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Altered thyroid hormone levels affect the capacity for temperature-induced developmental plasticity in larvae of *Rana temporaria* and *Xenopus laevis*

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ABSTRACT

Anuran larvae show phenotypic plasticity in age and size at metamorphosis as a response to temperature variation. The capacity for temperature-induced developmental plasticity is determined by the thermal adaptation of a population. Multiple factors such as physiological responses to changing environmental conditions, however, might influence this capacity as well. In anuran larvae, thyroid hormone (TH) levels control growth and developmental rate and changes in TH status are a well-known stress response to sub-optimal environmental conditions. We investigated how chemically altered TH levels affect the capacity to exhibit temperature-induced developmental plasticity in larvae of the African clawed frog (*Xenopus laevis*) and the common frog (*Rana temporaria*). In both species, TH level influenced growth and developmental rate and modified the capacity for temperature-induced developmental plasticity. High TH levels reduced thermal sensitivity of metamorphic traits up to 57% (*R. temporaria*) and 36% (*X. laevis*). Rates of growth and development were more plastic in response to temperature in *X. laevis* (+30%) than in *R. temporaria* (+6%). Plasticity in rates of growth and development is beneficial to larvae in heterogeneous habitats as it allows a more rapid transition into the juvenile stage where rates of mortality are lower. Therefore, environmental stressors that increase endogenous TH levels and reduce temperature-dependent plasticity may increase risks and the vulnerability of anuran larvae. As TH status also influences metabolism, future studies should investigate whether reductions in physiological plasticity also increases the vulnerability of tadpoles to global change.

1. Introduction

Anthropogenic alterations in abiotic and biotic environmental factors cause species and population declines worldwide due to habitat loss, disease, pollution, and increasing mean and extreme temperatures (Stuart et al., 2004; Gunderson and Stillman, 2015; Strong et al., 2017). Aquatic organisms, especially those with complex life cycles such as anuran larvae, often have a limited capacity to search for new, more favorable microhabitats (Yu et al., 2015; Searcy et al., 2015; Gutiérrez Pesquera et al., 2016). Larvae often must cope with the stress of being exposed to unfavorable environmental conditions (Schulte, 2014; Dantzer et al., 2014; Burraco and Gomez-Mestre, 2016). Plasticity in growth and development in response to changes in abiotic factors can be an effective mechanism to avoid negative impacts on organism fitness (Schlichting and Pigliucci, 1998; Boorse and Denver, 2004). Especially in anurans, individuals risk a significant reduction in fitness if they fail to reach a specific stage (e.g. the onset of metamorphosis) before a set time, especially in ephemeral waters (Rudolf and Rödel, 2007).

A multitude of environmental stressors affect growth and development of larval anurans by influencing physiological processes and hormone systems (Calich and Wassersug, 2012; Beachy et al., 1999). Environmental stress results in the enhanced production of stress

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hormones (Dantzer et al., 2014; Denver et al., 1998). Amphibian metamorphosis is under endocrine control by thyroid hormones (TH) governing the timing and speed of the morphological reorganization (Shi, 2000; Tata, 2006). Stress hormones can act to increase the production of TH (Laudet, 2011; Glennemeier and Denver, 2002) and thus, can accelerate developmental rate. However, environmental stressors may also decrease TH production in anuran larvae by obstructing TH production pathways resulting in decreased developmental rates (Ortiz-Santaliestra and Sparling, 2007; Bulaeva et al., 2015). Thus, environmental stress can lead to different ages and sizes at the onset of metamorphosis in anuran larvae by disrupting the natural endocrine control of metamorphosis (Wilbur and Collins, 1973; Denver et al., 1998). Whereas many natural changes in environmental conditions, such as increased crowding (Ding et al., 2015), the presence of predators (Relyea, 2007), low food quality and quantity (Courtney Jones et al., 2015; Carabio et al., 2017), long photoperiod (Laurila et al., 2001), high desiccation risk (Gervasi and Foufopoulos, 2008), and high temperature (Vences et al., 2002), are known to stimulate the production of TH, several studies have reported a decrease in production of TH in larvae exposed to municipal effluent, pesticides and herbicides, road salt, fertilizers, heavy metals and active pharmaceutical ingredients during amphibian metamorphosis (Lefcort et al., 1998; Ortiz-Santaliestra and Sparling, 2007; Fong et al., 2016).

From all environmental stressors affecting growth and developmental rate in ectotherms such as anuran larvae, temperature is the most pervasive and arguably the most important (Dalvi et al., 2009; Berg et al., 2017). In ectotherms, body temperature fluctuates with ambient temperatures and the rates of most biochemical reactions and many biological processes exponentially increase with temperature (Harkey and Semlitsch, 1988; Zuo et al., 2012; Theisinger et al., 2017). Larval amphibians are especially likely to encounter variation in temperature because they live in a variety of aquatic habitats and often in shallow ephemeral ponds (Walsh et al., 2008; Yu et al., 2015) and are known to exhibit temperature-induced plasticity in growth and developmental rate as a response to temperature variation (rev. Ruthsatz et al., 2018). However, an increase in temperature influences development stronger than growth (Smith-Gill and Berven, 1979; Zhao et al., 2014), which leads to a reduced size at metamorphosis under higher temperatures (Alvarez and Nicieza, 2002; Tejedo et al., 2010; Ruthsatz et al., 2018). This is consistent with the temperature-size rule (TSR; Atkinson, 1994) which anurans are known to follow (Ashton, 2002; Olalla- Tarraga and Rodriguez, 2007; Ruthsatz et al., 2018). Ectotherms reared at relatively lower temperatures typically mature later at larger sizes than conspecifics reared at higher temperatures (Atkinson, 1994; Laugen et al., 2005; Courtney Jones et al., 2015). Thus, there is an optimal thermal range for the development of amphibians which is buffered by a suboptimal range in which plasticity in age and size at metamorphosis does not occur (Walczyńska et al., 2016; Ruthsatz et al., 2018). This thermal range for growth and development and thus, the capacity for temperature-induced developmental plasticity is related to local adaptations and is thus, population-specific (Janzen, 1967; Seebacher et al., 2015; Ruthsatz et al., 2018).

Considering the current worldwide decline of amphibians (Alroy, 2015; Stuart et al., 2004), it is critical to investigate whether and how anuran larvae adjust their metamorphic traits in response to new thermal challenges and to endocrine disruption caused by natural or anthropogenic stressors (Strong et al., 2017). The role of environmental stress in amphibians and amphibian life-histories has recently received attention (Räsänen et al., 2003; Gabor et al., 2013, 2017; Kaiser et al., 2015) but little is known regarding the interactive effects of simultaneously and sequentially occurring stressors. Furthermore, global change is projected to continue and will generate novel combinations and severities of stressors (Williams and Jackson, 2007; Niinemets et al., 2017).

In this study, we examined how developmental temperature (T_{dev}) and altered TH level as caused by environmental stressors act to influence the survival and plasticity in metamorphic traits (age and size) at the onset of metamorphosis in larvae of two anuran species, the African clawed frog (*Xenopus laevis*) and the common frog (*Rana temporaria*). Investigating this capacity in anuran larvae may allow for more robust projections on the impacts of global change at both the individual and population level.

