^{-10}$;

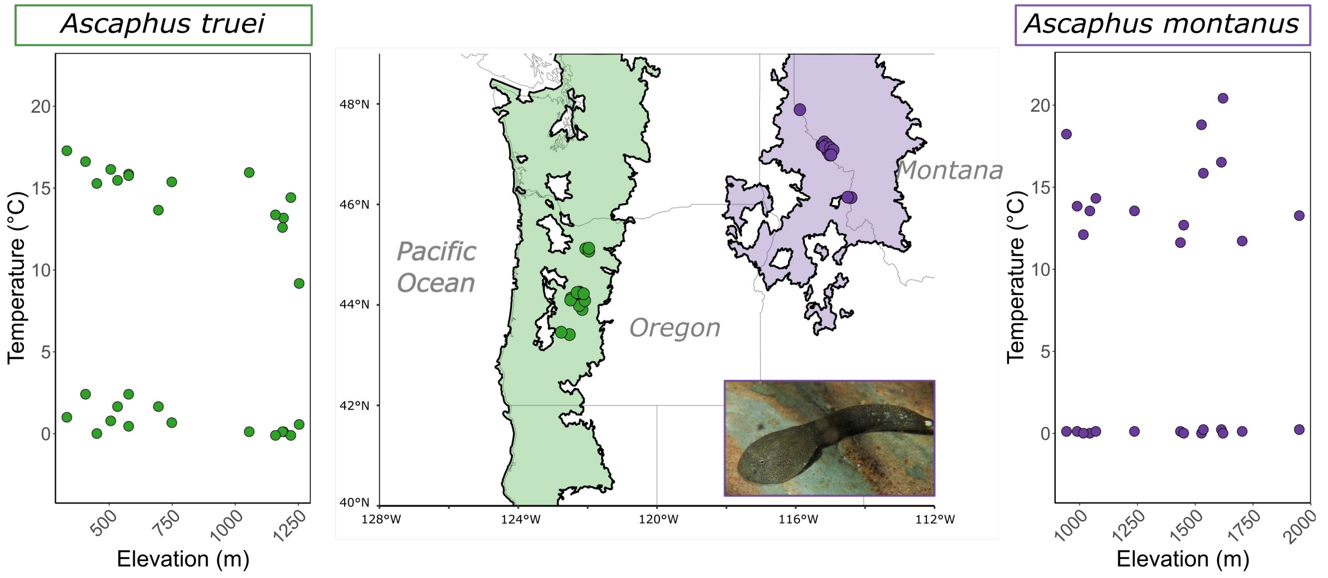

FIGURE 1 (Center map) Sampling locations for *Ascaphus truei* tadpoles from 15 populations in Oregon (USA species range in green) and *A. montanus* tadpoles from 14 populations in Montana (USA species range in violet). *A. montanus* tadpole shown in bottom right inset. (Outer panels) Maximum stream temperatures varied more than minimum stream temperatures within both species. Note that the scales of the *x*-axes differ between species.

maximum temperature: Pearson's $r = 0.99$, $df = 13$, $p = 4.01\text{e}^{-14}$) and Montana (minimum temperature: Pearson's $r = 0.49$, $df = 12$, $p = 0.08$; maximum temperature: Pearson's $r = 0.99$, $df = 12$, $p = 1.12\text{e}^{-11}$). 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 and 13 August 2019 (Table S1), which is typically the 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 (~100 m). We measured temperatures at a minimum of 100 points using a field temperature probe (ODO; YSI Inc.) 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 (<3 m wide: measurements every 0.5 m; >3 m wide: measurements every 1 m). For each measurement, we randomly offset the measurements from the interval along the stream transect using a random number table. For streams <3 m wide, the offset did not exceed 25 cm. For streams >3 m wide, the offset did not exceed 50 cm. For example, if a narrow (<3 m) stream at Transect 1, Interval 3 (1.5 m from stream bank) had an offset of −21, the measurement would be taken at 1.29 m 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.

2.3 | Thermal limit experiments

Tadpoles collected from each population were held and tested separately. Tadpoles were held in 142-L insulated containers (maximum 24 tadpoles per container) filled with water from their natal streams at 8°C (a commonly experienced temperature) for 3 days using a 1/10 HP recirculating water chilling unit (Coralife). We maintained high oxygenation of the holding water using standard aquarium air pumps and bubblers. During this 3-day holding period, food was withheld to ensure a similar post-absorptive state during experiments and standardise the condition of individuals before the experiments. We checked tadpoles daily for typical behaviour 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/min. 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 3 min 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., 1988; Delgado-Suazo & Burrowes, 2022; Mittan & Zamudio, 2019). 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 euthanised 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; Figure S3). 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 Table S1). 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 (>12× *SD*) and was also excluded from analyses. Therefore, the analysed data represent CTmax from 727 tadpoles (370 *A. truei*, 357 *A. montanus*).

2.4 | 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 characterised fine-scale variability within stream reaches by inspecting data density distributions, *SD*s 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 characterise the configuration of temperature variability. We used linear mixed effects (LME) 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 (Table S2), 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.

3 | RESULTS

3.1 | Temporal and spatial stream temperatures

Across all streams, temperatures reached near-freezing, although 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, $t_{23.69}=0.08$, $p=0.93$). 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 varied considerably (Figure 1).

