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Thermal tolerance and acclimation capacity in the European common frog (*Rana temporaria*) change throughout ontogeny

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Abstract

Phenotypic plasticity may allow ectotherms with complex life histories such as amphibians to cope with climate-driven changes in their environment. Plasticity in thermal tolerance (i.e., shifts of thermal limits via acclimation to higher temperatures) has been proposed as a mechanism to cope with warming and extreme thermal events. However, thermal tolerance and, hence, acclimation capacity, is known to vary with life stage. Using the common frog (*Rana temporaria*) as a model species, we measured the capacity to adjust lower (CT_{\min}) and upper (CT_{\max}) critical thermal limits at different acclimation temperatures. We calculated the acclimation response ratio as a metric to assess the stage-specific acclimation capacity at each of seven consecutive ontogenetic stages and tested whether acclimation capacity was influenced by body mass and/or age. We further examined how acclimation temperature, body mass, age, and ontogenetic stage influenced CT_{\min} and CT_{\max} . In the temperate population of *R. temporaria* that we studied, thermal tolerance and acclimation capacity were affected by the ontogenetic stage. However, acclimation capacity at both thermal limits was well below 100% at all life stages tested. The lowest and highest acclimation capacity in thermal limits was observed in young and late larvae, respectively. The relatively low acclimation capacity of young larvae highlights a clear risk of amphibian populations to ongoing climate change. Ignoring stage-specific differences in thermal physiology may drastically underestimate the climate vulnerability of species, which will hamper successful conservation actions.

KEYWORDS

acclimation response ratio, climate change, metamorphosis, phenotypic plasticity, thermal bottleneck, thermal limits

1 | INTRODUCTION

Environmental temperature directly affects numerous life-history traits including maintenance, growth rate, reproduction, and development in ectothermic animals (Angilletta & Angilletta, 2009; Angilletta et al., 2002; Huey & Stevenson, 1979), since the rates of most biochemical reactions and many biological processes increase

with temperature (Hochachka & Somero, 2002; Pörtner, 2001). Changes in environmental temperature can pose a challenge for wildlife, particularly for ectothermic animals whose body temperature fluctuates with environmental temperatures if behavioral thermoregulation is constrained (reviewed in Little & Seebacher, 2017). All animals have a range of tolerable temperatures that are constrained by upper and lower critical thermal limits (CT_{\min} and CT_{\max}) beyond

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which survival is not possible (Holzman & McManus, 1973; Little & Seebacher, 2017). The range in temperatures between CT_{min} and CT_{max} is defined as the thermal tolerance breadth (TTB; Paaijmans et al., 2013; Pörtner et al., 2006; Tewksbury et al., 2008). Since climate change has not only caused an increase in mean air and water temperatures but also an increased magnitude and frequency of extreme climatic events (Huey et al., 2012; IPCC, 2021; Pachauri et al., 2014; Pacifici et al., 2015), tolerance to temperature extremes and a shift in thermal limits via acclimatization to higher environmental temperatures are consequently among the most important traits defining survival and geographic distribution in ectotherms and might be key mechanisms that help species to cope with ongoing global warming (e.g., Comte & Olden, 2017; Dahlke et al., 2020; Duarte et al., 2012; Somero, 2010; but see Gunderson & Stillman, 2015; Morley et al., 2019).

As an adaptive response, thermal tolerance and acclimation capacity are found to be higher for species and populations experiencing greater diel and seasonal differences in environmental temperature as well as more pronounced thermal extremes (Janzen, 1967; Pither, 2003), with narrower tolerance ranges and higher thermal limits in tropical regions relative to temperate regions (Addo-Bediako et al., 2000; Deutsch et al., 2008; J. Sunday et al., 2019; J. M. Sunday et al., 2011, 2014; Gutiérrez-Pesquera et al., 2016; but see: Gunderson & Stillman, 2015; Sørensen et al., 2016). This geographic pattern can be explained by the *climate variability hypothesis* (Bozinovic et al., 2011; Ghalambor et al., 2006; Janzen, 1967; Stillman, 2003) since latitudinal gradients vary in mean temperature and seasonal changes in temperature. Besides these geographic patterns of thermal adaptation, thermal tolerance is also influenced by several extrinsic and intrinsic factors such as body mass (Ospina & Mora, 2004; Peralta-Maraver & Rezende, 2021; Ribeiro et al., 2012), thermal history (Nyamukondiwa & Terblanche, 2010; but not: Longhini et al., 2021), physiological condition (Rezende et al., 2014), phylogeny (Hoffmann et al., 2013; but not: Simon et al., 2015), and ontogeny (Cupp, 1980; Dahlke et al., 2020; Enriquez-Urzelai, Sacco, et al., 2019; Floyd, 1983; Turriago et al., 2015).

In species with complex life cycles such as amphibians (Brown & Cai, 2007; Shi, 2000), thermal tolerance and acclimation capacity are expected to change during the ontogeny according to physiological and morphological reorganizations and concomitant aerobic capacities in relation to oxygen demand (Leiva et al., 2019; Pörtner, 2002; Pörtner & Farrell, 2008; Pörtner & Knust, 2007). Furthermore, at some life cycle transitions, amphibian species undergo habitat shifts that result in profound changes in thermal environments (Enriquez-Urzelai, Sacco, et al., 2019; Shi, 2000; Werner, 1986; Wilbur, 1980). Stage-specific adaptations in thermal traits such as upper or lower thermal tolerance and acclimation capacity can be explained by the *adaptive acclimation hypothesis* (Huey et al., 1999) as thermal plasticity depends on the level of thermal variation to which each stage would be exposed (Angilletta & Angilletta, 2009; Chevin & Hoffmann, 2017; Gunderson & Stillman, 2015). For example, juvenile froglets are more likely to encounter more extreme temperatures as well as more variable thermal environments in their terrestrial habitats than aquatic larvae because air temperatures fluctuate more than water

temperatures (Feder & Hoffmann, 1999). However, to face thermal risk, terrestrial stages might use different thermoregulatory options such as behavioral thermoregulation by selecting favorable microhabitats or evaporative cooling (rev. in Navas et al., 2008; rev. in Little & Seebacher, 2017). In contrast, behavioral thermoregulation is constrained in aquatic amphibian larvae due to the lower thermal heterogeneity of water than air and soil, forcing the aquatic stages to adapt their thermal limits to microenvironmental extreme temperatures (Gunderson & Stillman, 2015). Thus, selection might promote stage-specific thermal adaptations such as specific thermal sensitivities, thermal preferences and tolerances, and acclimation capacity (Enriquez-Urzelai et al., 2018; Enriquez-Urzelai, Sacco, et al., 2019; Truebano et al., 2018), resulting in stage-specific sensitivity to climate change. Since species' vulnerability to climate change (i.e., survival) depends on the most temperature-sensitive life stages (Dahlke et al., 2020), improving our understanding of factors that shape thermal tolerance and acclimation capacity is increasingly important, given the rapidly changing climate.

