







RESEARCH ARTICLE

Phenology and plasticity can prevent adaptive clines in thermal tolerance across temperate mountains: The importance of the elevation-time axis

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Abstract

Critical thermal limits (CT_{max} and CT_{min}) decrease with elevation, with greater change in CT_{min} , and the risk to suffer heat and cold stress increasing at the gradient ends. A central prediction is that populations will adapt to the prevailing climatic conditions. Yet, reliable support for such expectation is scant because of the complexity of integrating phenotypic, molecular divergence and organism exposure. We examined intraspecific variation of CT_{max} and CT_{min} , neutral variation for 11 microsatellite loci, and micro- and macro-temperatures in larvae from 11 populations of the Galician common frog (*Rana parvipalmata*) across an elevational gradient, to assess (1) the existence of local adaptation through a P_{ST} - F_{ST} comparison, (2) the acclimation scope in both thermal limits, and (3) the vulnerability to suffer acute heat and cold thermal stress, measured at both macro- and microclimatic scales. Our study revealed significant microgeographic variation in CT_{max} and CT_{min} , and unexpected elevation gradients in pond temperatures. However, variation in CT_{max} and CT_{min} could not be attributed to selection because critical thermal limits were not correlated to elevation or temperatures. Differences in breeding phenology among populations resulted in exposure to higher and more variable temperatures at mid and high elevations. Accordingly, mid- and high-elevation populations had higher CT_{max} and CT_{min} plasticities than lowland populations, but not more extreme CT_{max} and CT_{min} . Thus, our results support the prediction that plasticity and phenological shifts may hinder local adaptation, promoting thermal niche conservatism. This may simply be a consequence of a coupled variation of reproductive timing with elevation (the “elevation-time axis” for temperature variation). Mid and high mountain populations of *R. parvipalmata* are more vulnerable to heat and cool impacts than lowland populations during the aquatic phase. All of this contradicts some of the existing predictions on adaptive thermal clines and vulnerability to climate change in elevational gradients.

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local adaption, microclimate, niche conservatism, phenotypic plasticity, thermal tolerance, warming tolerance

TAXONOMY CLASSIFICATION

Ecophysiology

1 | INTRODUCTION

Climate change is promoting fast increases in both mean temperatures and the frequency of extreme heat events and temporal anomalies, which may jeopardize biodiversity worldwide (IPCC, 2014; Pacifici et al., 2015; Parmesan, 2006). Species basically rely on four strategies to cope with this crisis: evolutionary changes in their tolerance limits, thermal acclimation (phenotypic plasticity), shifts in behavior and activity timing, and shifting geographical ranges in order to track historical climates (Habary et al., 2017; Hoffmann & Sgrò, 2011; Walther et al., 2002). Therefore, the study of population variation and phenotypic plasticity in physiological traits, and the correct characterization of thermal microenvironments can be decisive to predict the consequences of global warming (Camacho et al., 2015; Garland et al., 1991; Huey et al., 2012; Somero, 2010).

Spatial variation in thermal physiology (e.g., Critical Thermal Limits, CT_{max} and CT_{min}) in relation to latitude and elevation has been thoroughly described at the interspecific level being often associated with environmental thermal heterogeneity (Bozinovic et al., 2011; Pintanel et al., 2019, 2022; Shah et al., 2017; Stevens, 1989; Sunday et al., 2019). Compared with longer range climatic variation of latitudinal gradients, elevational gradients change climate over shorter distances. That results in predictable changes in air temperatures between $-6.5^{\circ}\text{C}/\text{km}$ and $-3.5^{\circ}\text{C}/\text{km}$, due to adiabatic cooling, and an increase in thermal variability, associated with higher solar radiation and the lowering of air density (Hodkinson, 2005). These steeper climatic gradients may select for thermal adaptations to local conditions and thermal plasticity, and act as physiological barriers to gene flow potentially producing genetic differentiation, particularly in non-seasonal tropical latitudes (Janzen, 1967; Polato et al., 2018). In turn, under moderate gene flow, populations living at divergent climates could introduce maladapted genotypes into each other, potentially decreasing the frequency of local adapted genotypes. This would determine a reduction in the steepness of the slope of adaptive clines, as it has been predicted theoretically (Endler, 1977; Slatkin, 1973), and demonstrated empirically in temperate amphibians (Bachmann et al., 2020). Recent intraspecific studies have revealed adaptive clinal variation with elevation in both CT_{max} and CT_{min} (Bishop et al., 2017; Klok & Chown, 2003; Sørensen et al., 2005) with more variation in CT_{min} than CT_{max} (Muñoz et al., 2014). In contrast, a number of studies did not reveal such elevational trend in physiological traits related to thermal tolerances (Buckley et al., 2013; Gvoždík & Castilla, 2001; Senior et al., 2019; Slatyer et al., 2016; Slatyer et al., 2019; Slatyer & Schoville, 2016; Tonione et al., 2020). Yet, reliable data to support adaptive clinal variation is still scant because of

the need and difficulty of integrating phenotypic (P_{ST}) and molecular divergence (F_{ST}) (Brommer, 2011; Leinonen et al., 2008).

Several biotic and abiotic factors may prevent adaptive differentiation in thermal physiology when analyzing mountain clines. Most animals experience climate at fine-scale patches, and microenvironmental temperatures actually faced by the organism can deviate greatly from recorded mean air temperatures obtained at larger spatial scales (Helmuth, 2009; Potter et al., 2013; Suggitt et al., 2011). In fact, recent analyses suggest that macroclimatic variables (e.g., WorldClim, Hijmans et al., 2005) may only weakly predict tolerance limits and physiological niches compared with microclimatic variables (Farallo et al., 2020; Gutiérrez-Pesquera et al., 2016; Katzenberger et al., 2018; Navas et al., 2013; Pintanel et al., 2019, 2022).

Spatiotemporal changes in microclimate may result, for example, from differences in topography and vegetation cover (Porter et al., 2002). Since microclimatic heterogeneity cannot be captured by downscaling regional climatic variation (Caillon et al., 2014; Diamond & Chick, 2018; Navas et al., 2013; Pincebourde et al., 2016), it becomes important to measure it. This is particularly important for assessing climatic tolerance in animals living at buffered microhabitats, like ponds (Ex. tadpoles, Gutiérrez-Pesquera et al., 2016; Katzenberger et al., 2018; Pintanel et al., 2022). This thermal heterogeneity at the microclimatic scales allows organisms for behavioral thermoregulation, which may preclude the evolution of physiological adaptations in performance by reducing the exposure of organisms to extreme temperatures (i.e., the Bogert effect; Huey et al., 2003, 2012; Kearney et al., 2009; Buckley et al., 2015; Farallo et al., 2018; Muñoz, 2022). Otherwise, organisms may be non-active year-round, adopting dormant physiological states such as diapause, hibernation or estivation, in order to escape stressful extreme temperatures (Ragland & Kingsolver, 2008), or because breeding habitats or resources are temporarily unavailable (e.g., ice covered aquatic habitats, and pond drying). Temporal adjustments of activity can be the result of two additive components, one seasonal (or phenological) and one at a finer temporal scale (24-h) that can be dependent on season and particular weather conditions. The limit for these adjustments will be imposed by the energy demand (Kearney & Porter, 2017, 2020).

Therefore, in absence of energetic constraints, populations can persist at their spatial locations despite change in thermal conditions, without changes in physiological traits. Thus, phenological adjustments in activity can modify the strength of directional selection over thermal tolerance limits through altitudinal gradients (Álvarez et al., 2012; Phillimore et al., 2010; Socolar et al., 2017). This is important because the physiological adjustment of thermal traits may be subjected to

severe constraints. For instance, extreme heat stress can occur even at high elevations (Sunday et al., 2014), so populations living in mountain areas should face both heat and cold extremes, which leads to an unlikely solution (“master-of-all” superorganism, Remold, 2012).

All these factors can contribute to buffer the realized thermal variation (i.e., the range of effective temperatures experienced by individuals) along elevational gradients and, ultimately, they can promote the pervasiveness and retention of climatic niches; hence, organisms would maintain their thermal niches unchanged while moving along an elevation gradient. In this context, behavioral tracking of the microclimatic niche over space and phenology can allow for retention of the microclimatic niche rather than adapting to the new local conditions with changes in physiology (Farallo et al., 2020; Huey et al., 2003; Kearney et al., 2009; Muñoz, 2022).

Populations living in highly variable thermal environments would express greater plasticity in their thermal tolerances (Angilletta, 2009; Gunderson & Stillman, 2015; Chevin & Hoffmann, 2017; Mallard et al., 2020; but see, for deeper discussion, Gilchrist, 1995, Enriquez-Urzelai et al., 2020). Besides behavioral thermoregulation and phenological adjustments, thermal acclimation may also prevent directional selection on physiological thermal traits, which would constrain thermal adaptation to the new climatic conditions. In fact, organisms can retain plasticity enough to allow for rapid shifts in thermal tolerances, thus precluding thermal stress, death and, likely, the effects of natural selection (Chevin et al., 2010; Huey et al., 2012; Levins, 1969).