2. Material and methods

2.1. Study species and experimental design

Xenopus laevis is the best studied amphibian species in terms of the TH system and development (Buchholz, 2017), providing solid physiological background for the patterns investigated in this study. Even though *X. laevis* is often used for laboratory experiments and, thus, cultured under constant thermal conditions, this is a tropical (Sub--Saharan Africa) species adapted to warm temperatures (Tinsley et al.,. 2015; Ihlow et al., 2016; Ruthsatz et al., 2018). *Rana temporaria* is a temperate species widely distributed throughout Europe (Lindgren and Laurila, 2009) and the duration of its larval period is known to be highly dependent on environmental conditions (Laurila et al., 2002). The plasticity of responses in age and size at metamorphosis of *R. temporaria* to variation in environmental factors is also known to be higher than in other anuran species (Laurila and Kujasalo, 1999). Whereas *X. laevis* completes its entire life cycle in aquatic habitats, *R. temporaria* switches to terrestrial habitats as juvenile, representing the typical biphasic amphibian life history strategy. As both species differ in their biology, they provide good model organisms to study how effects of global change on amphibian development might differ between very evolutionarily diverged species.

Five clutches of *R. temporaria* eggs were obtained from Waldpark Marienhöhe in Hamburg, Germany (53° 34′37.4″ N, 9° 46′57.5″ E). Three clutches of *X. laevis* eggs were obtained from the Universitätsklinikum Hamburg Eppendorf (UKE, Hamburg). Larvae were allowed to develop to Gosner stage 25 (free-swimming larvae; Gosner, 1960) at 19 °C for *X. laevis* and 14 °C for *R. temporaria* with a 12:12 light: dark (0900–2100) light regime. Before the start of the experiment, 675 (*X. laevis*) and 840 (*R. temporaria*) were intermixed before allocating them randomly to 45 (*X. laevis*) and 54 (*R. temporaria*) standard 9.5 L aquaria at five (*X. laevis*) and six (*R. temporaria)* different water temperatures (i.e. 9 aquaria/temperature) in a common-garden experiment. Fifteen larvae were kept each in an aquarium filled with 8 L of water (initial tadpole density: 1.87 tadpoles/L). The experiment was conducted in two climate chambers (Weiss Umwelttechnik GmbH, 35447 Reiskirchen, Germany) with a 12 to 12 light:dark (0900–2100) photoperiod and a mean ± SD air temperature of 16 ± 0.4 °C and 22 ± 0.1 °C for *X. laevis* and 10 ± 0.2 °C and 22 ± 0.1 °C for *R. temporaria*. Water temperatures were achieved by indirect heating elements beneath the aquaria (Tetra GmbH, Melle, Germany, adjustable heating element, Tetra HT100, 100 W) and by air temperature. Thus, the five mean (±SD) water temperatures for *X. laevis* were 16 (±0.4), 19 (±0.5), 22 (±0.1), 25 (±0.5) and 28 (±0.3) °C. The six mean (±SD) water temperatures for *R. temporaria* were 10 (±0.2), 14 (±0.5), 18 (±0.1), 22 (±0.1), 25 (±0.2) and 28 (±0.3) °C. At each developmental temperature, three aquaria (i.e. replicates) were exposed to each of the different treatments (L-thyroxine and sodium perchlorate) and the control group (*X. laevis*: 3 replicates x 3 treatments x 5 temperatures = 45 aquaria in total; *R. temporaria*: 3 treatments × 3 replicates/treatment × 6 temperatures = 54 aquaria in total). Hereafter, we refer to the L-thyroxine treatment as 'high TH level' treatment and to the sodium perchlorate treatment as 'low TH level' treatment.

Amphibian larvae were fed high-protein flaked fish food (Sera micron breeding feed for fish and amphibians, Sera, 52518 Heinsberg, Germany) and spirulina algae twice a day *ad libitum*. The amount of food was continuously adjusted during the entire experiment to control for differences in tadpole size and density between the aquaria since Miyata

and Ose (2012) indicated that a restricted feeding condition causes an atrophy of thyroid tissue similarly to TH agonists. The flakes were free of perchlorate according to the manufacturer. The aquaria were checked daily for dead or abnormal tadpoles, which were removed (Tietge et al., 2005). At 10 °C none of the *R. temporaria* larvae survived until the onset of metamorphosis. Therefore, we refer to larvae reared in a temperature range from 14° to 28 °C hereafter (i.e. 675 individuals in 45 aquaria: 5 water temperatures × 3 treatments × 3 replicates/treatment × 15 individuals).

The experiments were conducted over four weeks (*X. laevis*) and eleven weeks (*R. temporaria*), by which time all surviving larvae had reached the onset of metamorphosis (Gosner, 1960). At 10° none of the *R. temporaria* anuran larvae survived until the onset of metamorphosis and, from this point on, only the 14–28 °C treatments will be discussed.

2.2. T4 and sodium perchlorate exposures

We increased internal TH levels (i.e., high TH level) by exposing larvae to 10 µg/L exogenous L-thyroxine (T4, IRMM468 Sigma-Aldrich, Sigma-Aldrich, St. Louis, USA), a concentration which is known to influence amphibian metamorphosis (Lucas and Reynolds, 1967; Mann et al., 2009) and which is related to increases in T4 observed in larvae responding to stress (Denver, 1997, 1998). Larvae absorb exogenous T4 directly through their permeable skin (Shi, 2000; Tata, 2006; Coady et al., 2010). Exposing larvae to exogenous THs is an established method to simulate the proximate effects of environmental stressors on the TH system (Denver et al., 1998; Tata, 2006; Denver, 1998).

To achieve a decrease in TH levels (i.e., low TH level) we used sodium perchlorate (SP), an environmental relevant endocrine disruptor, which is a goitrogen that inhibits TH synthesis via competitive inhibition of the sodium-iodide symporter (Ortiz-Santaliestra and Sparling, 2007). Because iodide is essential for the production of both T4 and T3, perchlorate may act as a disrupter of amphibian metamorphosis (Ortiz-Santaliestra and Sparling, 2007). Perchlorate salts are strong oxidizers and are widely used as components of fireworks, airbags, and currently applied fertilizers (Trumpolt et al., 2005; Carr and Patino, 2011; Schmidt et al., 2012, Fig. 1). Contamination of surface and ground water occurs from military, aerospace, agriculture, and other commercial sources, but perchlorate also occurs naturally in arid places on the surface of the earth (Carr and Patiño, 2011). We used a concentration of 250 µg/L SP (99.99% trace metals basis, Aldrich, Sigma-Aldrich, St. Louis, USA) to achieve a decrease in TH levels. This concentration of SP is within environmental ranges measured in surface and ground waters of many industrial nations (Motzer, 2001; Tietge et al., 2005; Carr and Theodorakis, 2006; Mukhi and Patiño, 2007) and in bodies of water in which amphibians breed (Ortiz-Santaliestra and Sparling, 2007).

T4 and SP treatments were prepared in 0.1 N sodium hydroxide solutions (0.1 N, S2770 SIGMA, Sigma-Aldrich, St. Louis, USA) buffered with 0.1 N muriatic acid solutions as solvents. Solutions were added to the aquaria. To control for any effect of solvents addition, a solution of only 0.1 M sodium hydroxide solution buffered with 0.1 M muriatic acid solution was added to the control aquaria. Every second day, a 100% water change was made and new SP or T4 was added to each aquarium. This schedule was frequent enough to maintain a constant hormone and perchlorate level, in accordance with the standard procedure for chemical and hormonal addition (Miwa and Inui, 1987; Goleman et al., 2002a,b; Iwamuro et al., 2003; Rot-Nikcevic and Wassersug, 2004; Tietge et al., 2005; Ortiz-Santaliestra and Sparling, 2007; Bulaeva et al., 2015).