At a fine scale, sampled streams for both species were spatially homogenous in temperature (Figure S1). The *A. truei* streams ranged in *SD* from 0.05 to 0.64 and *A. montanus* streams ranged from 0.04 to 1.35. Only two *A. 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 (Figure S2).

3.2 | Upper thermal limits (CTmax)

Ascaphus 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–30.9°C) than *A. montanus* (2.5: 28.7°C–31.2°C), although *A. montanus* tadpoles exhibited the highest UTL estimates (Figure S4).

Results from the *A. truei* LME 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 1; Figure 2). Results from the LME 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 1; Figure 2). 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* LME 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 (Table S3).

4 | 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 demonstrate that closely related species occupying similar habitats may have different physiological relationships with their external thermal 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 with only a few 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 were similar (Shah et al., 2017). We expected to observe similar relationships within tailed frogs, yet 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. Adult *A. montanus* 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

TABLE 1 Linear mixed effects model estimate and results from ANOVA (type III, Satterthwaite's method) testing the effects of maximum stream temperature on critical thermal maximum (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 (1345.08)	<0.0001
Random effects	Variance ± *SD*			
Population	0.06 ± 0.24			
Residual	0.14 ± 0.37			

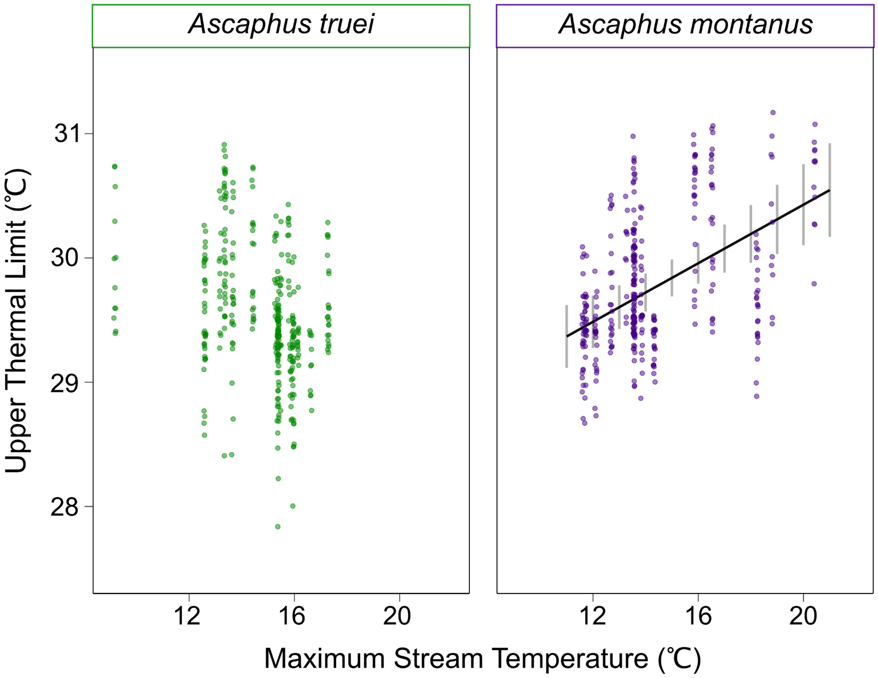

FIGURE 2 Upper thermal limits, measured as critical thermal maximum (shown here as raw data points), among populations were unrelated to maximum stream temperatures in *Ascaphus truei* (left) but were positively related in *A. montanus* (right). Prediction slope and 95% confidence intervals estimated by the linear mixed effects model for *A. montanus* (right, $p=0.0005$).

because our behavioural 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 that they typically experienced in nature.

Patterns of evolution in UTLs of terrestrial ectotherms generally have shown that they 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 other 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 populations. Within species, we found that CTmax varied among populations, but were related only 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 a consequence of 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 those studied here (Adams & Frisell, 2001; Dunham et al., 2007; Huff et al., 2005; Welsh Jr & Hodgson, 2008), although the full range of thermal conditions which 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 relationships between CTmax and stream temperatures between species, they 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 vulnerable to future warming in a similar way. 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 owing 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 high-elevation streams sampled 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 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 realised behavioural 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 ranged spatially over 1.6°C, the variation could be characterised as a gradient or clumped distribution. 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, therefore may be too small (i.e., <2°C) to provide adequate thermal refuge for inhabitants. Clumped distributions of thermal refuges can lead to inaccuracy in behavioural 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 our 100-m 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 that we uncovered may not be facilitating behavioural thermoregulation in this system but do 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 owing to the costs associated with behaviourally thermoregulating, such as increased predation risk and/or associated bioenergetic costs (e.g., Huey & 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 provide refuges through behavioural 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, 2019). 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 as a result of 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., 2023), 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.

AUTHOR CONTRIBUTIONS

Conceptualisation, developing methods, data interpretation, and writing: ASC, AAS, BRF, CKG, JBD, WCF. Conducting the research: ASC, AAS, BRF, WCF. Data analysis, preparation of figures and tables: ASC.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data are freely available from Dryad: https://doi.org/10.5061/dryad.qrfj6q5mh (Cicchino, 2023)

ORCID

Amanda S. Cicchino https://orcid.org/0000-0003-0170-829X

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SUPPORTING INFORMATION

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