Here we examined elevational clinal variation in the upper (CT_{max}) and lower (CT_{min}) critical thermal limits and their plasticity in 11 populations of the Galician common frog, *Rana parvipalmata* (Figure 1), across an altitudinal gradient from 40 to 1800 m a.s.l. We focus on the aquatic tadpole stage because the breeding aquatic habitat of many amphibian species exhibit low thermal heterogeneity. This limits tadpole ability for behavioral thermoregulation compared with terrestrial adult stages (see Feder & Hofmann, 1999) and, thus, determining that thermal selection on the aquatic phase could

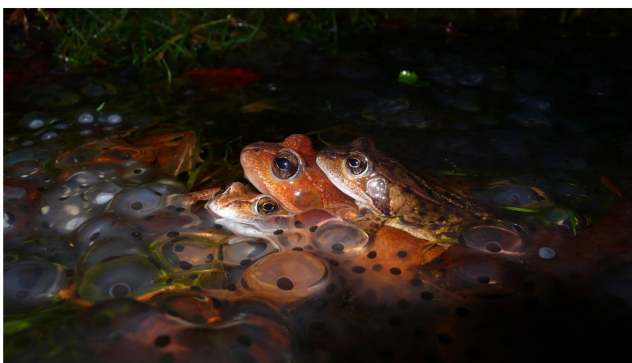


FIGURE 1 Amplexant pair of *Rana parvipalmata* and a satellite male surrounded by masses of recently spawned eggs and embryos in a breeding nucleus in the Color Valley (380 m a.s.l.). A second amplexus is under the egg masses. Breeding occurs in a series of small, shallow spring pools and ditches (maximum depth <12 cm) under complete canopy cover.

be a major driver of tadpole variation in critical thermal limits and its plasticity. In addition, recent research has identified maximum pond temperature as an important range-limiting factor for *R. temporaria* / *Rana parvipalmata* (Enriquez-Urzelai, Kearney, et al., 2019). Second, we analyzed population vulnerability to thermal stress by estimating warming and cooling tolerances (sensu Sunday et al., 2014; Gutiérrez-Pesquera et al., 2016). Since average temperatures usually decline with elevation, and thermal physiology limits are driven by peak environmental temperatures (Buckley & Huey, 2016; Gutiérrez-Pesquera et al., 2016; Overgaard et al., 2014; Pintanel et al., 2019), we posit the following two predictions: (1) greater heat impacts are expected at the lowlands (Pintanel et al., 2019; Sunday et al., 2014), whereas higher cold impacts are forecasted for mountain populations (Pintanel et al., 2019; Sunday et al., 2014) (*elevational thermal vulnerability hypothesis*); and, therefore, (2) under a scenario of selection in CTs, higher elevation populations will evolve lower tolerances to high temperatures and higher tolerances to low temperatures (*elevational thermal adaptive hypothesis*). In addition, we analyzed the among-populations variation in acclimation capacity (i.e., as a form of phenotypic plasticity) for thermal tolerances and the potential for local adaptation in thermal limits (CT_{max} and CT_{min}) by means of P_{ST} - F_{ST} comparisons across these populations. Considering that populations at different elevations may be exposed to different extreme temperatures and variable thermal ranges, we hypothesize that these populations will also differ in acclimation ability.

2 | MATERIALS AND METHODS

2.1 | Study system, population breeding dynamics, and sampling

The Galician common frog (*Rana parvipalmata*), formerly part of the European common frog (*Rana temporaria*) complex (Dufresnes et al., 2020), is endemic to the northwest Iberian Peninsula. Based on projected rates of climate change, there is growing evidence that common frogs (*Rana temporaria* and *Rana parvipalmata*) in northwestern Iberia may experience heat stress associated with heat waves in coming decades (Enriquez-Urzelai et al., 2020; Enriquez-Urzelai, Sacco, et al., 2019), although the impact on population growth would depend on the potential for behavioral thermoregulation of adults and, especially, juvenile individuals (Enriquez-Urzelai et al., 2018, 2020; Enriquez-Urzelai, Sacco, et al., 2019). Anyhow, recent forecasts based on climatic niche models (both correlative and mechanistic approaches), predicted alarming decreases in climatic suitability in genetic “hotspots” of the *R. temporaria* / *R. parvipalmata* complex (e.g., northern Iberian Peninsula and other glacial refuges in southern Europe), and, under the worst scenarios, the extinction of *R. parvipalmata* and the Cantabrian *R. temporaria* along with many populations in central Europe (Enriquez-Urzelai, Kearney, et al., 2019). In fact, under the most extreme climate change scenario, all mountain ranges but the Alps will also become thermally unsuitable by 2070 (Enriquez-Urzelai, Kearney, et al., 2019).

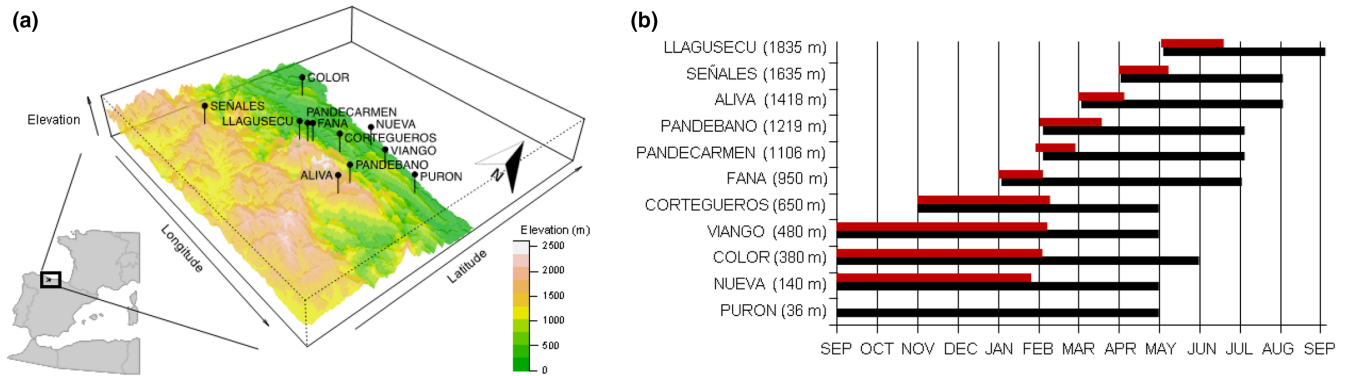


FIGURE 2 (a). Study area and geographic locations of 11 populations of *Rana parvipalmata* (sample points for embryos). (b) Observed temporal variation in the breeding and larval period of *Rana parvipalmata* along the altitudinal gradient for the analyzed populations. Bars marked in red indicates population adult breeding activity; black bars show the presence of larvae in the water. Purón adult breeding activity is not marked because the scarcity of observations.

Reproductive timing is strongly conditioned by elevation which results in a wide, sequential breeding period over most of the year (Figure 2; Álvarez et al., 2012). The observed breeding phenology seems to be the result of physical constrains (e.g., pond desiccation, winter severity at high elevation) derived from climatic conditions, and selection on adults to prevent breeding out of time that renders reproductive success, rather than the product of natural selection acting on larval thermal physiology. In the lowlands, breeding take place in autumn, extends over weeks or a few months with spawning peaks associated with heavy rains, and metamorphs leave the aquatic habitat by late-winter and early spring, before the risk of pond desiccation increases. In contrast, above 1400 m a.s.l. the onset of reproduction delays until snow melting in spring, populations show explosive breeding (lasting 1–2 weeks), and the larval phase can extend until the end of summer (Figure 2). For mountain top populations (1800–2200 m a.s.l.), the seasonal activity window may be less than 5 months to carry out larval development, juvenile growth, and fat storage before the onset of hibernation. Sampling was carried out between 2012 and 2014 in Picos de Europa National Park and surrounding areas, covering a relatively reduced geographical area (Figure 2, Table S2). We selected a total of 11 populations along an elevational transect between 40 and 1800 m a.s.l. All of them belong to the T2 (eastern) lineage of *Rana parvipalmata* (Dufresnes et al., 2020) and were assigned to a maximum of five genetic clusters (Choda, 2014).

For each site, we haphazardly collected 5–7 recently fertilized clutches of *R. parvipalmata* to obtain a representative sample of the population. Embryos were transported to the Doñana Biological Station (EBD-CSIC), and maintained inside climatic chambers (FitoClima, Aralab) under constant conditions of photoperiod (12:12L:D) and temperature (15°C) until hatching. Thereafter, tadpoles were kept in plastic containers at a larval density of 20 individuals \cdot L⁻¹ and constant photoperiod (12:12L:D) and temperature (15°C), until they reached stage 26 (Gosner, 1960) and the tests were conducted. Since we assumed no cross-generational effects, this common garden approach allowed us to disentangle the effects of environment and genetics on physiological thermotolerance.