Here, we studied the thermal tolerance and acclimation capacity of upper and lower thermal limits in different life stages of a central European lowland population of the European common frog (*Rana temporaria*) reared at six, constant acclimation temperatures (10–28°C) in a laboratory experiment. Specifically, we examined how acclimation temperature, body mass, age, and ontogenetic stage influenced thermal tolerance (i.e., CT_{min} , CT_{max} , and TTB) and whether acclimation capacity was determined by ontogenetic stage, body mass, and age. We calculated the acclimation response ratio (ARR; Claussen, 1977) as a metric to assess the stage-specific acclimation capacity at each of seven consecutive ontogenetic stages. We predicted that thermal tolerance and acclimation capacity would vary with life stage and be reduced during metamorphic climax due to increased metabolic demands associated with morphological and physiological reorganization (Cupp, 1980; Hourdry et al., 1996; Menke & Claussen, 1982; Ruthsatz, Dausmann, Reinhardt, et al., 2019). Also, we predicted that thermal tolerance would be lower in juvenile froglets than in late larvae since terrestrial stages might have the capacity to behaviorally regulate body temperature, whereas selection of favorable microhabitats by earlier stages might be constrained in aquatic thermal environments (Enriquez-Urzelai et al., 2018, 2020; Köhler et al., 2011; Ludwig et al., 2015; Vences et al., 2002). Our study aimed to identify the most sensitive and, thus, most vulnerable ontogenetic stage to temperature variation in the life cycle of this temperate amphibian and to determine whether this population had the capacity to shift thermal limits via acclimation to higher environmental temperatures as a mechanism to cope with global warming.

2 | MATERIALS AND METHODS

2.1 | Animal husbandry, experimental design, and acclimation temperatures

Five clutches of *R. temporaria* were obtained from the Waldpark Marienhöhe (53°34'37.4" N 9°46'57.5" E) in Hamburg, Germany on April 12th in 2018 and immediately transferred to climate chambers

(Weiss Umwelttechnik GmbH) at the Institute of Zoology at Universität Hamburg. All aquaria contained tap water that was dechlorinated by continuous aeration.

At Gosner stage 25 (free-swimming larvae; Gosner, 1960), 270 of the larvae were randomly allocated to one of six different temperatures treatments (i.e., mean (\pm SD) of 10.0 (0.2), 14.0 (0.5), 18.0 (0.1), 22.0 (0.1), 25.0 (0.2), and 28.0 (0.3) $^{\circ}$ C; three replicates/temperature, 18 tanks) at an initial tadpole density of 1.87/L (9 L tank with 8 L of water and 15 larvae).

Water temperatures during larval development (i.e., 14, 18, 25, and 28 $^{\circ}$ C) were achieved by indirect heating elements beneath the tanks (Tetra GmbH; adjustable heating element, Tetra HT100, 100 W) or by stable air temperature for 10 and 22 $^{\circ}$ C in controlled environment rooms. Throughout larval and juvenile rearing, animals received a 12:12 light:dark (09:00 a.m. to 21:00 p.m.) light regime corresponding to the natural condition when the clutches were collected. Larvae were reared in glass tanks and fed ad libitum rations of 50% high-protein flaked fish food (Sera micron breeding feed for fish and amphibians; Sera) and 50% spirulina algae twice a day to guarantee that food was available in abundance. Ration size was adjusted to account for changes in body size and the number of individuals in each aquarium. After completing metamorphosis at Gosner stage 46 (complete resorption of the tail; Gosner, 1960), froglets were transferred into separate tanks containing a small amount of water to avoid desiccation. All surviving animals were placed in a climate chamber maintained at $22 \pm 0.1^{\circ}$ C, representing an average temperature commonly experienced in the field (reviewed in Drakulic et al., 2017). Froglets were fed ad libitum with adult *Drosophila melanogaster* for 7 days. The tanks were checked daily for dead animals, which were removed. At the end of the experiment, froglets were euthanized with 200 mg/L of tricaine methanesulfonate (MS-222, ethyl 3-aminobenzoate methanesulfonate, E10521 ALDRICH; Sigma-Aldrich) dissolved in water, buffered with 200 mg/L of sodium bicarbonate (S5761 SIGMA; Sigma-Aldrich) (Cecala et al., 2007) and transferred into ethanol (70%).

2.2 | Life-history variables and ontogenetic staging

After hatching, we measured snout-vent length (SVL) and body mass and determined the ontogenetic stage every other day. The age of all individuals at each measurement was known (age = days since hatching). The SVL of the larvae and froglets was measured with a caliper to the nearest 0.5 mm. Specimens were dry blotted and their mass was determined to the nearest 0.001 g with an electronic balance (Professional Digital Jewelry Gold Scale Balance; GandG).

The ontogenetic stage was determined by evaluating the status of key morphological features as detailed in Gosner (1960). Each animal was assigned an ontogenetic stage group according to the procedure of Ortiz-Santaliestra and Sparling (2007), Ruthsatz, Dausmann, et al. (2019), resulting in seven consecutive groups: (1) pre-limb (absence of hind limbs; Gosner stages 24–26), (2) limb bud (hind limb visible, but no clear joint formed; Gosner stages 27–34), (3)

middle hind limb (knee joint apparent, but toes not completely separated; Gosner stages 35–37), (4) late hind limb (hind limb tubercles and subarticular patches formed; Gosner stages 38–41), (5) onset of metamorphic climax (at least one forelimb present; Gosner stage 42), (6) end of the metamorphic climax (complete resorption of the tail; Gosner stage 46), and (7) juvenile (Gosner stage 46 + 7 days; Ruthsatz, Dausmann, et al., 2019, 2020; Ruthsatz, Dausmann, Reinhardt, et al., 2020). Hereafter, we refer to stages 1–5 as tadpoles and to stages 6–7 as froglets.

2.3 | Thermal tolerance

Thermal tolerance of *R. temporaria* was evaluated at ontogenetic stage groups 1–7. Both critical thermal minimum (CT_{min}) and maximum (CT_{max}) endpoints were defined as the temperature at which locomotor activity became disorganized and the animal lost the ability to right itself (Holzman & McManus, 1973; Lutterschmidt & Hutchison, 1997; Turriago et al., 2015; Figure 1). The CT_{min} and CT_{max} limits were determined using the dynamic method according to Cowles and Bogert (1944) and Hutchison (1961), except for the endpoint (Wu and Kam, 2005). In anuran larvae, the occurrence of spasms is difficult to determine, and thus we decided to use the loss of the righting response after being flipped on the back in the water with a probe as our criterion for the CT_{min} and CT_{max} endpoints (Lutterschmidt & Hutchison, 1997; Turriago et al., 2015; Wu & Kam, 2005) in tadpoles and froglets.