2.3. Growth and development measurements

Developmental stage was determined by evaluating the status of key morphological features typical of specific developmental stages, as detailed in Gosner (1960). The developmental stage of each tadpole was recorded according to the procedure of Ortiz-Santaliestra and Sparling (2007): Gosner stage group 1–5: 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 limbs (hind limb tubercles and subarticular patches formed, Gosner stages 38–41), and 5. Metamorph (at least one forelimb present, Gosner stage 42 and above) (Gosner, 1960; Ortiz-Santaliestra and Sparling, 2007). Onset of metamorphosis was defined by the emergence of at least one forelimb (Gosner stage 42). The snout vent length (SVL) of larvae was measured with a caliper to the nearest 0.5 mm. Larvae were weighed to the nearest 0.001 g with an electronic balance (digital gold scale, Smart Weigh). Growth rate (mg/d) was calculated from mass at

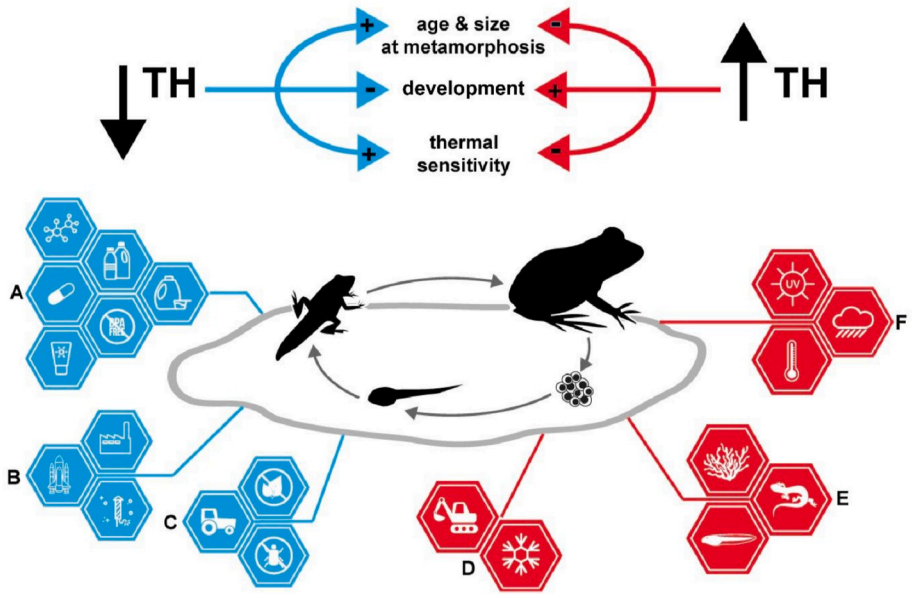

Fig. 1. Environmental stressors in the habitat of anuran larvae that affect developmental rate and, thus, age and size at the onset of metamorphic climax by influencing endogenous thyroid hormone (TH) levels (Kashiwagi et al., 2008; Mann et al., 2009; Carr and Patiño, 2011; Boas et al., 2012). Altered TH levels influence the thermal reaction norm (i.e. sensitivity) of growth and developmental rate during metamorphosis. Blue symbols: stressors acting as endocrine disruptors inhibiting TH production pathways resulting in low endogenous TH levels. Red symbols: stressors increasing TH production by the activation of the neuroendocrine stress axis. **A** Household chemicals and pharmaceuticals: Artificial steroid hormones (testosterone) and hormonal contraceptives (estrogen and gestagen), analgesic agents (e.g., ibuprofen, diclofenac), chemicals from sunscreen, microplastics from packaging and clothes, bisphenol A (BPA) from packaging, phosphates from washing agents. **B** Byproducts of industry (persistent organic products), aerospace (ClO_4^-), and fireworks (ClO_4^-). **C** Chemicals from agriculture: fertilizer (NO_3^-), herbicides, pesticides (Atrazine, Malathion). **D** Habitat fragmentation and road salt. **E** Biotic stressors: Food availability, competition (i.e. crowding), predator pressure. **F** Climatic stressors: UV-radiation, temperature variation, precipitation and desiccation risk.

the onset of metamorphosis minus the mass directly after hatching, divided by the days from hatching to metamorphosis (i.e. 'age'). Developmental rate was calculated from Gosner stage (GS) at the onset of metamorphosis (i.e. 42) minus the Gosner stage at hatching (i.e. 23), divided by age (GS/day).

2.4. Statistical analyses

All statistical tests were carried out using R (R 3.6.3) and plots were constructed using ggplot2 (Wickham, 2009) and Adobe Illustrator CS6.

2.4.1. Effects of altered TH levels and T_{dev} on metamorphic traits

For each species, SVL andTL data were analyzed using separate linear mixed-effect models [lme, Type III model, covariance type: variance components, REML (restricted maximum likelihood) method for parameter estimation, 100 iterations (Bates et al., 2007)]. Probability (p) values were obtained from likelihood-ratio tests, which compared the models with the respective null-model (Crawley, 2007). Residuals of each model were visually checked for normal distribution (QQ-plots). Generalized Linear Mixed Models (GLMM) in R using the *glmer ()* function in the lme4 package (https://cran.r-project.org/package=lme4) in R (Bates et al., 2007), with Poisson distribution were used to analyze developmental rate, growth rate, and mass data in separate models. To address dependencies in the data, the variable 'aquarium' was included as a random factor in both LMMs and GLMMS (Appendix Table 1; Figs. 2 and 3).

For testing the effect of altered TH levels ('treatment': high TH, low TH, and control), developmental temperatures ('T_{dev}'), and the interaction of treatment × T_{dev}, we used 'T_{dev}' as a covariate and 'treatment: T_{dev}' as fixed factors.

Pairwise multiple comparisons were made using Mann-Whitney-U-Test as post hoc test with Bonferroni correction.

2.4.2. Temperature effects on metamorphic traits and plasticity index (PIX)

To determine the thermal reaction norm (i.e. sensitivity) of metamorphic traits (as measured by age, mass, and SVL) to temperature variation, we performed linear regressions of T_{dev} (independent variable) and metamorphic traits (dependent variables) across treatment groups within species for each aquarium independently (Table 2, Fig. 4). The slope of each regression describes the change in a metamorphic trait with a given change in developmental temperature and was used as a plasticity index (PIX) according to Ruthsatz et al. (2018). Higher absolute values of PIX correspond to higher plasticity (e.g. greater sensitivity of growth and/or developmental rate to temperature).