Animal captures were carried out under permits granted by Government of the Principality of Asturias (2010/000371), and Picos de Europa National Park (CO/09/121/2012, CO/09/0125/2013, CO/09/012/2014). All procedures complied with the country legal requirements on animal welfare (RD 53/2013) and were conducted in accordance with the guidelines of the Research Ethics Committee of the University of Oviedo under authorization #8-INV-2012. The members of the research team have approved licenses by the Service of Animal Welfare and Production of the Principality of Asturias to design and conduct experimental protocols with animals (license types C and D to A.G.N). This study was carried out in compliance with the ARRIVE guidelines (Animal Research: Reporting in Vivo Experiments) for how to report animal research in scientific publications (<https://arriveguidelines.org/arrive-guidelines>).

2.2 | Environmental data

To better characterize potential selective pressures that might lead to thermal local adaptation, we obtained micro- and macro-environmental thermal data for all the sampled populations. To define the thermal profile at each site, we took into account the population's phenology, encompassing both the breeding and larval periods (Figure 2; Tables S1–S5). In order to characterize the macroclimatic thermal environments, we used the 'extract' function in the R package RASTER (Hijmans & van Etten, 2014; R Core Team, 2019) to obtain temperature data from WorldClim 2 layers (the temperature of shaded air at around 2 m off the ground, 30 s or 1 km² spatial resolutions; records from 1970 to 2000) (Fick & Hijmans, 2017). For each population, we assessed monthly maximum (TMAX) and minimum (TMIN) temperatures restricted to the time period with presence of larvae in the ponds (Tables S2 and S3). In addition, we calculated seasonal temperature range (SR) as the difference between TMAX and TMIN (Supporting Information, Table S4). Since most animals experience climate at fine-scale patches (Porter et al., 2002; Suggitt et al., 2011), macroclimatic data may be poorer predictors of thermal physiology

than microclimatic variables (Gutiérrez-Pesquera et al., 2016; Katzenberger et al., 2018; Pintanel et al., 2019; Sunday et al., 2014). Hence, we gathered microclimatic data by placing HOBO Pendant temperature dataloggers that recorded temperature every 10–30min at each pond (Table S5). All the Hobo data-loggers were placed underwater on the bottom of the ponds at a depth of 15–25 cm. One datalogger (Aliva population, 1420m) was lost and we employed the information from a nearby population with similar elevation and pond characteristics (Pandébano, 1220m). For each location, we calculated maximum daily temperature (tmax), minimum daily temperature (tmin), seasonal thermal range (sr) ($sr = tmax - tmin$) and average daily temperature range (dr) for the period when tadpoles are present in the ponds (Figure 1, Table 1, Table S5).

2.3 | Estimation of critical thermal limits and warming and cooling tolerances

Previous research with tadpoles showed that there is not significant variation in CTs across the larval period and only later on, at the verge of metamorphosis climax (Gosner stage 42, Gosner, 1960), both CTs exhibit a strong decline (e.g., Floyd, 1983). Therefore, to determine the CT_{max} and CT_{min} of *R. parvipalmata* populations, we haphazardly selected 32 larvae within Gosner stages 26–39 from each population pool (a mix of 5–7 families per population). Then, each population sample was split into two groups of 16 tadpoles for the estimation of CT_{max} and CT_{min} , respectively. Tadpoles were kept individually in 400ml plastic cups and acclimated inside environmental chambers (FitoClima, Aralab) to a constant temperature of 20°C with a photoperiod of 12L:12D for 4 days before conducting the tolerance assays. This is the time required for adult amphibians and in tadpoles (J. Turriago and M. Tejedo, unpublished data) to stabilize CT_{max} after a large change in environmental temperature, such as field and laboratory conditions (Hutchison, 1961). Tadpoles were fed ad libitum with Purina rabbit chow. Oxygen saturation in the vessels was daily monitored with a laboratory multi-parameter probe (WTW CellOx® 325) and recorded values were always over 60%. Thermal tolerance limits (CT_{max} and CT_{min}) were determined using the Hutchison's dynamic method (Gutiérrez-Pesquera et al., 2016; Lutterschmidt & Hutchison, 1997b). Tadpoles were weighed immediately before the beginning of the test and placed in individual 100 ml containers with dechlorinated tap water inside a thermal bath of 15 L (HUBER K15-cc-NR) previously stabilized to 20°C (acclimation temperature and start temperature) for five minutes. Afterwards, each animal was exposed to a constant heating / cooling rate ($\Delta T = 0.25^\circ C \text{ min}^{-1}$; CT_{max} and CT_{min} , respectively) until the endpoint was attained. The endpoint for both thermal limits was defined as the temperature at which tadpoles become motionless and failed to respond to external stimuli (10 consecutive gentle prods with a wooden stick applied in 2s intervals). Since tadpoles were small in size, we assumed that body temperature was equivalent to water temperature (Gutiérrez-Pesquera et al., 2016; Lutterschmidt &

TABLE 1 Critical thermal limits (CT_{max} and CT_{min} , N, Mean and Standard Error), estimated at a pre-assays acclimation temperature of 20°C. Maximum (TMAX, tmax) and minimum (TMIN, tmin) environmental temperatures (in caps, WorldClim estimator, in lowercase microclimate estimators). Average seasonal (SR: TMAX-TMIN; sr: tmax-tmin) and diel (dr: daily tmax-tmin) temperature ranges.

Population	Elevation (m)	CT_{max} (°C)		CT_{min} (°C)		SE	N	\bar{X}	SE	TMAX	tmax	TMIN	tmin	SR	sr	dr	WT	wt	CT	ct
		N	\bar{X}	N	\bar{X}															
Llagusecu	1835	14	37.0	0.1	14	-1.9	0.1	14.1	29.7	1.4	0.5	5.4	29.2	5.5	22.9	7.3	3.3	3.3	2.4	
Señales	1635	14	36.8	0.1	16	-1.8	0.1	15.6	22.6	-1.2	4	7.0	18.6	4.9	21.2	14.2	0.6	0.6	5.8	
Aliva	1418	14	37.5	0.1	16	-1.9	0.1	16.3	24.5	-0.5	4.3	6.3	20.2	6.5	21.2	13.0	1.4	1.4	6.2	
Pandébano	1219	16	36.7	0.1	16	-1.9	0.1	15.4	28.9	-0.8	0.6	6.9	28.3	7.8	21.3	7.8	1.1	1.1	2.5	
Pandecarmen	1106	16	37.0	0.1	16	-1.8	0.1	16.8	33	-0.3	0.2	7.5	34.6	13.1	20.2	4.0	1.5	1.5	2.0	
Fana	950	16	36.5	0.1	16	-1.3	0.1	17.2	29.3	-0.2	1.5	7.4	27.8	8.9	19.3	7.2	1.1	1.1	2.8	
Cortegueros	650	16	36.8	0.1	12	-1.6	0.1	18.9	28.4	1.8	1	7.5	27.4	6.2	17.9	8.4	3.3	3.3	2.6	
Viangio	480	16	37.1	0.1	15	-2.1	0.2	19.3	25.6	1.9	2.4	7.0	23.2	5.0	17.8	11.5	4	4	4.5	
Color	380	16	36.9	0.1	16	-2.3	0.1	19.1	22.5	3	3.8	6.7	18.7	1.0	17.8	14.4	5.3	5.3	6.1	
Nueva	140	16	36.8	0.1	13	-1.9	0.1	21.1	15.7	5	6.4	7.1	9.3	1.7	15.7	21.1	6.9	6.9	8.3	
Purón	36	16	36.4	0.1	16	-0.9	0.1	21.7	11.4	4.7	9.9	7.6	1.5	0.4	14.7	25.0	5.6	5.6	10.8	

Warming tolerance (WT, wt; $WT = CT_{max} - TMAX$, $wt = CT_{max} - tmax$) and cooling tolerance (CT, ct; $CT = TMIN - CT_{min}$, $ct = tmin - CT_{min}$) for eleven populations of *R. parvipalmata*. Data on geographical locations appear in Tables S1–S3. All environmental data were obtained only during the breeding period of each population

Hutchison, 1997a). Hence, CT_{max} and CT_{min} were recorded as the water temperature beside the tadpole, using a Miller & Weber quick-recording thermometer (to the nearest 0.1°C). After the tolerance limit was determined, we immediately transferred tadpoles to water at 20°C, to allow for recovery. Each tadpole was tested only once, and its survival was assessed a few minutes and 24 hours after the experimental procedure. Only those individuals who remained alive and exhibited normal behavior 24 h after the test were included in subsequent analyses.

For each population, warming tolerances (Deutsch et al., 2008; Duarte et al., 2012) were defined as the difference between CT_{max} and the maximum exposure environmental temperature (mean maximum temperature for the warmest month) taken at either the micro- (pond; $wt = CT_{max} - t_{max}$) or the macro-scale (air; $WT = CT_{max} - T_{MAX}$). By doing so, we expect to reduce noise associated with extremely low or high temperatures during periods when larval habitat is lacking (dry or frozen ponds) and thus obtain more plausible estimates of thermal hazard (see also Duarte et al., 2012; Gutiérrez-Pesquera et al., 2016; Hoffmann et al., 2013). Similarly, cooling tolerances (ct , CT) were determined as the difference between the minimum environmental temperature taken at either the micro scale pond temperature (t_{min}), or the macro scale minimum air temperature (T_{MIN}), and population CT_{min} ($t_{min} - CT_{min}$ and $T_{MIN} - CT_{min}$, respectively) (Gutiérrez-Pesquera et al., 2016; Pintanel et al., 2019; Slatyer et al., 2016; Sunday et al., 2014). We used both WorldClim air temperature and microenvironmental water temperatures, recorded only during the larval period of each population.