This method involves linearly decreasing (for CT_{min}) or increasing (for CT_{max}) test temperatures by a specific rate until an appropriate endpoint is reached (Lutterschmidt & Hutchison, 1997). Each individual was placed in a 250-ml flask with 200 ml (i.e., tadpoles) and 10 ml (i.e., froglets) of water, which was then placed in a temperature-controlled water bath. The heating and cooling rates were $\pm 0.1^{\circ}$ C \times min^{-1} , and the water temperature served as a proxy for body temperature in small froglets (Enriquez-Urzelai, Sacco, et al., 2019; Fan et al., 2021, Hutchison, 1961). The initial temperature in the water bath was set at the individual's acclimation temperature. The TTB was equal to the difference between CT_{max} and CT_{min} . Thermal tolerance measurements were carried out between 1200 and 1700 h to control for the influence of natural circadian rhythms and photoperiod (Agudelo-Cantero & Navas, 2019; Floyd, 1985). During this period, animals experience the highest diurnal temperatures in their natural habitats.

At ontogenetic stages 1–6, 10 larvae from each aquarium ($N = 5$, CT_{min} and $N = 5$, CT_{max}) were tested at set time intervals. One week after completing the metamorphic climax (ontogenetic stage 7), all remaining animals ($N = 159$) were tested for both, individual CT_{min} and CT_{max} . Froglets were allowed to recover for 24 h between CT_{min} and CT_{max} measurements (Fan et al., 2021). In half of the froglets, CT_{min} was measured first, whereas in the other half CT_{max} was measured first. Froglets were randomly assigned to each cohort. Hereafter, we refer to CT_{min} , CT_{max} , and TTB as measures of thermal tolerance.

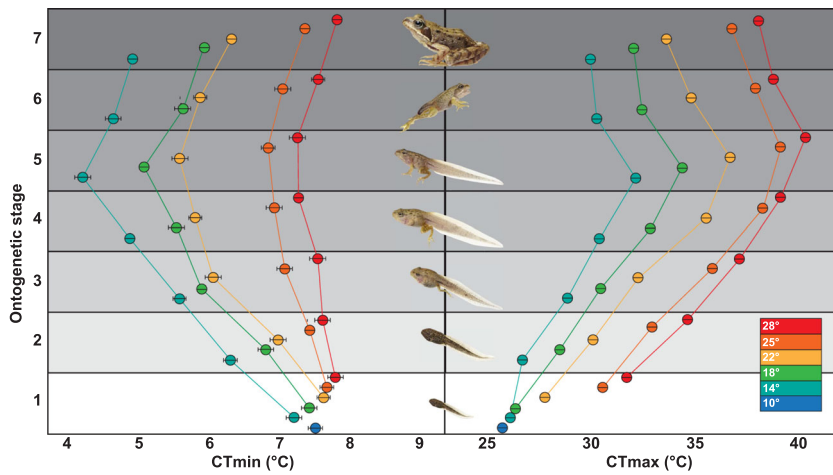


FIGURE 1 Mean thermal tolerance + 2 SE [i.e., CT_{min} (left panel) and CT_{max} (right panel)] of seven consecutive ontogenetic stages of *Rana temporaria* (larvae and froglets) at six acclimation temperatures.

2.4 | Statistical analysis

For all statistical tests, R 4.0.5 (R Development Core Team, 2007) for Windows was used. All plots were constructed using ggplot2 (Wickham, 2009) and Adobe Illustrator CS6.

2.4.1 | Acclimation capacity

A well-established method to measure acclimation capacity in ectothermic animals is the calculation of the ARR, that is, the slope of the linear function describing the change in thermal tolerance (as measured by CT_{min} and CT_{max}) with a given change in acclimation temperature (e.g., Claussen, 1977; Gunderson & Stillman, 2015; Morley et al., 2019).

To determine the acclimation capacity of thermal limits to temperature variation at each ontogenetic stage in *R. temporaria*, we calculated the ARR for CT_{max} and CT_{min} using the equation according to Claussen (1977):

$$\text{ARR} = \frac{\begin{array}{l} [\text{CT}_{\text{max}} \text{ at highest acclimation temperature} \\ \text{(e. g. , 18}^\circ\text{C)} \\ - \text{CT}_{\text{max}} \text{ at lowest acclimation temperature} \\ \text{(e. g. , 16}^\circ\text{C)}] \\ \text{°C change between the two different acclimation} \\ \text{temperatures (e. g. , 2}^\circ\text{C)} \end{array}}{\text{°C change between the two different acclimation temperatures (e. g. , 2}^\circ\text{C)}}$$

The calculations were performed for both CT_{max} and CT_{min} across temperature treatments within each ontogenetic stage independently for each aquarium (Table A1). Higher absolute values of ARR correspond to higher plasticity in thermal tolerance limits (i.e., greater acclimation capacity; Claussen, 1977; Gunderson & Stillman, 2015; Kingsolver & Huey, 1998; van Heerwaarden et al., 2016). An acclimation response ratio of 1.00 indicates a 100% acclimation in

thermal tolerance to a temperature increase of 1°C (Gunderson & Stillman, 2015; Morley et al., 2019). One hundred percent acclimation in thermal tolerance traits indicates an increase of 1°C in CT_{min} and CT_{max} to a temperature increase of 1°C.

2.4.2 | Effects of acclimation temperature, mass, and ontogenetic stage on thermal tolerance

We tested the effect of acclimation “temperature” and “ontogenetic stage” as fixed factors on thermal tolerance (measured as CT_{min}, CT_{max}, and TTB) in three separate generalized mixed-effect models (GLMM) using the *glmer()* function in the lme4 package (<https://cran.r-project.org/package=lme4>) in R (Bates et al., 2018), with gaussian distribution (Table 1). “Age” (Terblanche et al., 2007) and “mass” (Leiva et al., 2019) were included as covariates to GLMMs. To investigate whether the impact of acclimation temperature on thermal tolerance depended on the ontogenetic stage, we also included the interactive effect of “temperature” and “ontogenetic stage” as fixed factors. The replicate unit for the GLMM analyses was aquarium (the mean value for individuals in an aquarium). *P* values were obtained from quasi-likelihood-ratio tests (Bolker, 2008). To address dependencies in the data, “aquarium” was included as a random factor in all models. Since the individual larvae per aquarium could not be marked, the ID of a single larvae could not be included as a random factor to the models to account for statistical independence of the repeated measurements. *N* refers to the total number of animals and *n* to the total number of aquaria.