3. Results

3.1. Effects of altered TH levels and temperature on survival, age, and size at the onset of metamorphosis

In *R. temporaria* mean (±SD) survival in the control, low TH, and high TH treatment was 76.4 (11.7), 77.1 (11.4), and 66.3 (13.5) %, respectively, and none of the larvae reared at 10 °C survived until the onset of metamorphosis (Table 1). Thus, survival was lowest at high TH

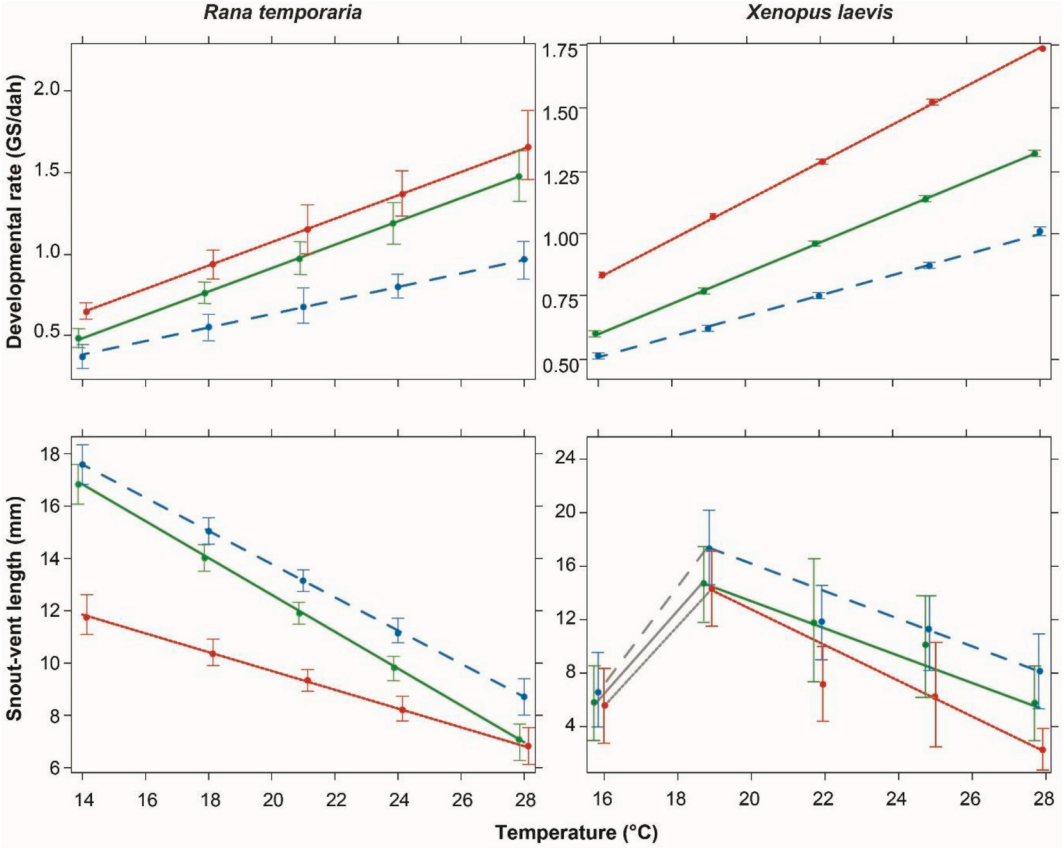

Fig. 2. Effects of altered TH levels and different developmental temperatures on age (days after hatching) and snout-vent length (mm; SVL) at the onset of metamorphosis in *Rana temporaria* and *Xenopus laevis*. Mean (±confidence interval) values are shown. Trend line for interactive effect of developmental temperature and altered TH levels. T4 = high TH levels. SP = low TH levels. Grey lines show the lack of plasticity between 16° and 18 °C. Blue dashed: Low TH levels = SP treatment. Green: Control group. Red dotted: High TH levels = T4 treatment.

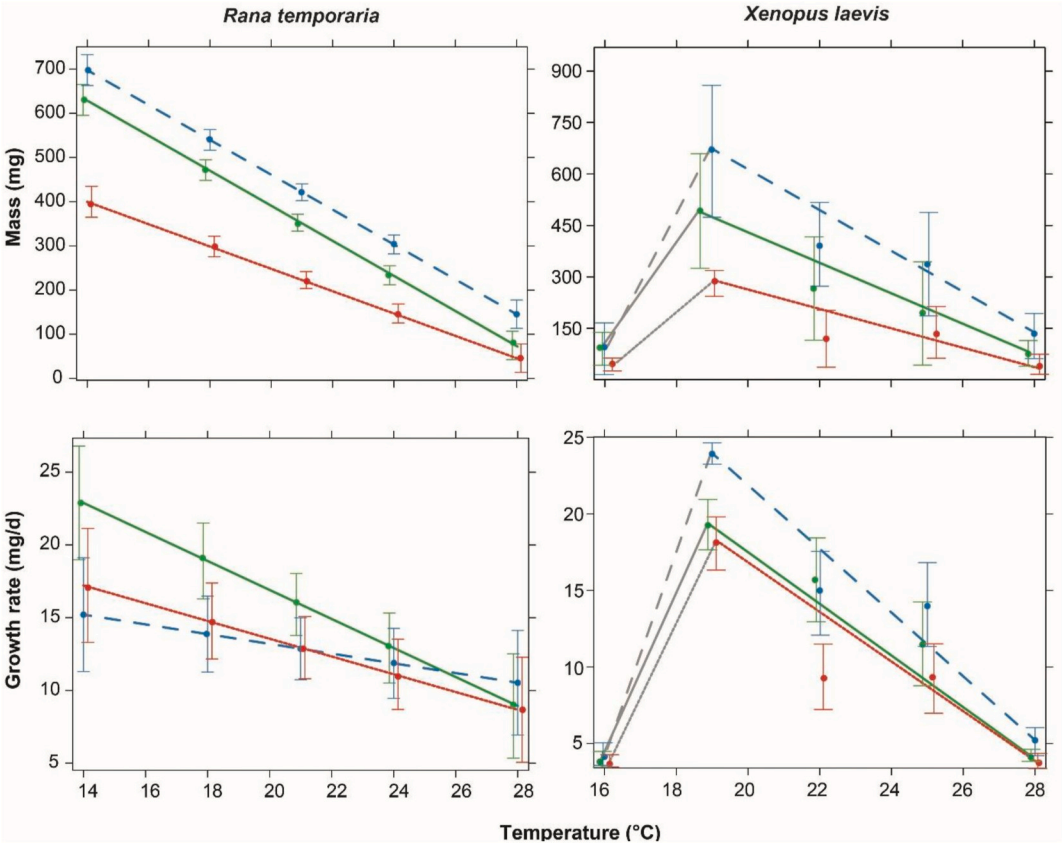

Fig. 3. Effects of altered TH levels and different developmental temperatures on mass (mg) and growth rate (mg/d) at the onset of metamorphosis in *Rana temporaria* and *Xenopus laevis*. Points show mean values and bars for confidence interval. Trend line for interactive effect of developmental temperature and altered TH levels. Grey lines show the lack of plasticity between 16° and 18 °C. Blue dashed: Low TH levels = SP treatment. Green: Control group. Red dotted: High TH levels = T4 treatment.

Table 1
Mean survival (%) and mean age (days after hatching) (±SD) at the onset of metamorphosis in anuran larvae of *R. temporaria* and *X. laevis* exposed to different combinations of developmental temperature (T_{dev}) and altered thyroid hormone (TH) levels. Low TH levels = SP treatment. High TH levels = T4 treatment.