2.4 | Phenotypic plasticity in CT_{max} and CT_{min}

We studied temperature acclimation in both thermal limits (CT_{max} and CT_{min}) of five populations, corresponding to low: Nueva (140 m), Cortegueros (650 m); medium: Pandecarmen (1106 m), Aliva (1418 m), and high: Llagusecu (1835 m) elevations. For each population, tadpoles were individually maintained in 400 ml plastic vessels and randomly separated into four batches, totaling 32 tadpoles per batch. Each batch was then acclimated to a specific constant temperature (6, 13, 20 or 27°C) with a 12L:12D photoperiod, for four days (Table S6). These temperatures are relevant because they cover the thermal range of tadpole exposure along the elevation gradient (Álvarez et al., 2012). Although this range can be exceeded in natural conditions at some point, it represents a reasonable adjustment for acclimation. We set the target temperature treatments in a Binder thermal chamber for 6°C treatments, and FitoClima chambers to obtain the rest of thermal treatments. Additionally, we employed Portable Fluid Heaters with Regulation Adjustment, (patent licensing U201431698) to reduce variability in water temperatures within thermal treatments (see caption in Figure 6). CT_{max} and CT_{min} were determined following the above protocols with a start temperature of 20°C for all acclimation temperatures.

2.5 | Molecular markers. DNA extraction

In order to examine the potential for local adaptation in thermal limits (CT_{max} and CT_{min}), we conducted P_{ST} - F_{ST} comparisons across the 11 populations by using 11 microsatellite loci to assess neutral variation (Table S7). We chose microsatellites because these markers are suitable to define population structure at the fine scale (Camacho-Sánchez et al., 2020; DeFaveri et al., 2013; Lemopoulos et al., 2019; Saint-Pé et al., 2019). To reduce the likelihood of including closely related individuals, we obtained the material for genetic analyses from breeding adults, either as buccal swabs (Pidancier et al., 2003) or by cutting the tip of a toe on the foot. In the few cases where tadpoles or embryos were sampled, each tadpole was collected at a different pool to avoid sampling of highly related individuals. All samples were stored at low temperature in 99% EtOH. Whole genomic DNA was isolated from samples with either standard Chelex extraction (500 μ l of a 10% Chelex solution [Chelex-100, Bio-Rad] incubate with 7 μ g Proteinase K at 55°C for 60 min and 100°C for 20 min) or an E.Z.N.A kit for DNA extraction. We selected 11 polymorphic microsatellite loci whose primers were developed for *Rana temporaria*. These markers included different degrees of polymorphism (Supporting Information S7).

2.6 | Estimates of neutral genetic and phenotypic divergences (F_{ST} and P_{ST})

We used the MICRO-CHECKER software (Oosterhout et al., 2004) to check for genotyping errors and null alleles. No evidence of scoring alleles and large alleles dropout was found, but RtU7 was excluded due to the possibility of null alleles. In addition, four markers (Rtemp μ 1, RtU4, Rt μ H, and Rt μ B) were discarded due to failed amplifications. The remainder six markers (Rtemp μ 2, Rtemp μ 4, BFG072, BFG093, BFG183, and BFG241) were quantifiable for the 11 experimental populations and therefore used to estimate F_{ST} values. Exact tests for departure from Hardy-Weinberg equilibrium (HWE) and tests for linkage disequilibrium were performed for each population across all loci, and at each locus individually, using GENEPOP v2.1 (Raymond & Rousset, 1995). Significance was evaluated using the Markov chain methods (Guo & Thompson, 1992) with 5000 dememorizations steps and 1000 batches of 10,000 interactions per batch for HWE, and 5000 interactions for linkage disequilibrium tests.

To test whether local adaptation or neutral divergence drive the elevational variation in CT_{max} and CT_{min} , we compared the genetic differentiation (F_{ST}) and the genetic divergence of quantitative traits (Q_{ST}) among populations, which allow to discern whether trait differentiation is due to genetic drift or natural selection (Leinonen et al., 2008). F_{ST} estimates were calculated according to Weir and Cockerham (1984), using FSTAT 2.9.3.2 (Goudet, 2002). As a surrogate for Q_{ST} data, we used a measure of phenotypic divergence of a trait (P_{ST}) (Brommer, 2011), which can be calculated by the equation:

$$P_{ST} = \frac{c\sigma_B^2}{c\sigma_B^2 + 2h^2\sigma_W^2}$$

where σ_B^2 is the phenotypic variance between populations, σ_W^2 the phenotypic variance within-population, and h^2 is the character heritability. The constant c represents the proportion of the total variance due to additive genetic effects across populations (Leinonen et al., 2006).

To obtain the P_{ST} values for each pair of populations, we used a linear mixed-effects model (LMM), with population defined as a random factor, using the LME4 R-package: $CT_{max} \sim 1 + (1|Population)$, and $CT_{min} \sim 1 + (1|Population)$ (Bates et al., 2015). We used the error variance as a proxy for σ_W^2 (within-population variance) and the intercept variation for σ_B^2 (variance between populations). Thus, P_{ST} estimates depend on the ratio $\frac{c}{h^2}$. Since these parameters are extremely challenging to obtain in the wild and usually unknown (Pujol et al., 2008), we considered a set of values to calculate P_{ST} (Brommer, 2011). We constructed several matrices for the P_{ST} values obtained for different values of c and h^2 . For each possible combination, the overall mean values (overall P_{ST}) and their 95% confidence intervals were calculated using a nonparametric bootstrap and compared with the upper limit of the confidence interval for overall F_{ST} (Supporting Information S7–S9). The value of the c/h^2 ratio at which the lower confidence interval for P_{ST} and the upper F_{ST} estimates overlap, can be interpreted as a measure of the robustness of the difference between F_{ST} and P_{ST} estimates (see Brommer, 2011). The use of P_{ST} presents several caveats, since non-additive genetic variance (epistasis or dominance effects), maternal effects or environmental factors and genotype-environment interaction, can lead to a distorted picture of additive genetic variation when studying only phenotypic variation in natural conditions (Brommer, 2011; Leinonen et al., 2008; Pujol et al., 2008). However, because experimental individuals were raised and analyzed under the same environmental conditions, we can assume a lower risk of unwanted effects.

2.7 | Statistical analyses

To test for geographic variation in CT_{max} and CT_{min} among populations of *R. parvipalmata*, we fitted two separate ANCOVA models for CT_{max} and CT_{min} , as dependent variables, including populations as categorical factor, and body mass as a covariate. Tukey HSD post-hoc tests were conducted to identify which populations differed in their thermal limits.

To test for covariation between CT_{max} and CT_{min} , thermal variation and elevation, we assessed linear and quadratic regression models using elevation and the thermal data as independent variables and CT_{max} , CT_{min} , WT, wt, CT, ct as dependent variables. This allowed to determine the relationship between species' physiological limits and environmental thermal predictors, including elevation. Paired t-Test and non-parametric test of Kolmogorov–Smirnov (KS) were used to assess differences between estimates of vulnerability of exposure to extreme temperatures, determined using macroclimate (WT, CT) or microclimate (wt, ct) thermal data. Finally, we

ran Mantel tests with 999 permutations in the R-package 'vegan' (Oksanen et al., 2018) to test the correlations between P_{ST} and F_{ST} pairwise population matrices.

In order to evaluate whether acclimation effects differ among populations, we used a two-way analysis of variance of CT_{max} and CT_{min} , with temperature (6, 13, 20, and 27°C) and population as fixed factors. We estimated the Acclimation Response Ratio (ARR) that measures the change in thermal tolerance relative to change in acclimation temperature (Claussen, 1977; Ruthsatz et al., 2022). In the case of ARR for upper thermal tolerance, $ARR = [(highest\ CT_{max} - lowest\ CT_{max}) / \Delta^\circ C]$. Then it provides a metric of thermal plasticity capturing acclimation capacity, thus allowing standardized comparisons between populations and critical thermal limits, (e.g., whether beneficial acclimation is greater for CT_{min} than CT_{max}). During CT_{min} estimates at the lower acclimation temperatures (6 and 13°C), water often reached crystallization (exothermic freezing reaction) before the immobility endpoint was attained. Most of the tadpoles briefly exposed to the freezing point recovered activity within a few seconds and survived the 24-h period, indicating resistance to these extreme cold temperatures, but the actual CT_{min} was inestimable (i.e., in these cases CT_{min} was lower [cooler] than the observed freezing point of water). Thus, in order to avoid bias associated with either biased sample or a biased CT_{min} estimates, we restricted the statistical analyses of CT_{min} acclimation scope to the 20–27°C range. All the statistical analyses were performed in R 3.6.1 (R Core Team, 2019).