2.4.3 | Effects of the ontogenetic stage, mass, and acclimation duration on acclimation capacity

To examine the effect of the ontogenetic stage, mass, and acclimation duration on the acclimation capacity of CT_{min} and CT_{max} in

TABLE 1 Effects of acclimation temperature, ontogenetic stage, mass, and age on thermal tolerance traits (i.e., CT_{min} , CT_{max} , and TTB) during the development of the common frog (*Rana temporaria*)

| Dependent variable | Fixed effects and covariate | Estimate | SE | t value | p value | N (n) |
|--------------------------------|---------------------------------|----------|--------|---------|---------|----------|
| CT_{min} (°C) | (Intercept) | 7.361 | 0.171 | 43.022 | <0.001 | 624 (18) |
| | Temperature | 0.014 | 0.006 | 2.271 | 0.023 | |
| | Ontogenetic stage | -0.860 | 0.054 | -15.660 | <0.001 | |
| | Mass | -0.003 | 0.000 | -20.417 | <0.001 | |
| | Age | 0.035 | 0.004 | 8.135 | <0.001 | |
| | Temperature × ontogenetic stage | 0.027 | 0.001 | 14.663 | <0.001 | |
| CT_{max} (°C) | (Intercept) | 0.026 | 0.731 | 35.703 | <0.001 | |
| | Temperature | 0.024 | 0.0276 | 8.711 | <0.001 | |
| | Ontogenetic stage | 2.847 | 0.243 | 11.704 | <0.001 | |
| | Mass | 0.004 | 0.001 | 7.443 | <0.001 | |
| | Age | -0.337 | 0.002 | -17.725 | <0.001 | |
| | Temperature × ontogenetic stage | -0.035 | 0.008 | -4.284 | <0.001 | |
| Thermal tolerance breadth (°C) | (Intercept) | 18.999 | 0.783 | 24.255 | <0.001 | |
| | Temperature | 0.223 | 0.029 | 7.562 | <0.001 | |
| | Ontogenetic stage | 3.724 | 0.260 | 14.308 | <0.001 | |
| | Mass | 0.008 | 0.000 | 11.293 | <0.001 | |
| | Age | -0.037 | 0.020 | -18.526 | <0.001 | |
| | Temperature × ontogenetic stage | -0.064 | 0.008 | -7.171 | <0.001 | |

Note: Results were obtained from separate GLMMs, using “temperature” and “ontogenetic stage” as well as the interactive effect of both as the fixed factors; “aquarium” as the random factor; “age” and “mass” as the covariates. *N* is the total number of analyzed data points across ontogeny; *n* to the total number of analyzed tanks. Significance was set at $p < 0.05$.

Abbreviations: CT_{min} , critical thermal minimum; CT_{max} , critical thermal maximum; GLMMs, generalized linear mixed-effects models; TTB, thermal tolerance breadth.

R. temporaria, data were analyzed using two separate GLMMs using the *glmer()* function (Bates et al., 2018) in the lme4 package (<https://cran.r-project.org/package=lme4>) in R, with Gaussian distribution. The ARR of CT_{min} and CT_{max} were used as dependent variables in two separate models (Table 2). To avoid pseudoreplication, the categorical predictor “aquarium” was included as a random factor. We used age in days after hatching as an estimate/metric for “larval/acclimation duration” since tadpoles were allocated to different acclimation temperatures after hatching (Terblanche et al., 2007). Consequently, larval duration was equivalent to the animals' age and time exposed to acclimation temperatures.

Using the *lmer.glmulti* wrapper function implemented in the *glmulti* package (<https://cran.r-project.org/package=glmulti>; Calgano), we then selected the best-fit model with three predictors or less for each of the dependent variables. The best GLMMs were selected based on the lowest Akaike Information Criterion (AIC). We used “ontogenetic stage,” “mass,” and “larval duration” as fixed factors, considering the random factor as specified above. Best GLMMs were followed by post hoc comparisons (Tukey's test; Tukey's honestly significant difference function, *multcomp* package, vers. 1.2–13) to compare all

possible pairwise combinations of ontogenetic stages when overall tests were significant (Table A2).

3 | RESULTS

The experiment was conducted for 9 weeks. At the end of the experiment, all animals had reached the end of metamorphic climax + 7 days at that time (i.e., ontogenetic stage 7; Gosner, 1960; Ruthsatz, Dausmann, Drees, et al., 2020). Mean survival (\pm SD) was high in all treatments between 14 and 28°C (14°C: $91.1 \pm 3.3\%$; 18°C: $95.5 \pm 3.3\%$; 22°C: $91.1 \pm 3.3\%$; 25°C: $84.4 \pm 3.3\%$; 28°C: $91.1 \pm 6.6\%$). At 10°C, however, none of the larvae survived to ontogenetic stage 2. Therefore, stages 2–7 only include animals at developmental temperatures of 14, 18, 22, 25, and 28°C.

At all acclimation temperatures, body mass increased until ontogenetic stage 4, decreased during the metamorphic climax (i.e., ontogenetic stages 5–7), and increased again after the completion of metamorphosis (i.e., ontogenetic stage 7; Figure A1). Body mass of larvae and froglets was negatively correlated with acclimation temperature within and across all seven ontogenetic stages (Figure A1).

| Dependent variable | AIC best model | Fixed effects | Estimate | SE | t value | p value | N (n) |
|-----------------------|----------------|---------------|----------|-------|---------|---------|--------|
| ARR CT _{min} | -139.9 | GS1 | 0.023 | 0.003 | 7.06 | <0.001 | 21 (3) |
| | | GS2 | 0.069 | 0.004 | 15.05 | | |
| | | GS3 | 0.119 | 0.004 | 25.97 | | |
| | | GS4 | 0.151 | 0.004 | 32.92 | | |
| | | GS5 | 0.201 | 0.004 | 43.63 | | |
| | | GS6 | 0.182 | 0.004 | 39.50 | | |
| | | GS7 | 0.182 | 0.004 | 39.65 | | |
| ARR CT _{max} | -123.5 | GS1 | 0.333 | 0.004 | 68.12 | | |
| | | GS2 | 0.206 | 0.006 | 31.91 | | |
| | | GS3 | 0.261 | 0.006 | 40.30 | | |
| | | GS4 | 0.302 | 0.006 | 46.69 | | |
| | | GS5 | 0.232 | 0.006 | 35.83 | | |
| | | GS6 | 0.293 | 0.006 | 45.19 | | |
| | | GS7 | 0.235 | 0.006 | 36.39 | | |

TABLE 2 Best GLMMs using the AIC as a selection criterion and showing the influence of ontogenetic stage on acclimation capacity in upper and lower thermal tolerance (i.e., acclimation response ratio of CT_{min} and CT_{max}, ARR) during the development of the common frog (*Rana temporaria*)

Note: GLMMs used the "ontogenetic stage" as the categorical fixed factor; "aquarium" as the random factor. N is the total number of analyzed data points; n to the total number of aquaria. Significance was set at $p < 0.05$.