		Rana temporaria						*Xenopus laevis*				
		10 °C	14 °C	18 °C	22 °C	25 °C	28 °C	16 °C	19 °C	22 °C	25 °C	28 °C
Survival (%)	low TH	0	95.5 ± 3.1	91.1 ± 3.1	97.7 ± 3.1	77.7 ± 3.3	93.3 ± 6.6	66.6 ± 0	60.6 ± 6.6	68.8 ± 4.4	57.7 ± 8.8	75.5 ± 4.4
± SD	Control	0	91.1 ± 3.3	95.5 ± 3.3	91.1 ± 3.3	84.4 ± 3.3	91.1 ± 6.6	91.1 ± 8.8	86.6 ± 6.6	91.1 ± 8.8	97.7 ± 2.2	93.3 ± 0
	high TH	0	86.6 ± 0	93.3 ± 0	84.4 ± 3.3	68.8 ± 3.3	62.2 ± 3.3	51.1 ± 2.2	68.8 ± 4.4	48.8 ± 4.4	55.5 ± 4.4	42.2 ± 4.4
Age (days)	low TH	NA	59.2 ± 9.7	48.6 ± 6.7	26.0 ± 3.2	24.9 ± 1.3	18.0 ± 1.3	31.0 ± 0	30.0 ± 0	27.0 ± 0	23.0 ± 0	18.0 ± 0
± SD	Control	NA	35.8 ± 2.7	28.4 ± 1.7	20.9 ± 2.0	15.0 ± 1.2	13.1 ± 1.5	28.0 ± 0	25.0 ± 0	17.0 ± 0	15.0 ± 0	13.0 ± 0
	high TH	NA	30.0 ± 2.0	22.4 ± 1.8	16.0 ± 2.3	12.2 ± 1.7	10.3 ± 1.8	21.0 ± 0	16.0 ± 0	14.0 ± 0	11.0 ± 0	10.0 ± 0

levels. Survival to the onset of metamorphosis in *R. temporaria* decreased over the temperature range from 14 °C to 25 °C and then increased between 25 °C and 28 °C (Table 1). The survival of *R. temporaria* was lowest in the 25°C-control, 25°C-low TH and 22°C-high TH treatments and was highest in the 18°C-control, 14°C-low TH, and 18°C-high TH treatments (Table 1). In *X. laevis*, mean (±SD) survival from the start of the experiment (Gosner stage 25) to the onset of metamorphosis (Gosner stage 42) in the control, low TH and at high TH treatments was 92.0 (10.0), 65.7 (13.3) and 53.3 (13.3) %, respectively. Survival was lowest in the 19°C-control, 25°C-SP and 28°C-T4 treatments and highest in the 25°C-control, 28°C-low TH, and 19°C-high TH treatments (Table 1).

Age at the onset of metamorphosis was different among all groups and, thus, influenced by both TH status and temperature treatment during development in both species Table 1). In both species, developmental rate was slower at low TH and faster at high TH levels (Table A1; Fig. 2). Differences among TH treatments were more pronounced at extreme temperatures. The interactive effect of TH status and temperature was strongest at the coldest temperature and at low TH level and at the warmest temperature and high TH level (Fig. 2). In *R. temporaria*, larvae from the 14°C-low TH group developed slowest (59.2 ± 9.7 days), whereas larvae from the 28°C-high TH group developed most rapidly (10.3 ± 1.8 days) (Table 1, A1). In *X. laevis,* development was slowest (31 days) in the 16°C-low TH group and was fastest (10 days) in the 28°C-high TH group (Table 1, A1). Developmental rate was significantly higher at all temperatures in both species. In contrast, low TH levels reduced developmental rate significantly at all temperatures (Fig. A1, A2). Developmental rate increased with increasing temperature (Fig. 2; Table A1), whereas growth rate decreased respectively (Fig. 3; Table A1).

Size at the onset of metamorphosis was expressed as mass, SVL, and

Table 2
Effects of altered TH levels at five different developmental temperatures during development on snout-vent length (SVL), total length (TL), mass, and growth rate (mg per day after hatching), developmental rate (Gosner stages per day after hatching; DR), and mass in tadpoles of the African clawed frog (*Xenopus laevis*) at the onset of metamorphosis (Gosner stage 42) (Gosner, 1960). SP = low TH levels. T4 = high TH levels. Pairwise multiple comparisons were made using Mann-Whitney-U-Test as post hoc test with Bonferroni correction. Significance was set at $P < 0.05$. N is the total number of analyzed individual animals.

Xenopus laevis		Mann-Whitney-Test					
Temperature	Dependent variable	Control - SP			Control - T4		
		z	P	N	z	P	N
16°	SVL (mm)	−1.126	0.260	69	−1.93	0.053	63
	TL (mm)	−5.59	< **0.001**		−6.64	< **0.001**	
	GR (mg/d)	−0.40	0.686		−6.61	< **0.001**	
	DR (GS/d)	−8.26	< **0.001**		−7.87	< **0.001**	
	Mass (mg)	−6.58	< **0.001**		−6.61	< **0.001**	
19°	SVL (mm)	−3.44	**0.001**	66	−5.73	< **0.001**	70
	TL (mm)	−6.08	< **0.001**		−7.12	< **0.001**	
	GR (mg/d)	−6.87	< **0.001**		−6.39	< **0.001**	
	DR (GS/d)	−8.06	< **0.001**		−8.31	< **0.001**	
	Mass (mg)	−6.87	< **0.001**		−7.16	< **0.001**	
22°	SVL (mm)	−2.54	**0.011**	72	−6.71	< **0.001**	63
	TL (mm)	−7.27	< **0.001**		−6.55	< **0.001**	
	GR (mg/d)	−7.23	< **0.001**		−6.51	< **0.001**	
	DR (GS/d)	−8.42	< **0.001**		−7.87	< **0.001**	
	Mass (mg)	−7.23	< **0.001**		−6.51	< **0.001**	
25°	SVL (mm)	−6.21	< **0.001**	70	−4.51	< **0.001**	69
	TL (mm)	−6.55	< **0.001**		−6.94	< **0.001**	
	GR (mg/d)	−6.97	< **0.001**		−6.89	< **0.001**	
	DR (GS/d)	−8.31	< **0.001**		−8.27	< **0.001**	
	Mass (mg)	−6.79	< **0.001**		−6.89	< **0.001**	
28°	SVL (mm)	−7.23	< **0.001**	78	−5.92	< **0.001**	62
	TL (mm)	−2.81	< **0.001**		−6.30	< **0.001**	
	GR (mg/d)	−7.61	< **0.001**		−6.34	< **0.001**	
	DR (GS/d)	−8.77	< **0.001**		−7.81	< **0.001**	
	Mass (mg)	−7.61	< **0.001**		−6.34	< **0.001**	

TL. SVL and TL were significantly influenced by the TH level and T_{dev} (Table A1), but not by the interactive effect of both in *X. laevis*. In *R. temporaria* mass, SVL, and TL were significantly affected by high TH levels, T_{dev}, and the interactive effect of both (Table A1). SVL was significantly higher in low TH animals at all temperatures except 16 °C in *X. laevis* (Table 2). In *R. temporaria*, SVL was significantly higher in low TH animals at 18 °C, 22 °C, 25 °C, and 28 °C (Table 3; Fig. A1, A2). High TH levels did not significantly decrease SVL in *X. laevis* at 16 °C but at all other developmental temperatures (Table 2). SVL was significantly lower at 14 °C, 22 °C, and 25 °C in *R. temporaria* (Table 3; Fig. A1, A2). TL was significantly higher in low TH animals at all temperatures in both species but not at 14 °C in *R. temporaria* (Tables 2 and 3). High TH levels decreased TL significantly at all temperatures in both species (Fig. A1, A2). Mass was significantly increased in low TH animals at all temperatures in both species, whereas high TH levels decreased mass significantly at all temperatures (Tables 2 and 3; Fig. A1, A2). Growth rate was significantly affected by T_{dev} in *R. temporaria* and in *X. laevis*. Growth was delayed in the high TH individuals (Fig. 3; Fig. A1, A2), whereas low TH individuals grew faster at 22 °C, 25 °C, and 28 °C in *R. temporaria* (Table 3; Fig. 3; A2) and at 19 °C, 25 °C, and 28 °C in *X. laevis* (Table 2; Fig. 3; A1).