3 | RESULTS

Pond minimum temperatures (TMIN, tmin) changed with altitude according to a quadratic function (Figure 3b; Table 2). However, for maximum temperatures we found a contrasting pattern of variation between macro- and microclimatic indicators along the elevation gradient (TMAX vs tmax, $t = -3.1944$, $df = 19$, $p < .01$; $D = 0.64$, $p < .05$). While TMAX monotonically decreased with altitude ($R^2 = 0.97$, $p < .01$; Figure 3a), tmax increased with elevation and peaked at mid-altitude ($R^2 = 0.77$, $p < .01$; Figure 3a, Table 2). Similarly, both seasonal and diel temperature ranges increased with elevation and peaked at mid-altitude populations (Table 2, Figure 3c,d).

A preliminary analysis revealed that tadpoles from populations between 900 and 1700m were smaller than tadpoles from low elevation (<700m) and the highest elevation (1800m) (ANOVA; $F_{10,325} = 57.26$, $p < .0001$). In general, critical thermal limits were unaffected by tadpole size. The effect of tadpole mass on CT_{max} was not significant in any of the populations (Color: $p = .07$; all the rest $Ps > 0.26$). Tadpole mass has a negative effect on CT_{min} in Nueva ($F_{1,11} = 8.91$; $p = .012$), Pandecarmen ($F_{1,12} = 8.10$; $p = .015$), and Pandéban ($F_{1,14} = 6.54$; $p = .023$) (that is, larger tadpoles reached cooler temperatures), but not for Fana ($F_{1,14} = 3.45$; $p = .084$) and the rest of the populations (all $Ps > 0.17$).

Critical thermal limits significantly differed between populations (CT_{max} , ANCOVA $F_{10,149} = 8.83$, $p < .001$; CT_{min} , ANCOVA $F_{10,149} = 15.39$, $p < .001$) (Tables 1 and Table S1) although they were

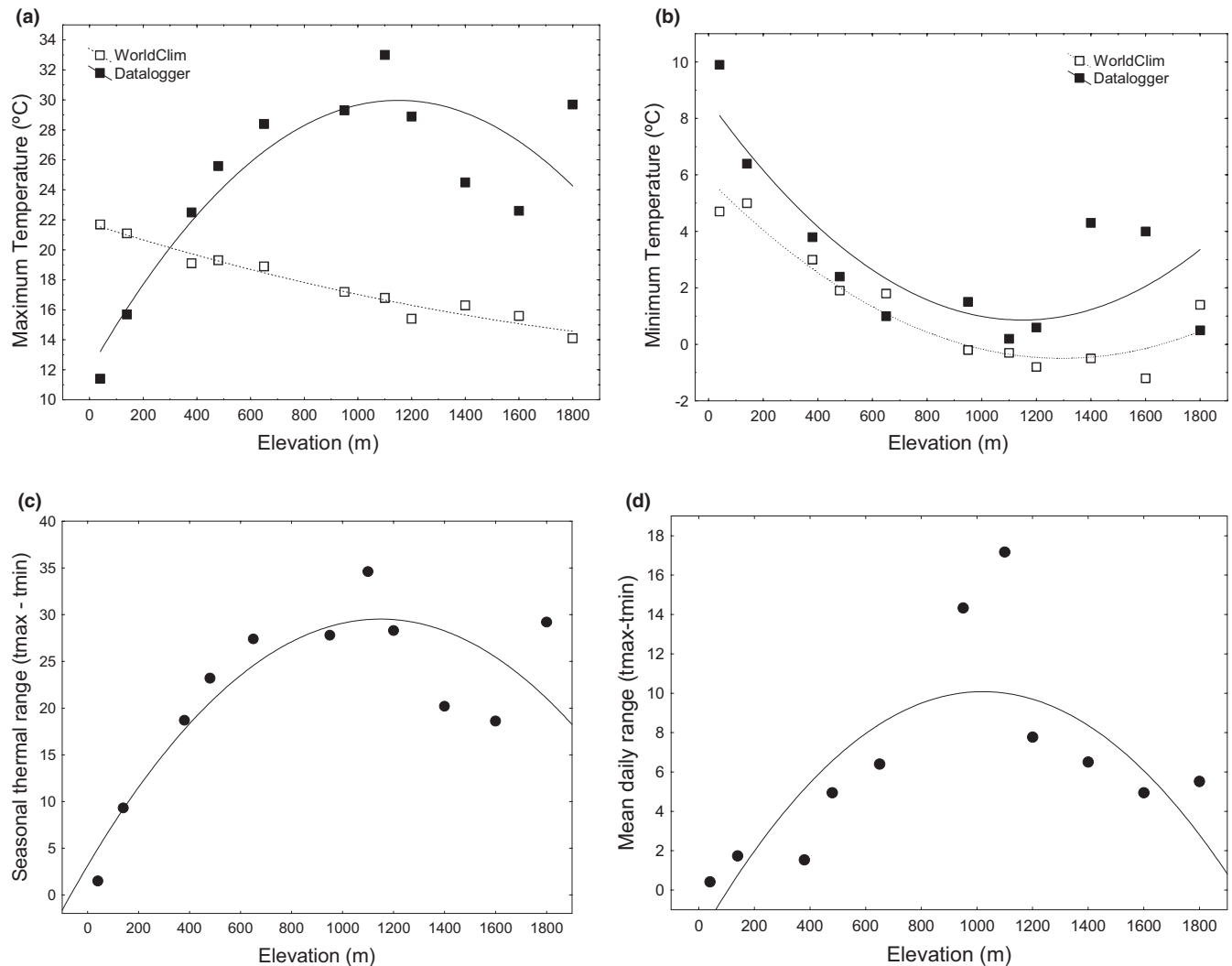


FIGURE 3 Elevational variation in: Absolute maximum (a) and minimum temperatures (b) using the WorldClim database (TMAX and TMIN), and microenvironmental pond datalogger information (tmax and tmin). Absolute seasonal temperature range (c) ($st = tmax - tmin$) and mean daily temperature range (dr) (daily $tmax - tmin$) (d), based in microenvironmental pond datalogger information. Thermal information corresponds only to the larval period for each studied location (see Figure 1b).

not affected by either elevation or any or macro- and microclimatic predictor (Figure 4; Table 2). Regarding vulnerability to heat stress, we also found contrasting differences between warming tolerance estimates based on macro- (WT; WorldClim) and micro-climate data (wt) ($t = 2.74$, $df = 10$, $p = .02$; $D = 0.82$, $p < .01$). These differences were remarkable in two ways. First, WT was consistently higher than wt, except for two populations at the lowest elevation (Figure 5a). Second, WT increased linearly with altitude ($R^2 = 0.95$, $p < .01$), whereas wt showed a minimum at mid-altitude sites ($R^2 = 0.71$, $p < .01$) (Table 2, Figure 5a). In contrast, cooling tolerances exhibit a non-linear decrease with elevation using both micro and macro-climatic estimators, Table 2, Figure 5b. Finally, both warming (wt) and cooling (ct) tolerances decrease with increasing daily temperature range (dr) (Table 2).

The overall value of neutral differentiation (F_{ST}) of *R. parvipalmata* was 0.066 (95% CI: 0.058–0.075) and revealed significant genetic differentiation among the 11 populations (Table S8). Under the

null hypothesis $c = h^2$, both CT_{max} and CT_{min} showed higher overall P_{ST} values than the upper confidence interval for F_{ST} ($P_{ST} > CT_{max}$, 95% CI: 0.12–0.21; $P_{ST} > CT_{min}$, 95% CI: 0.17–0.30) (Figure S1, Tables S9 and S10). However, the significance of this difference was not very robust, as the lower confidence estimate of P_{ST} overlaps with the upper limit of F_{ST} when $c/h^2 = 0.51$ for CT_{max} , and when $c/h^2 = 0.29$ for CT_{min} . The pairwise P_{ST} and F_{ST} matrices were not correlated for either CT_{max} or CT_{min} under the null hypothesis ($c = h^2$) (CT_{max} $r = 0.066$, $p = .40$; CT_{min} $r = -0.1695$, $p = .75$).

We found significant divergence among populations in the level of plasticity of CT_{max} and CT_{min} to variation in acclimation temperature (population \times acclimation interaction; Table 3). Acclimation to warm temperatures resulted in higher CT_{max} and CT_{min} (Table 4, Figure 6). Mid and high-altitude populations showed the highest phenotypic plasticity for CT_{min} (ARR; mean \pm 1SD, 0.32 ± 0.05 , $n = 3$, Table 4), being twice greater in magnitude when compared with the ARR of lowland populations (mean \pm 1SD, 0.16 ± 0.01 , $n = 2$;

TABLE 2 Results for the linear and quadratic regressions between temperature data, critical thermal limits elevation, warming tolerance (WT, wt) and cooling tolerance (CT, ct).