Abbreviations: AIC, Akaike Information Criterion; ARR, acclimation response ratio; CT_{min}, critical thermal minimum; CT_{max}, critical thermal maximum; GLMMs, generalized linear mixed models.

Consequently, larvae and froglets that had developed at 14°C were the heaviest throughout the experiment, whereas animals that developed at the warmest temperature (28°C) were the lightest indicating a reduced growth (Figure A1).

3.1 | Effects of acclimation temperature, mass, and ontogenetic stage on thermal tolerance

3.1.1 | Temperature

We found a positive effect of acclimation temperature on thermal tolerance traits (Table 1). At all ontogenetic stages, thermal tolerance increased with increasing acclimation temperature (Figures 1 and 2; Table 1). Animals acclimated to 14°C and 28°C revealed the lowest and highest upper thermal tolerance, respectively (Figures 1 and 2). Differences in thermal tolerance between animals from different acclimation temperatures were largest at the onset of metamorphic climax (i.e., ontogenetic stage 5) and lowest at ontogenetic stage 1 (Figure 1). At each acclimation temperature, there was a decrease in CT_{max} during metamorphosis, whereas CT_{min} increased (Figure 1). The effect of acclimation temperature was more pronounced on CT_{max} than on CT_{min}, resulting in an increase of TTB with acclimation temperature (Table 1). Across all ontogenetic stages, CT_{max} and TTB increased until an acclimation temperature of 25°C, but then started to decrease so that both variables were lower at 28°C than at 25°C (Figure 2).

3.1.2 | Body mass

There was a negative effect of mass on CT_{min} (Figure 2 and Table 1). In contrast, CT_{max} and TTB increased slightly with increasing body mass (Figure 2 and Table 1). Larger animals have thus a higher cold and heat tolerance than smaller animals.

3.1.3 | Age

CT_{min} increased with increasing age (Figure 2 and Table 1). In contrast, age affected CT_{max} and TTB negatively (Figure 2 and Table 1). At the same ontogenetic stage, older animals have thus a lower cold and heat tolerance than younger animals. Since age at an ontogenetic stage decreased at higher acclimation temperatures (Figure 2), animals reared at higher temperatures are thus younger at a respective ontogenetic stage and display a higher tolerance to thermal extremes. The effect of age was more pronounced on CT_{max} than on CT_{min} (Table 1).

3.1.4 | Ontogenetic stage

The ontogenetic stage had a negative effect on CT_{min} (Figure 2 and Table 1). In contrast, CT_{max} and TTB increased with the ontogenetic stage (Figure 2 and Table 1). The effect of the ontogenetic stage was more pronounced on upper thermal tolerance and TTB than on CT_{min} (Table 1). We also found an interactive effect of ontogenetic stage

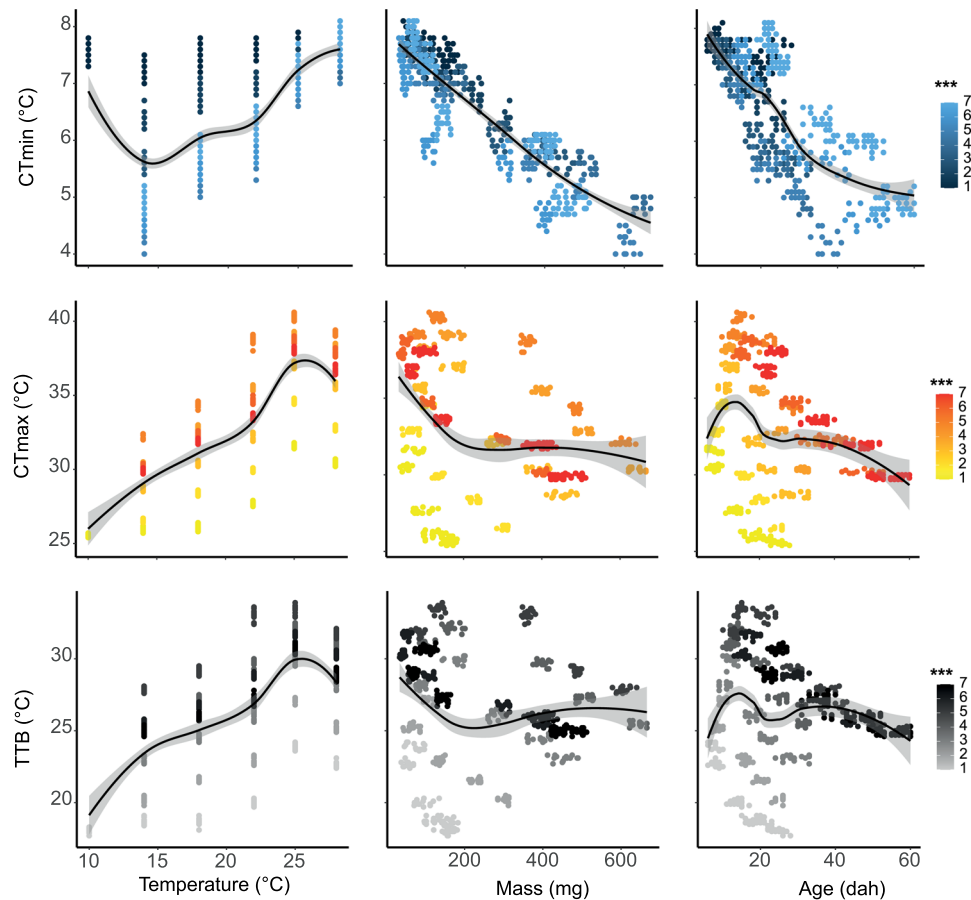


FIGURE 2 Relationships between thermal tolerance traits (i.e., CT_{min} , CT_{max} , and TTB) and their constant acclimation temperature ($^{\circ}C$), body mass (mg), and age (days after hatching) left to right, respectively, during the development of *Rana temporaria* at seven consecutive ontogenetic stages (see text for further details). Points show raw data used to fit models. Ontogenetic stages are indicated by shades from dark blue (stage 1) to light blue (stage 7) for CT_{min} , from yellow to red for CT_{max} , and from light gray to dark gray for TTB. Smooth curves are based on means with $\pm SE$ across ontogenetic stages. All models are significant at a p value of 0.05. CT_{min} , critical thermal minimum; CT_{max} , critical thermal maximum; TTB, thermal tolerance breadth.

and temperature on thermal tolerance traits, indicating that the effect of temperature on thermal tolerance depends on the ontogenetic stage (Table 1).

3.2 | Acclimation capacity depends on the ontogenetic stage and differs between CT_{min} and CT_{max} in *R. temporaria*

We found that the ontogenetic stage explained the acclimation capacity in CT_{min} and CT_{max} (Table 2). Body mass and acclimation duration were not included in the best models.