3.2. Capacity for temperature induced developmental plasticity

The data collected on *X. laevis* larvae reared at 16 °C were excluded from the analysis because this temperature is presumably outside the species-specific thermal range for development as larvae showed a lack of plasticity (Figs. 2 and 3). Thus, results presented for *X. laevis* refer to the temperature range from 19° to 28 °C (Table 4).

Lower TH levels increased the thermal reaction norm of metamorphic traits whereas higher TH levels decreased the thermal reaction norm, respectively (Fig. 4). Within all treatments, age, mass and SVL at the onset of metamorphosis were significantly, linearly correlated to temperature (Table 4). Plasticity indices (PIX) of age and mass were highest in animals with low TH levels, followed by animals from control and animals with high TH level revealing the lowest PIX. For SVL, PIX was highest in control animals, followed by high TH level in *X. laevis* and by low TH level in *R. temporaria*. PIX for body size (SVL and mass) at the onset of metamorphosis was higher in larvae of tropical *X. laevis*, whereas PIX for age was higher in larvae of temperate *R. temporaria* within the selected temperature range (Table 4). Since high PIX values (i.e. value of) indicate a high thermal sensitivity of growth and developmental rate to increasing temperature, the thermal sensitivity of growth and developmental rate was highest at low TH level (Table 4). Effect of TH level was temperature-dependent as high TH levels have strong effects on thermal reaction norms of metamorphic traits at lower temperatures and low effects at higher temperatures in both species (Fig. 4). Therefore, at higher temperatures effects of altered TH levels are less intense (Fig. 4). Consequently, the thermal sensitivity of metamorphic traits is reduced at high TH levels and increased at low TH levels (Figs. 1 and 4).

4. Discussion

Changing environmental conditions elicit plastic responses in most organisms, especially in those living in temporally and spatially heterogeneous environments. In amphibians, for example, the timing of and size at metamorphosis (i.e. plastic growth and developmental rate) may be adjusted (Wilbur and Collins, 1973; Pechenik et al., 1998; Rudolf and Rödel, 2007; Laudet, 2011; Ruthsatz et al., 2018), allowing for increased fitness in later life stages (Schlichting and Pigliucci, 1998; Boorse and Denver, 2004) as a short larval period and a large size at metamorphosis

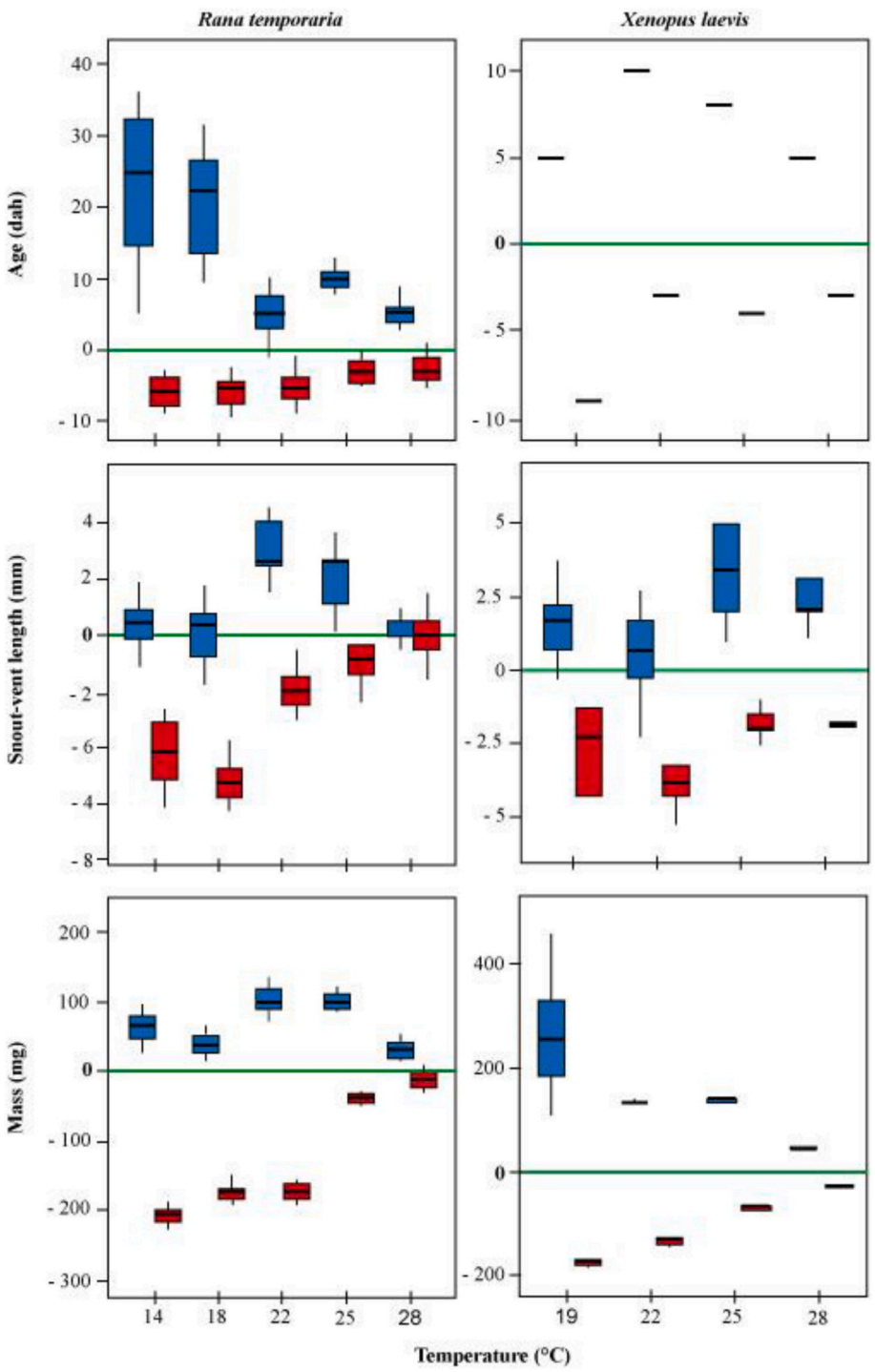

Fig. 4. Mean species-specific differences in thermal reaction norm of age, mass, and snout-vent length at the onset of metamorphosis in *Rana temporaria* and *Xenopus laevis* exposed to different endocrine disruptive treatments. Shown is the mean deviation for each hormone treatment from the control group in age (days after hatching), snout-vent length (mm), and mass (mg) at the onset of metamorphosis. The data collected on *X. laevis* larvae reared at 16 °C were excluded from the analysis as larvae showed a lack of plasticity in length and mass. Positive and negative values indicate an increase and decrease, respectively, in thermal sensitivity of metamorphic traits. Blue: Low TH levels = SP treatment. Red: High TH levels = T4 treatment. The green line indicates the level of the control group.

are assumed to confer greater fitness (Wilbur and Collins, 1973; Beck and Congdon, 2000). Most studies of phenotypic plasticity investigate the effects of single environmental factors on organism phenotypes (Stillwell et al., 2007). However, organisms exist in an ecologically complex world where multiple environmental factors interact to affect growth, development and life histories. Furthermore, ongoing (anthropogenic) global change will result in multiple, simultaneously occurring environmental stressors. Nevertheless, studies investigating interactive effects of those environmental stressors are still rare. In this study, we examined the independent and interactive effects of two important environmental factors, temperature and altered TH levels (i.e. proximate effects of different thyroid-affecting stressors), using a multifactorial experimental design, on life history traits and survival in two anuran species which differ in their thermal ecology and life history strategy. Our results suggest that altered TH levels as caused by environmental stressors in *R. temporaria* and *X. laevis* larvae (1) alter survival, growth and developmental rate and (2) modify the capacity for plasticity in these metamorphic traits across temperatures. Effects of altered TH levels were more pronounced at extreme temperatures.