Model	Dependent	Predictors	Equation	F-value	df	R ²	p-value
	TMAX~	Elevation+Elevation ²	$y = 21.76 - 0.006x + 9.25E-7x^2$	162.17	2,8	0.97	.0000001
	tmax~	Elevation+Elevation ²	$y = 12 + 3.12E-2x - 1.36E-05x^2$	13.77	2,8	0.77	<.01
	TMIN~	Elevation+Elevation ²	$y = 5.85 - 9.81E-3x + 3.79E-6x^2$	49.44	2,8	0.93	.0026
	tmin~	Elevation+Elevation ²	$y = 8.63 - 1.35E-2x + 5.89E-6x^2$	8.12	2,8	0.67	.012
	SR~	Elevation+Elevation ²	$y = 7.03 + 1.14E-3x - 1.01E-6x^2$	5.81	2,8	0.59	.028
	sr	Elevation+Elevation ²	$y = 3.14 + 0.046x - 2.01E-5x^2$	66.68	2,8	0.74	.00001
	dr~	Elevation+Elevation ²	$y = -2.44 + 0.024x - 1.16E-5x^2$	15.71	2,8	0.60	.002
	CT _{max} ~	Elevation	$y = 36.7 + 1.92E-4x$	1.61	1,9	0.15	.236
	CT _{max} ~	TMAX	$y = 37.55 - 0.04x$	0.99	1,9	0.10	.346
	CT _{max} ~	tmax	$y = 36.48 + 0.01x$	1.06	1,9	0.11	.330
	CT _{max} ~	SR	$y = 38.62 - 0.26x$	3.72	1,9	0.29	.086
	CT _{max} ~	dr	$y = 36.76 + 0.02x$	0.44	1,9	0.05	.524
	CT _{min} ~	Elevation	$y = -1.62 - 1.5E-4x$	0.55	1,9	0.06	.477
	CT _{min} ~	TMIN	$y = -1.80 + 0.03x$	0.25	1,9	0.03	.629
	CT _{min} ~	tmin	$y = -1.91 + 0.05x$	1.69	1,9	0.16	.226
	CT _{min} ~	SR	$y = -3.77 + 0.29x$	2.75	1,9	0.23	.132
	CT _{min} ~	dr	$y = -1.75 - 0.002x$	0.004	1,9	<0.01	.951
	WT~	Elevation	$y = 15.38 + 4.18E-3x$	157.50	1,9	0.95	.000001
	WT~	SR	$y = 37.06 - 2.60x$	6.64	1,9	0.42	.03
	wt~	Elevation+Elevation ²	$y = 24.63 - 3.08E-2x + 1.34E-5x^2$	13.26	2,8	0.71	.003
	wt~	dr	$y = 20.34 - 1.47x$	24.49	1,9	0.73	.0008
	CT	Elevation+Elevation ²	$y = 7.38 - 9.31E-3x + 3.59E-6x^2$	24.36	2,8	0.86	.0004
	CT	SR	$y = 1.38 + 0.25x$	0.05	1,9	0.01	.828
	ct	Elevation+Elevation ²	$y = 10.15 - 1.32E-2x + 5.68E-6x^2$	8.17	2,8	0.67	.012
	ct	dr	$y = 8.70 - 0.71x$	24.89	1,9	0.73	.0007

TMAX and TMIN (maximum and minimum temperatures from macroclimate); tmax and tmin (maximum and minimum temperatures from dataloggers); sr, seasonal temperature range (tmax-tmin); dr, diel temperature range

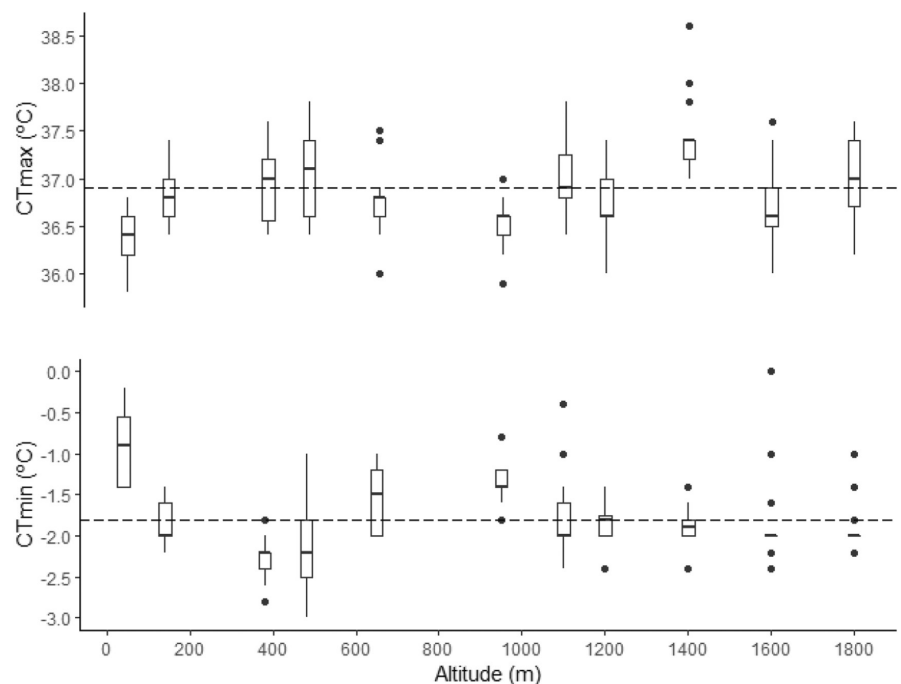


FIGURE 4 Boxplot showing the variation of thermal tolerance limits along the elevational gradient. The first and third quartiles ("hinges") and the 95% confidence interval of the median ("notches") are shown. The dashed line indicates the mean CT values of *R. parvipalmata* for the overall data, and dots placed past the line edges indicate outliers.

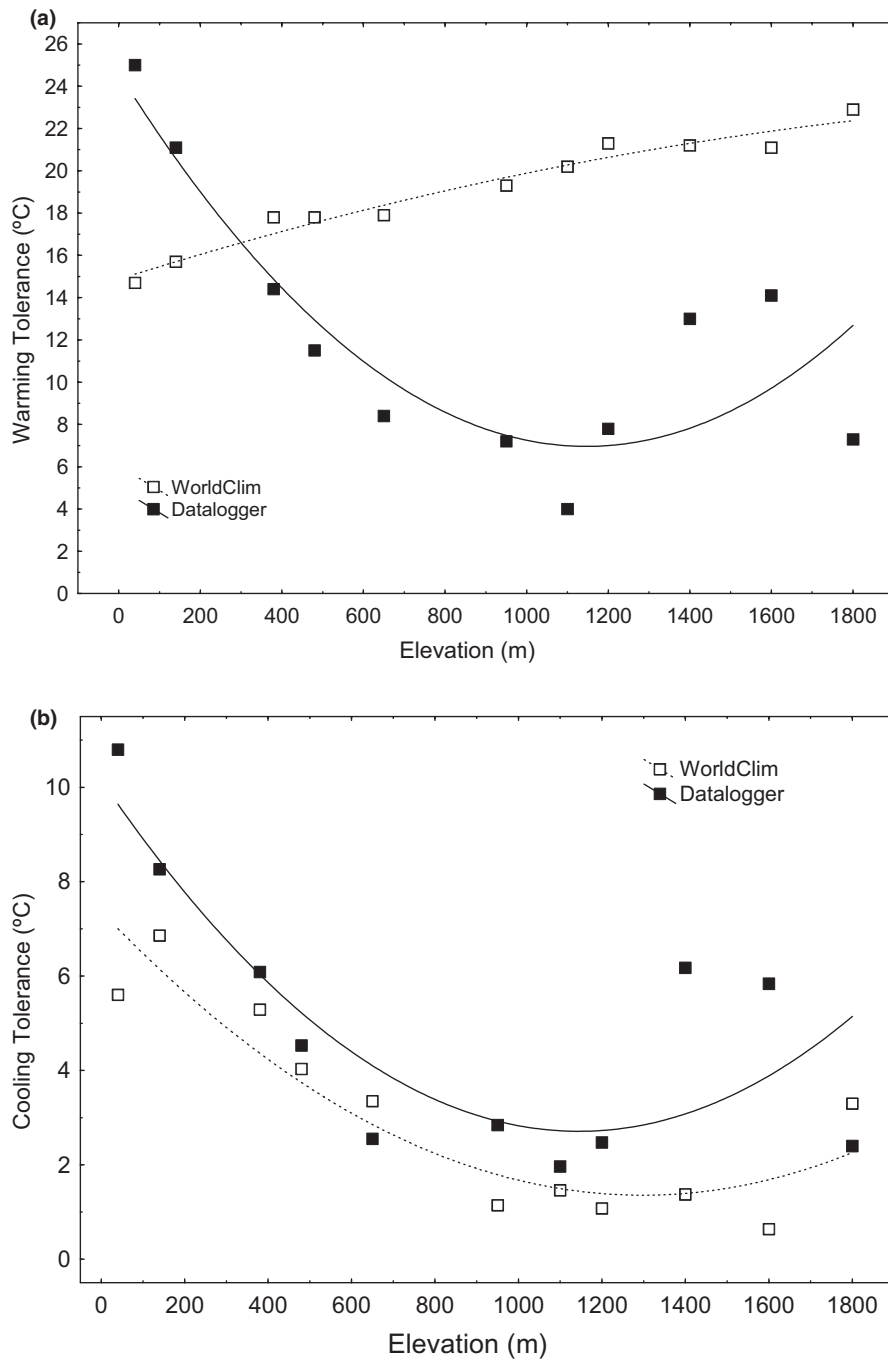


FIGURE 5 Estimates of Warming Tolerance: CT_{\max} minus TMAX or tmax, for either WorldClim or microclimate pond maximum temperature estimates (datalogger), respectively (a), and Cooling Tolerances: TMIN or tmin, for either WorldClim or microclimate pond minimum temperatures (datalogger), respectively, minus CT_{\min} (b), for 11 populations of *R. parvipalmata*, considering only the larval period. Triangles: estimates based on air temperature from WorldClim. Squares: estimates based on water temperatures registered in ponds with dataloggers.