ARR of CT_{min} was highest in animals at the onset of metamorphic climax (i.e., ontogenetic stage 5; Table A1 and Figures 3 and 4). At the onset of metamorphosis, CT_{min} increased by $0.224^{\circ}C$ for every $1^{\circ}C$ increase in acclimation temperature. The acclimation capacity of lower thermal tolerance was lower in animals after completion of metamorphosis (i.e., ontogenetic stage 6) than at the onset of metamorphosis (Table A1). In juvenile froglets (i.e., ontogenetic stage 7), CT_{min} increased

by $0.205^{\circ}C$ for every $1^{\circ}C$ increase in acclimation temperature. ARR of CT_{min} was between 0.224 (maximum) and 0.023 (minimum).

In contrast, ARR and thus, acclimation capacity of CT_{max} was highest in animals before the onset of metamorphic climax (i.e., ontogenetic stage 4; Table A1 and Figures 3 and 4) and lowest in young larvae (i.e., ontogenetic stages 1 and 2; Table A1 and Figures 3 and 4). The acclimation capacity of upper thermal tolerance was higher in animals after completing metamorphosis (i.e., ontogenetic stage 6) than at the onset of metamorphosis (Table A1). Before the onset of metamorphosis, CT_{max} increased by $0.635^{\circ}C$ for every $1^{\circ}C$ increase in acclimation temperature. In juvenile froglets (i.e., ontogenetic stage 7), ARR of CT_{max} increased by $0.569^{\circ}C$ for every $1^{\circ}C$ increase in acclimation temperature, respectively. The ARR of CT_{max} was between 0.635 (maximum) and 0.333 (minimum).

Mean ARR and thus acclimation capacity of CT_{min} across ontogenetic stages were 0.152, whereas mean ARR of CT_{max} was 0.551. Consequently, we found CT_{max} to be more sensitive (i.e., plastic) to temperature variation than CT_{min} (Figure 3). At the beginning of the experiments, the acclimation capacity of both thermal tolerance traits

was lowest (Figure 4 and Table A1). The acclimation capacity of both thermal traits was well below 100% at all ontogenetic stages (Figure 4).

4 | DISCUSSION

The capacity for acclimation of thermal tolerance is expected to be a primary trait dictating the vulnerability of amphibians and other ectotherms to warming and the increased frequency of extreme events

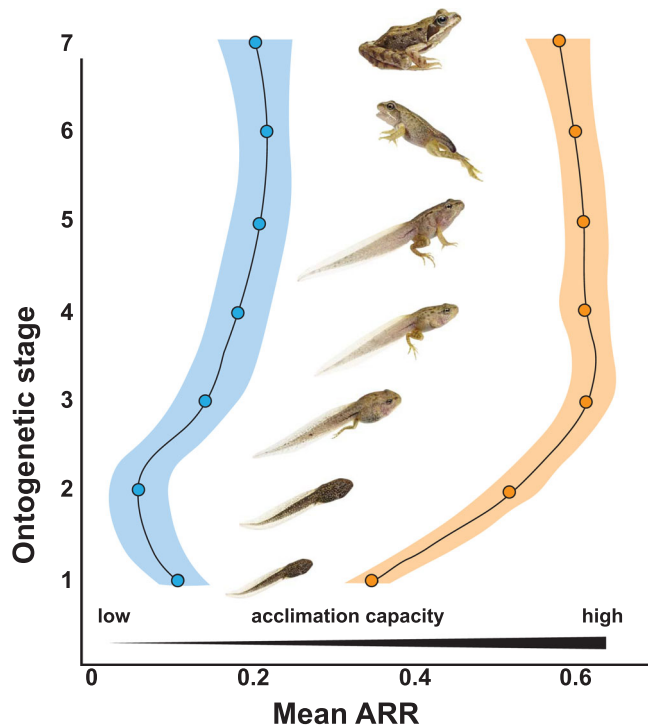


FIGURE 3 Mean ARR of CT_{min} (blue) and CT_{max} (orange) in *R. temporaria* indicating acclimation capacity at seven consecutive ontogenetic stages (see text for further details). Dots for mean values of ARR. Smooth curves show a 95% confidence interval of mean ARR. ARR, acclimation response ratio; CT_{min} , critical thermal minimum; CT_{max} , critical thermal maximum

expected due to climate change (Gunderson & Stillman, 2015; Pachauri et al., 2014; Seebacher et al., 2015; Gutiérrez-Pesquera et al., 2016). Here, we examined the thermal tolerance and acclimation capacity of *R. temporaria* at different life stages during larval and early juvenile ontogeny and assessed the potential of acclimation in thermal tolerance as a coping mechanism to changing environmental temperatures. Our key findings suggest that thermal tolerance and acclimation capacity of *R. temporaria* changes throughout ontogeny. We identified young larvae as the most temperature-sensitive stages from the seven life stages tested here (i.e., not encompassing older juveniles or adults) in the life cycle of *R. temporaria* since their tolerance to extreme temperatures as well as their acclimation capacity is low. We thus emphasize that early life stages might define the climate change vulnerability of amphibian populations. However, our results also highlight the need for more studies testing the acclimation capacity of amphibians across life stages that would allow for more robust projections to be made based on life stage-specific sensitivity of amphibians to climate change and the potential for adaptive capacity via acclimation.

4.1 | Stage-specific thermal tolerance and acclimation capacity in *R. temporaria*

Here, we used *R. temporaria* as a model species representing the life-history strategy and habitat characteristics of a broad range of temperate, lentic amphibians. The larvae of *R. temporaria* commonly inhabit shallow ponds where thermal conditions are quite homogeneous in contrast to deeper ponds and (juvenile) terrestrial habitats (Feder & Hoffmann, 1999; Livingstone et al., 1999). The larvae are relatively small and, thus, their body temperature is equal to the surrounding water temperature (Enriquez-Urzelai, Kearney, et al., 2019; Lutterschmidt & Hutchison, 1997). Consequently, lentic larvae are highly susceptible to extreme pond temperatures (i.e., minimum in spring and maximum in early summer). Accordingly, a high cold tolerance (i.e., a low CT_{min}) directly after hatching might be advantageous in all temperate lentic amphibian species and other limnic ectotherms reproducing in early spring to cope with potential cold