Table 3
Effects of altered TH levels at five different developmental temperatures during development on snout-vent length (SVL), total length (TL), mass, and growth rate (mg per day after hatching), developmental rate (Gosner stages per day after hatching; DR), and mass in tadpoles of the common frog (*Rana temporaria*) at the onset of metamorphosis (Gosner stage 42) (Gosner, 1960). SP = low TH levels. T4 = high TH levels. Pairwise multiple comparisons were made using Mann-Whitney-U-Test as post hoc test with Bonferroni correction. Significance was set at $P < 0.05$. N is the total number of analyzed individual animals.

Rana temporaria		Mann-Whitney-Test					
Temperature	Dependent variable	Control - SP			Control - T4		
		z	P	N	z	P	N
14°	SVL (mm)	−1.86	0.063	84	−7.78	< **0.001**	80
	TL (mm)	−0.50	0.960		−7.52	< **0.001**	
	GR (mg/d)	−7.49	< **0.001**		−7.39	< **0.001**	
	DR (GS/d)	−7.89	< **0.001**		−6.90	< **0.001**	
	Mass (mg)	−7.81	< **0.001**		−7.70	< **0.001**	
18°	SVL (mm)	−8.44	< **0.001**	96	−0.13	< **0.001**	97
	TL (mm)	−8.37	< **0.001**		−3.96	< **0.001**	
	GR (mg/d)	−8.35	< **0.001**		−7.19	< **0.001**	
	DR (GS/d)	−8.37	< **0.001**		−3.45	**0.001**	
	Mass (mg)	−8.35	< **0.001**		−8.41	< **0.001**	
22°	SVL (mm)	−9.28	< **0.001**	114	−8.73	< **0.001**	104
	TL (mm)	−9.20	< **0.001**		−8.74	< **0.001**	
	GR (mg/d)	−0.67	0.501		−7.01	< **0.001**	
	DR (GS/d)	−7.50	< **0.001**		−7.66	< **0.001**	
	Mass (mg)	−9.21	< **0.001**		−8.78	< **0.001**	
25°	SVL (mm)	−6.71	< **0.001**	73	−4.54	< **0.001**	69
	TL (mm)	−5.92	< **0.001**		−4.92	< **0.001**	
	GR (mg/d)	−0.59	0.551		−2.53	**0.011**	
	DR (GS/d)	−7.41	< **0.001**		−5.84	< **0.001**	
	Mass (mg)	−7.35	< **0.001**		−7.11	< **0.001**	
28°	SVL (mm)	−2.67	**0.008**	83	−0.76	0.444	**69**
	TL (mm)	−4.18	< **0.001**		−5.88	< **0.001**	
	GR (mg/d)	−1.68	0.093		−0.56	0.574	
	DR (GS/d)	−7.84	< **0.001**		−5.21	< **0.001**	
	Mass (mg)	−7.69	< **0.001**		−3.83	< **0.001**	

4.1. Effects of altered TH levels during metamorphosis go from bad to worse at extreme temperatures – but one stressor is not like the other

Environmental stress is of great importance for anuran larvae in ephemeral habitats, proximately causing alterations in the endocrine control of metamorphosis (Räsänen et al., 2003; Burraco and Gomez-Mestre, 2016) and influencing growth, development, and survival. In the present study, the fastest development was observed in larvae exposed to high TH levels at the warmest temperature tested. As biochemical processes speed up at higher temperatures, high TH levels also have a greater effect on TH-dependent processes during larval development (e.g. limb development) (Little and Seebacher, 2016; Shi, 2000) at higher temperatures. In contrast, SP, a TH inhibiting factor (Sparling and Fellers, 2007; Goleman et al., 2002a; b) caused reduced developmental rates in the larvae resulting in older and larger metamorphs in both species over the entire range of temperatures tested. These results are in accordance with studies on endocrine disruption and TH control of amphibian metamorphosis (Denver, 1997; Heimeier and Shi, 2010; Bulaeva et al., 2015; Li et al., 2016) indicating an obvious disruptive effect of environmental stressors on the TH system in larval anurans with ramifications for growth, development, and survival.

Altered growth and developmental rate due to the impact of environmental stressors on the TH system can cause differences in the age and size at the onset of metamorphosis. Large body size at metamorphosis is associated with high fitness in amphibians and it is beneficial to reach a large size quickly, e.g. to reduce the time spent in habitats with greater risks of mortality via predation and desiccation (Ståhlberg et al., 2001). Although reduced rates of growth and development caused by endocrine disruptors such as SP may increase the duration of exposure of larvae to higher risks of mortality, the probability of post-metamorphic survival may be increased. Large body size obtained via slow growth was associated with reduced predation pressure and better performance during the juvenile and adult stages (Berven, 1990; Ståhlberg et al., 2001). On the other hand, larvae exposed to stress that increases TH levels may suffer from the disadvantage of a small body size resulting from increased developmental rates but will likely face less risks within heterogeneous larval habitats (Richter-Boix et al., 2011; Orizaola et al., 2013). However, any imbalance of growth and developmental rate due to environmental stressor may impair survival and, thus, overall fitness. Furthermore, a shorter larval period allows for a longer accumulation of energy reserves in juveniles if food is abundant in the terrestrial habitat, which is particularly important in hibernating species such as *R. temporaria*.

In this study, larvae had lower survival when TH levels were altered (regardless of the direction) and, although the effect was generally most pronounced at the highest temperatures (25 and 28 °C), the overall response at different temperatures was species-specific. The warmest temperatures used in this study are commonly encountered by the larvae of *X. laevis* in the tropics but are rarely encountered in the breeding ponds of the temperate *R. temporaria* (Drakulić et al., 2017). Interestingly and in contrast to our results, Ruhmekorf reported that pond temperatures between 21 and 26 °C are favorable for growth and development in *R. temporaria* larvae in nature. Nevertheless, Kingsolver et al. (2015) emphasized that aquatic ectotherms such as anuran larvae experience daily variation in temperature and that general performance can differ between fluctuating and constant thermal environments. Future studies should examine the response of the larvae of these and other species to fluctuating versus constant temperatures, particularly responses to heating experienced for short time periods at different stages of larval development (e.g. to mimic responses to heatwaves). Furthermore, both species are very evolutionarily diverged which also might contribute to these species-specific differences in thermal

Table 4
Thermal reaction norm of age and size at the onset of metamorphosis in *Rana temporaria* and *Xenopus laevis* exposed to different combinations of developmental temperature (T_{dev}) and endocrine disruptive treatments leading to lower and higher TH levels compared to control. Plasticity indices (PIX) were equal to the slopes of trait versus temperature regressions. Regressions were calculated independently for each aquarium. Low TH levels = SP treatment. High TH levels = T4 treatment. $P < 0.001$ for all linear regressions.