Table 4). Greater ARR indexes for CT_{\max} were obtained for the mid-elevation populations (Table 4).

4 | DISCUSSION

Thermal limits (CT_{\max} , CT_{\min}) of tadpoles varied between *Rana parvipalmata* populations across the elevation gradient. However, CT_{\max} and CT_{\min} did not correlate with elevation nor with any of the macro- and microclimate predictors, suggesting niche conservatism. We did find some indications of directional selection as the divergence in thermal physiology limits (P_{ST}) tended to be slightly greater than the neutral

differences among populations (F_{ST}). Yet, since the differentiation matrices F_{ST} - P_{ST} were not correlated, and thermal physiology limits were not related to elevation or any of the environmental variables, our data did not support the hypothesis of local adaptation in thermal limits. The neutral genetic differentiation observed among the studied populations of *R. parvipalmata* ($F_{ST}=0.066$) is weak but well within the values of global F_{ST} reported for other amphibians over a wide spectrum of geographical scales and structured systems (Seppä & Laurila, 1999: 0.068; Luquet et al., 2015: 0.046, 0.024, and 0.011; Lenhardt et al., 2017: 0.041, 0.0159, 0.0215 and 0.0987).

The prevalence of intraspecific adaptive clinal variation with elevation in thermal traits is still a contentious topic in current

TABLE 3 Two-way ANOVA for changes in critical thermal limits (CT_{max} and CT_{min}) in five populations acclimated to several temperatures (6, 13, 20°C and 27°C for CT_{max} ; 20 and 27°C for CT_{min})

	CT_{max}				
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Population	4	6.298	1.574	10.957	<0.001
Acclimation temperature	3	222.322	74.107	515.7394	<0.001
Population × temperature	12	4.382	0.365	2.5413	0.003
Residuals	274	39.371	0.144		
	CT_{min}				
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Population	4	10.342	2.585	11.087	<0.001
Acclimation temperature	1	116.51	116.51	499.652	<0.001
Population × temperature	4	11.105	2.776	11.906	<0.001
Residuals	130	30.314	0.2333		

literature. Although several studies have found support for this pattern in both Critical Thermal Limits (Bishop et al., 2017; Klok & Chown, 2003; Miller & Packard, 1977; Sørensen et al., 2005) with greater variation in CT_{min} than CT_{max} (Gilbert & Miles, 2019; Muñoz et al., 2014), many others did not (Buckley et al., 2013; Gvoždík & Castilla, 2001; Slatyer et al., 2016; Slatyer & Schoville, 2016; Senior et al., 2019; Slatyer et al., 2019; Tonione et al., 2020; Enriquez-Urzelai et al., 2018, 2020). The lack of clinal variation in *R. parvipalmata* contrasts with the observation of local adaptation in larval life history traits of other temperate amphibians in seasonal thermal gradients (Berven et al., 1979; Luquet et al., 2015; Richter-Boix et al., 2015), including the closely related *Rana temporaria* (Laugen et al., 2003; Lind et al., 2011; Muir et al., 2014). However, our findings were consistent with the absence of elevational variation in thermal sensitivity of locomotion and thermotolerance reported for post-metamorphic and adults of *Rana parvipalmata* in the same study system (Enriquez-Urzelai et al., 2018, 2020). In that case, the terrestrial stages of amphibians can thermoregulate via micro-habitat selection or activity timing, sheltering underground in crevices and rodent burrows to avoid peak temperatures (Enriquez-Urzelai et al., 2020). This is parallel to the known Bogert effect in many ectotherms, (Bogert, 1949; Buckley et al., 2015; Farallo et al., 2018; Huey et al., 2003; Muñoz, 2022; Muñoz & Losos, 2018). However, the scope for behavioral thermoregulation in water is much more limited than in the terrestrial environment due to its high specific heat capacity and conductivity that reduces spatial thermal heterogeneity. Amphibian tadpoles, although able to thermoregulate (Hutchison & Dupré, 1992), can be exposed to unavoidably thermal stress, particularly in sunlit ponds without canopy cover (Duarte et al., 2012). Exposure to sunlight is prevalent in the breeding habitats of mid- and high-elevation populations of *R. parvipalmata*, which are exposed to relatively stressful high temperatures with warming tolerances <8°C (Figure 5a) and a wide range of both seasonal and short-term thermal variation (Figure 3d).

Shifts in the reproductive period and microclimatic conditions that are conditioned by elevation may dampen temperature changes along the gradient and prevent the expected linear decrease in both critical thermal limits. Therefore, thermal conservatism in tolerance limits together the absence of local adaptation may simply be a consequence of a coupled variation of reproductive timing with elevation (the “elevation-time axis” for temperature variation, in contrast with the elevation axis). In montane areas, altitudinal variation in the timing of reproduction seems to be constrained by hydroperiod rather than temperature itself, with higher altitude populations delaying breeding until snow melting (Álvarez et al., 2012; Corn, 2003). In addition, the time of spawning may be genetically determined in mountain populations (Álvarez et al., 2012; Phillimore et al., 2010; Wilczek et al., 2010). In this sense, it appears that during warm winters, when early snow melting occurs, frogs still delay reproduction until a threshold time is reached (Álvarez et al., 2012).

Phenotypic plasticity of thermal limits matched the observed variability in temperature through the elevation gradient. Populations from mid and high-elevations showed higher levels of plasticity than low-elevation populations, especially for CT_{min} . These populations, particularly those from mid-elevation, are also exposed to greater seasonal and daily thermal ranges, which supports the idea that phenotypic plasticity in critical thermal limits can be a response to the increased environment thermal variability. Similar adaptive plasticity in thermal tolerances are shown by populations of two toad species exposed to more variable climates (Alveal-Riquelme et al., 2016; McCann et al., 2018). Furthermore, since mid-elevation populations showed the lowest warming and cooling tolerances, and both thermal limits showed no clear clines, our data suggest the existence of a trade-off between phenotypic plasticity and tolerance to environmental temperatures (Stillman, 2003). Acclimation to low temperatures allowed tadpoles to achieve tolerance to extreme cold beyond the physical freezing point of water. The common frogs are among the most cold-tolerant amphibians of Europe (Gutiérrez-Pesquera et al., 2016; see also Enriquez-Urzelai et al., 2020) and, in northern

TABLE 4 Variation in the thermal physiology limits in five populations of *R. parvipalmata*, acclimated to constant temperatures (CT_{max} : 6, 13, 20, 27°C; CT_{min} : 20, 27°C). Plasticity for CT_{max} is estimated as the difference between mean CT_{max} values at the 27 and 6°C acclimation treatments, whereas in CT_{min} is estimated as the difference between mean CT_{min} values at the 27 and 20°C acclimation treatments. For CT_{max} , $ARR = [(highest\ CT_{max} - lowest\ CT_{max}) / \Delta^{\circ}C]$. For CT_{min} , $ARR = [(CT_{min}\ 27^{\circ}C - CT_{min}\ 20^{\circ}C) / \Delta^{\circ}C]$.