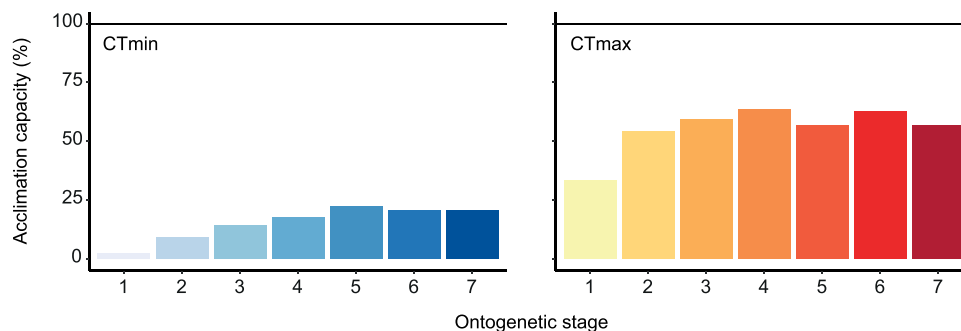


FIGURE 4 Acclimation capacity of CT_{min} (blue shades) and CT_{max} (red shades) in percent. One hundred percent acclimation in thermal tolerance traits indicates an increase of 1°C in CT_{min} and CT_{max} to a temperature increase of 1°C. CT_{min} , critical thermal minimum; CT_{max} , critical thermal maximum

snaps Before the onset of metamorphosis in late spring or early summer, however, lentic larvae are likely to experience heat waves in their natural habitats or increasing mean temperatures due to decreasing water levels. Therefore, heat tolerance and acclimation capacity in CT_{max} before the onset of metamorphosis would also appear advantageous.

In our analysis, cold tolerance as well as heat tolerance and acclimation capacity to different temperatures was lowest in early larvae. Cold spells in early spring might, therefore, pose a serious threat to larvae directly after hatching if early larvae are exposed to temperatures below their CT_{min} . False springs can consequently reduce tadpole survival if a selection of warmer microhabitats is not possible. Young larvae can, therefore, be considered as the most-temperature-sensitive life stage during early larval through early juvenile ontogeny of *R. temporaria*. In contrast, heat tolerance and acclimation capacity were highest in late larvae before the onset of metamorphosis. Since the major risks affecting tadpole's survival to metamorphosis are high temperatures and high desiccation in ephemeral ponds, the heat tolerance and acclimation capacity to warmer temperatures might be an important mechanism to cope with heat events in early summer. Our finding is in line with the pattern found by Gunderson and Stillman (2015), who demonstrated a higher thermal plasticity of organisms (including amphibians) in aquatic versus terrestrial habitats. As aquatic habitats tend to have less spatial variability in operative thermal conditions than terrestrial habitats (Gunderson & Stillman, 2015), behavioral thermoregulation is constrained, and amphibian larvae are more likely to use thermal plasticity to buffer themselves against changing thermal conditions. Furthermore, larvae just before the onset of metamorphosis are known to select higher temperatures (Drakulic et al., 2017) to increase their developmental rate to avoid desiccation risk in shallow ponds. We also found that both heat tolerance and acclimation capacity to warmer temperatures were reduced during metamorphosis and in newly metamorphosed froglets, very likely due to energetic costs of metamorphosis (Kirschman et al., 2017; Orlofske & Hopkins, 2009; Ruthsatz, Dausmann, et al., 2019), as was demonstrated by several studies examining thermal tolerance (e.g., Cupp 1980; Enriquez-Urzelai, Sacco, et al., 2019; Navas & Otani 2007). Whereas terrestrial froglets may select favorable microhabitats to escape from extreme temperatures, the ability for behavioral thermoregulation is constrained in metamorphs and larvae (Enriquez-Urzelai, Sacco et al., 2019; Enriquez-Urzelai, Kearney, et al., 2019; Kingsolver et al., 2011; Sinclair et al., 2016). As a consequence, extreme temperatures can reduce metamorphs' survival and lead to offspring losses if in situ temperatures exceed CT_{max} .

Since *R. temporaria* had the lowest tolerance to extreme temperatures as well as lowest acclimation capacity in thermal limits at the early larval stage, extreme temperatures in the larval habitat may, thus, impose limits to survival and the ability to cope with global warming in temperate lentic amphibians and other ectotherms. A recent meta-analysis on marine and freshwater fish found a similar pattern for thermal tolerance and further concluded that these thermal bottlenecks during ontogeny define the climate vulnerability

of different fish species (Dahlke et al., 2020). Furthermore, Katzenberger et al. (2021) found that CT_{max} differs across species in temperate wetland communities (i.e., insect predators, amphibian larvae, zooplankton, and amphipods) and suggested that future climate change may not only affect the amphibian larvae but also the whole community structure.

4.2 | Shrinking body sizes in response to climate change might impair tolerance to extreme temperatures in amphibians

Recent studies have suggested that shrinking body sizes in ectotherms such as amphibians may constitute a general response to global warming (e.g., Daufresne et al., 2009; Gardner et al., 2011; Ohlberger 2013; Ruthsatz et al., 2018; Ruthsatz, Dausmann, Drees, et al., 2020; Sheridan & Bickford, 2011; Verberk et al., 2021). In our study, body size at all ontogenetic stages was also lowest at warmest developmental temperatures and vice versa. Body size is of fundamental importance for the ecophysiology of amphibians through its effect on metabolic rate (Kingsolver & Huey, 2008), water loss (Tracy & Christian, 2005), locomotor performance (Altwegg & Reyer, 2003; Ruthsatz, Dausmann, et al., 2019; Yagi & Green, 2018), and thermoregulation (Enriquez-Urzelai, Sacco, et al., 2019; Rohr et al., 2018) at all life stages. Shrinking body sizes in response to global warming might, therefore, result in implications for thermal tolerance and acclimation capacity. Although this trend has become known as a "universal response" (Daufresne et al., 2009; Gardner et al., 2011), findings on possible effects of shrinking body sizes on thermal physiology are still controversial (e.g., Enriquez-Urzelai, Sacco, et al., 2019; Leiva et al., 2019; Ospina & Mora, 2004; Peralta-Maraver & Rezende, 2021; Ribeiro et al., 2012; Rohr et al., 2018; Rubalcaba & Olalla-Tárrage, 2020).

In our analysis, smaller individuals of *R. temporaria* had a lower tolerance to thermal extremes than larger individuals, but the effect of size was more pronounced on heat than on cold tolerance. A recent study on ontogenetic thermal tolerance in *R. temporaria* found an effect of size on heat tolerance in juvenile froglets, but not in larvae or metamorphs (Enriquez-Urzelai, Sacco, et al., 2019). Besides its influence on thermal tolerance, body size has been suggested to be an important driver of thermal acclimation (Kingsolver and Huey, 2008; Pörtner et al., 2017). Recent evidence suggests that acclimation capacity is positively correlated with body size across amphibian species (Rohr et al., 2018). In the present study, however, we found no effect of size on acclimation capacity across ontogeny in *R. temporaria*. Nevertheless, body mass might affect acclimation capacity in aquatic life stages (i.e., larvae) more than in terrestrial life stages (i.e., juveniles and adults) due to oxygen limitation (Rubalcaba et al., 2020; Verberk et al., 2021), but this was beyond the scope of our study. Based on the relationship between body mass and thermal tolerance reported here for *R. temporaria*, we conclude that shrinking body sizes as a response to global warming may comprise a crucial morphological constraint on adaptive responses that ultimately

reduce survival at thermal extremes in amphibians. In particular, small newly metamorphosed froglets might suffer most from a size effect on heat tolerance and acclimation capacity since they are predominantly diurnal (Vences et al., 2000), and are thus more likely to encounter extreme temperatures than are the aquatic larvae or nocturnal adults (Enriquez-Urzelai et al., 2020; Navas & Otani 2007).