CT_{max} (°C)		Nueva			Cortegueros			Pandecarmen			Aliva			Llagusecu		
Acclimation	N	\bar{X}	SE	N	\bar{X}	SE	N	\bar{X}	SE	N	\bar{X}	SE	N	\bar{X}	SE	
6°C	11	36.06	0.13	16	35.91	0.10	17	35.64	0.07	15	36.21	0.14	14	36.26	0.10	
13°C	12	36.37	0.08	16	36.16	0.09	17	36.02	0.09	14	36.65	0.08	14	36.53	0.10	
20°C	16	36.84	0.08	16	36.76	0.09	16	37.04	0.11	14	37.46	0.11	14	37.03	0.12	
27°C	16	38.21	0.05	16	38.11	0.11	17	38.30	0.09	12	38.32	0.11	14	38.43	0.11	
Plasticity		2.15			2.20			2.70			2.11			2.17		
ARR 6–27°C	0.102			0.105			0.127			0.100			0.103			
ARR 6°–20°C	0.051			0.064			0.1			0.093			0.05			
ARR 20–27°C	0.196			0.194			0.18			0.123			0.2			
CT_{min} (°C)		Nueva			Cortegueros			Pandecarmen			Aliva			Llagusecu		
Acclimation	N	\bar{X}	SE	N	\bar{X}	SE	N	\bar{X}	SE	N	\bar{X}	SE	N	\bar{X}	SE	
20°C	13	-1.86	0.06	12	-1.55	0.11	16	-1.76	0.13	16	-1.87	0.06	14	-1.90	0.08	
27°C	12	-0.77	0.15	12	-0.38	0.18	17	0.49	0.11	14	-0.04	0.14	14	0.64	0.20	
Plasticity		1.09			1.17			2.25			1.83			2.54		
ARR (20–27°C)	0.156			0.167			0.321			0.261			0.363			

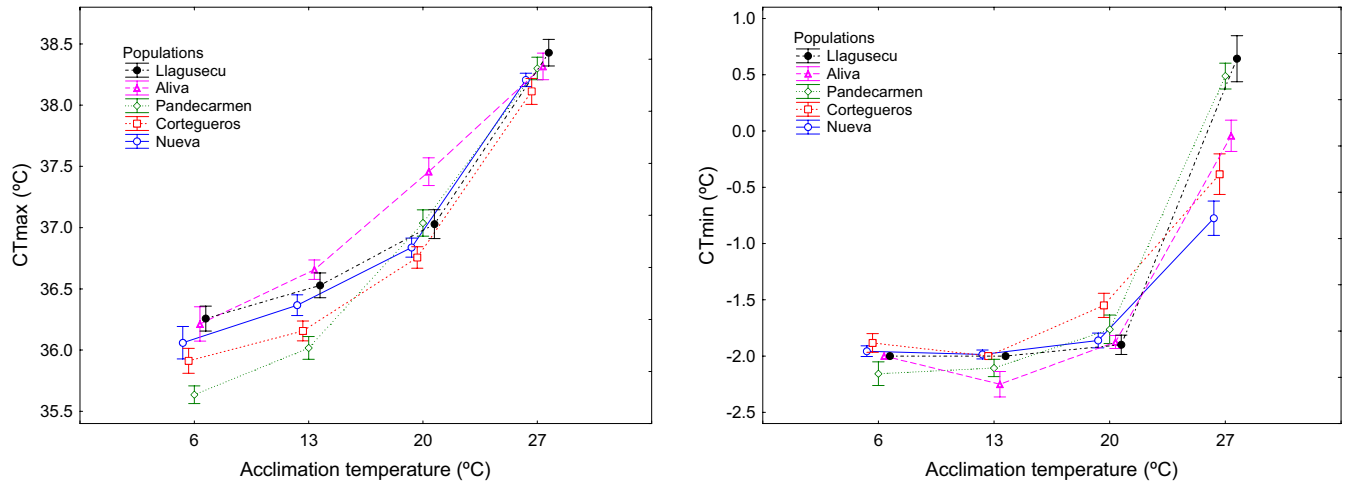


FIGURE 6 Phenotypic plasticity of critical thermal limits, CT_{max} (left) and CT_{min} (right), in five *Rana parvipalmata* populations: Nueva (140m), Cortegueros (650m), Pandecarmen (1106m), Aliva (1418m) and Llagusecu (1835m), acclimated to different constant temperatures. Data of plasticity in CT_{min} at 6°C and 13°C were excluded from statistical analyses because in most occasions water was frozen once it reaches crystallization point. Here, they are shown as an upper bound for CT_{min} ('true' CT_{min} were below the values showed for 6°C and 13°C acclimation temperature). Values for each population / acclimation treatment are Means \pm 1 SE.

Iberia, likely expanded during the cold glacial cycles (Dufresnes et al., 2020). Thus, it is possible that these extremely low CT_{min} are remnants of adaption to environmental conditions from that period, resulting in no current micro-geographic adaptive differentiation along elevation gradients. This hypothesis of 'evolutionary anachronism' (Janzen & Martin, 1982; see also Qu & Wiens, 2020, Moreira et al., 2021) was supported by our finding that lowland populations showed extreme cold tolerances (e.g., Color, 380m) although they presented the higher minimum temperature recorded.

The contrasting estimates of warming tolerances derived from macro- and microclimate data highlight the importance of monitoring the microhabitat when assessing vulnerability to global warming (Baudier et al., 2015; Gutiérrez-Pesquera et al., 2016; Katzenberger et al., 2018; Enriquez-Urzelai, Kearney, et al., 2019; Pintanel et al., 2019, 2022; see also Sunday et al., 2014). Warming tolerances estimated from macroclimate data supported the elevational thermal vulnerability hypothesis (risk of heat stress at low elevations and risk of cold stress at high elevations) (Pintanel et al., 2019, 2022; Sunday et al., 2014). Cooling tolerance (CT and ct) and warming tolerance estimated with microclimate data (wt), showed that mid-elevations populations are more likely to suffer both cold and heat acute stress than populations from low and high elevations. These deviations from the expected patterns arise from two factors. First, despite both CT_{max} and CT_{min} can vary between populations this variation was very weak (1.1 and 1.4°C for CT_{max} and CT_{min} , respectively). Besides, there was no distinct pattern across the elevation gradient and neither CT_{max} nor CT_{min} were related to any of the macro- and microclimate data (see also Richter-Boix et al., 2015; Schou et al., 2017; Enriquez-Urzelai, 2018; Enriquez-Urzelai et al., 2020). Second, because of such weak variation in CT_{max} and CT_{min} , the elevation variation in warming and cooling tolerances matched the pattern of variation in environmental temperatures, with only minor influence of thermal

physiology limits. Only TMAX, used to determine WT, varied with elevation as expected, being lower at high elevation populations. However, both the lower minimum temperatures (TMIN and tmin) and higher tmax were found in mid-elevation populations and not at the high-peak populations. This a priori unexpected outcome is likely the result of phenological shifts in these local populations and differences in habitat structure (i.e., canopy cover, topographical shadow) between low-, mid-, and high-elevation wetlands. In the study area, the breeding habitat of lowland (below 500–700m) common frogs consists of very small and shallow waters scattered on a rather humanized landscape (e.g., track pools, ditches, and less often small temporary ponds), and located in valley bottoms with dense canopy cover, which prevents direct beam solar radiation. In contrast, breeding habitats in mid-altitude areas (700–1200m) are small, shallow, temporary ponds, most often located on high plains and hills without canopy cover, and therefore, exposed to high levels of direct solar radiation and a low thermal buffering. Finally, although high altitude wetlands (1300–2100m) can be affected by topographic shading, most often they lack canopy cover, which besides a thinner atmosphere leads to low thermal buffering. This, along with the change in reproductive phenology (the elevation-time axis), may reduce the actual differences in temperatures experienced by larvae at different elevations.

Phenological shifts and microgeographic variation in habitat structure can determine the thermal regimes experienced by populations along mountain gradients (see Muñoz & Losos, 2018). Seemingly, frog populations have responded to natural selection on breeding phenology, likely due to low reproductive success of too late breeders (high risk of pond drying in late winter/spring) in the lowlands and both early and late breeders in medium and high elevation populations (high risk of crushing due to late snowfall, pond drying in summer, and time constraints for the year recruits). In turn, this pattern of temporal displacement with elevation

has reduced the thermal differences between populations, thus hindering physiological evolution (see also Enríquez-Urzelai et al., 2018; Muñoz, 2022).

5 | CONCLUSIONS

Directional changes in reproductive phenology and phenotypic plasticity can block local thermal adaptation by lowering selective pressures for population differentiation, (consistent with niche conservatism hypothesis; Muñoz & Losos, 2018). Therefore, although “phenological buffering” may override thermal stress under current conditions, it could hinder long-term adaptation to climate change, potentially compromising long-term population sensitivity (Buckley et al., 2015; Enriquez-Urzelai et al., 2018; Kearney et al., 2009). This agrees with the idea that phenotypic responses do not occur in selective vacuums, and therefore, any adjustment in one response can cause an evolutionary ripple in others (Muñoz, 2022). For instance, if the timing of breeding is under genetic control, rapid climate change could cause temporal mismatches between physiological traits and the new thermal conditions with still unknown consequences. Our previous work on *R. parvipalmata* using biophysical models of thermal exposure indicated that the risk of reaching body temperatures beyond the species' thermal tolerance is similar across elevations, but mountain populations can face the worst climatic scenario because of their narrow seasonal activity windows (Enriquez-Urzelai et al., 2018, 2020) and conflicting selection on breeding phenology. Present results reinforce this view: mountain populations of *R. parvipalmata* are also the most vulnerable during the aquatic phase. Therefore, future research should focus on the genetic component of reproductive phenology, the physiological responses of mountain populations, and the effects of space-time covariations in biological processes that can determine how the species face climate change.

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DATA AVAILABILITY STATEMENT

Data are deposited on Figshare (<https://doi.org/10.6084/m9.figshare.21187024.v1>).

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