4.3 | Thermal acclimation might be an important component among *R. temporaria*'s repertoire of buffering mechanisms against global warming

In our study species (*R. temporaria*), the capacity for phenotypic plasticity to variation in environmental factors such as temperature is known to be higher than in other amphibian species (Laugen et al., 2003; Laurila & Kujasalo, 1999; Laurila et al., 2002; Lindgren & Laurila, 2005; Merilä et al., 2000, 2004; Ruthsatz, Dausmann, Drees, et al., 2020). As thermal acclimation is a form of phenotypic plasticity, we expected our study species to reveal a relatively high acclimation capacity in thermal limits. Across all ontogenetic stages, the acclimation capacity of CT_{max} was above 55%, but *R. temporaria* revealed far less capacity to adapt to critically cold temperatures. Our results are contradictory to those of Gunderson and Stillman (2015), who reported a generally low acclimation capacity in amphibians across life stages, but a higher acclimation capacity for CT_{min} (24%) than in CT_{max} (15%). In addition, results from a recent meta-analysis including amphibians indicate an average acclimation capacity of 15% in upper thermal tolerance (Morley et al., 2019). Generally, wide-ranging species exhibit a greater capacity for thermal acclimation of upper thermal tolerance than narrowing-ranging species, suggesting that selection for acclimation ability may have been a key factor enabling geographic expansion into areas with greater thermal variability (Markle & Kozak, 2018). As *R. temporaria* is the most widespread anuran in Europe with a distribution covering northern Spain to northernmost Norway (Gasc et al., 1997; Günther, 1996; Sørensen et al., 2009), the high acclimation capacity of the temperate population studied here might reflect an adaptation to increased thermal variability to enable survival in heterogeneous environments, especially during the larval stage. Markle and Kozak (2018) confirmed this pattern for 16 species of North American plethodontid salamanders. Given that wide-ranging species such as *R. temporaria* might have both a high acclimation capacity and higher thermal tolerance to warm temperatures, they are likely to be less susceptible to environmental warming associated with anthropogenic climate change. However, even if the acclimation capacity of *R. temporaria* was high compared to the amphibian average, it is well below 100%, and thus too low to buffer this species from the impact of climate change (Gunderson & Stillman, 2015; Morley et al., 2019). Furthermore, populations of *R. temporaria* adapted to other macroclimates might reveal a lower or higher acclimation capacity as this is the case for developmental plasticity (Laugen et al., 2003;

Laurila & Kujasalo, 1999; Laurila et al., 2002; Lindgren & Laurila, 2005). Enriquez-Urzelai et al. (2020) suggest that amphibians might most likely escape from thermal extremes by behavioral thermoregulation. This is particularly true for the terrestrial stage, but amphibian larvae are very limited in their ability for behavioral thermoregulation, especially in shallow ponds where the bottom water temperatures are similar or the same as water surface temperatures. Rather, larvae *R. temporaria* might cope with direct and indirect effects of global warming by exhibiting phenotypic plasticity in both metamorphic and physiological traits and, in this respect, thermal acclimation might be an important component among this species' repertoire of buffering mechanisms.

5 | CONCLUSION

How organisms cope with thermal variation and extreme temperature exposure is a central research topic in conservation physiology, evolutionary biology, and ecology. Acclimation capacity might serve as an important mechanism allowing individuals to cope with global warming, particularly in animals with restricted dispersals such as amphibians (Sinsch, 2014) and freshwater fish (Comte and Olden, 2017) and is thus expected to be a primary factor that dictates the vulnerability of taxa to climate change (Huey et al., 2012; Somero, 2010; Stillman, 2003). The present study is, to our knowledge, the first to analyze the thermal physiology of different life stages in amphibians to identify the most temperature-sensitive stage and consequently highest vulnerability to climate-driven changes in environmental temperature. We demonstrated large ontogenetic changes in thermal tolerance and acclimation capacity in a temperate population of *R. temporaria*. This study identifies young larvae as the most-temperature-sensitive life stages in the life cycle of *R. temporaria* and highlights that early life stages might define the climate change vulnerability of amphibian populations. Given that this widely distributed species possesses a high capacity for developmental (Laurila & Kujasalo, 1999; Lind & Johansson, 2007; Merilä et al., 2004), morphological (Ruthsatz, Giertz, et al., 2019), and physiological plasticity (present study; Burraco et al., 2017; Ruthsatz, Dausmann, Reinhardt, et al., 2020), compared to other amphibian species (Laurila & Kujasalo, 1999; Ruthsatz et al., 2018; Vences et al., 2017), this bottleneck in the life cycle might be more pronounced in the vast majority of amphibian populations and species. Much of what is currently known about acclimation capacity in amphibians and other ectotherms has been gained from studies conducted on adults or from meta-analyses that pool data across life stages (rev. in Bodensteiner et al., 2021; Gunderson & Stillman, 2015; Morley et al., 2019). Ignoring stage-specific differences in thermal physiology may drastically underestimate the climate vulnerability of species with consequences for successful conservation actions. Examining life-stage specific thermal tolerance and acclimation capacity across a broader range of taxa from different latitudes and elevations is needed to understand the risks imposed by global warming on local populations and to create robust strategies for conserving biodiversity.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ETHICS STATEMENT

The authors have no ethical conflicts to disclose. The experiments were conducted under permission from the *Amt für Verbraucherschutz, Lebensmittelsicherheit und Veterinärwesen* in Hamburg, Germany (Billstraße 80, 20539 Hamburg; Gz. V1305/591-00.33, Nr. 03/16). The permission for extraction of spawn of the Common frog (*Rana temporaria*) was granted by the *Amt für Naturschutz, Grünplanung und Energie* (Neuenfelder Straße 19, 21109 Hamburg; Gz. NGE3102/897.20-90).

AUTHOR CONTRIBUTIONS

Katharina Ruthsatz, Kathrin H. Dausmann, Myron A. Peck, and Julian Glos conceived and designed the study. Katharina Ruthsatz conducted the experiments. Katharina Ruthsatz performed the statistical analysis. Katharina Ruthsatz led the writing of the manuscript. All authors participated in manuscript editing and final approval.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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