Dependent Variable	Treatment	Aquarium	*Rana temporaria*			*Xenopus laevis*		
			PIX	Intercept	R^2	PIX	Intercept	R^2
Age (d)	Control	1	−1.77	61.06	0.94	−0.70	35.80	0.86
		2	−1.62	57.48	0.92	−0.68	35.55	0.87
		3	−1.66	57.99	0.94	−0.69	35.54	0.86
		mean	**−1.68**	**58.84**		**−0.69**	**35.63**	
	high TH	1	−1.44	49.27	0.90	−0.74	41.67	0.98
		2	−1.46	49.40	0.91	−0.72	41.35	0.98
		3	−1.45	49.45	0.90	−0.72	41.27	0.98
		mean	**−1.45**	**49.37**		**−0.72**	**41.43**	
	low TH	1	−3.05	100.61	0.82	−1.38	41.16	0.97
		2	−3.18	103.80	0.85	−1.36	40.81	0.97
		3	−2.99	99.14	0.82	−1.33	40.43	0.97
		mean	**−3.07**	**101.18**		**−1.35**	**40.80**	
Mass (mg)	Control	1	−39.71	1182.70	0.96	−41.34	1194.10	0.96
		2	−39.67	1183.70	0.96	−42.23	1216.90	0.96
		3	−39.56	1181.90	0.96	−42.23	1215.90	0.95
		mean	**−39.64**	**1182.76**		**−41.93**	**1208.96**	
	high TH	1	−25.43	756.93	0.97	−60.35	1777.30	0.92
		2	−25.41	757.26	0.97	−63.38	1856.30	0.89
		3	−25.60	761.04	0.97	−63.33	1852.10	0.91
		mean	**−25.14**	**78.41**		**−62.35**	**1828.56**	
	low TH	1	−39.42	1246.20	0.94	−26.32	743.68	0.89
		2	−39.26	1241.70	0.94	−26.91	756.79	0.89
		3	−39.52	1247.90	0.94	−26.69	750.46	0.89
		mean	**−39.40**	**1245.26**		**−26.64**	**750.31**	
SVL (mm)	Control	1	−0.71	26.88	0.90	−1.01	34.47	0.84
		2	−0.70	26.67	0.90	−1.06	35.57	0.85
		3	−0.69	26.71	0.90	−1.07	35.81	0.87
		mean	**−0.70**	**26.75**		**−1.04**	**35.28**	
	high TH	1	−0.38	17.41	0.81	−0.89	33.63	0.85
		2	−0.35	16.85	0.76	−0.85	32.32	0.80
		3	−0.35	16.80	0.77	−0.91	34.40	0.86
		mean	**−0.36**	**17.02**		**−0.88**	**33.45**	
	low TH	1	−0.63	26.45	0.86	−0.91	29.51	0.90
		2	−0.61	25.91	0.85	−0.89	29.01	0.88
		3	−0.64	26.75	0.88	−0.93	30.11	0.90
		mean	**−0.62**	**26.37**		**−0.91**	**29–54**	

sensitivity. The reduced survival before metamorphosis may be also caused by a low toxicity of SP itself (Coady et al., 2009) whereas the reduced survival in larvae exposed to TH is probably due to metabolic stress (Sheridan, 1994; Coady et al., 2009) as THs are also responsible for energetic homeostasis (Frieden, 1981; McNabb and King, 1993).

High endogenous TH levels lead to high standard metabolic rates and energy expenditure (Rowe et al., 1998; Steyermark et al., 2005; Ruthsatz et al., 2018, 2019). Thus, when endogenous TH levels are increased in anuran larvae as a consequence of endocrine disruption, larvae need higher rates of feeding to cover their energetic needs (i.e. maintenance costs) (Beck and Congdon, 2000; Orlofske and Hopkins, 2009), even more so at warm temperatures. On the other hand, larvae exposed to TH inhibitors are likely to have lower maintenance costs (Orlofske and Hopkins, 2009) even at warm temperatures. As anuran larvae stop eating during metamorphic climax due to the remodeling of oral and intestinal structures (Orlofske and Hopkins, 2009), high TH levels are more likely to be associated with failure to complete metamorphosis, because high maintenance costs reduce the energy available for development (Beck and Congdon, 2000).

4.2. *Proximate effects of environmental stress modify the thermal reaction norm of metamorphic traits*

Consequences of human activities including climate change not only alter temperature regimes, but also lead to a multitude of interacting environmental stressors such as higher desiccation risks (Richter-Boix et al., 2011; Gervasi and Foufopoulos, 2008), chemical contamination of larval habitats (Shenoy et al., 2009), and altered presence of predators (Mikó et al., 2017; Blaustein et al., 2011). Hence, understanding how temperature and endocrine-induced stress responses interact to affect the growth and development of anuran larvae is critical to understand and project future impacts on populations.

Our results show that altered TH levels change the capacity to express temperature-induced developmental plasticity as larvae exposed to high levels of THs did not adjust their rates of growth and developmental to higher temperatures (Fig. 4). Since a plastic response in growth and developmental rate is beneficial in heterogeneous larval habitats as it allows for faster escape, environmental stressors which increase endogenous TH levels may result in more vulnerable anuran larvae due to a reduced capacity for temperature-induced developmental plasticity (i.e. thermal reaction norm of metamorphic traits). In warmer habitats larvae with high TH will be unable to display the same degree of plasticity in growth and developmental rate and thus, will be

more vulnerable to climate-driven warming. On the other hand, if environmental stressors inhibit or delay TH production during metamorphosis, the capacity for temperature-induced developmental plasticity in larvae can increase as demonstrated in our study. Inhibition of TH pathways is usually caused by environmental pollution and, therefore, chemical contamination of the larval habitat. Consequently, larvae in habitats polluted by agricultural fertilizers and pesticides (Garriga et al., 2017; Mikó et al., 2017), industrial chemicals (Lefcort et al., 1998; Ossana et al., 2017) or fireworks (Sparling and Harvey, 2006; Bulaeva et al., 2015), often have a slower rate of development, are larger at the onset of metamorphosis but maintain a higher developmental plasticity even at warm temperatures. In this one respect, larvae from these polluted sites may be less vulnerable to the impacts of climate change.

Within the range of temperatures tested, size (i.e. SVL and mass) at the onset of metamorphosis was more plastic in larvae of the entirely aquatic *X. laevis*, whereas age was more plastic in larvae of *R. temporaria*. In *R. temporaria*, where metamorphs switch to terrestrial habitats, we suggest that plasticity in developmental rate is more adaptive than plasticity in growth rate as the former allows individuals to emerge from ponds more quickly if habitat quality decreases. Consequently, selection may favor plasticity in developmental rate more than growth rate in *R. temporaria* (Newman, 1992; Van Buskirk and Relyea, 1998; Laurila et al., 2002). Similarly, since *X. laevis* completes its entire life cycle in aquatic habitats and does not have the option to escape ponds, we suggest that plasticity in developmental rate is less important. Moreover, species at very low and high latitudes (e.g. tropics and poles) generally display less plasticity to temperature (are more stenothermic) compared to those at mid-(temperate) latitudes (Janzen, 1967; Ghalambor et al., 2006). This is demonstrated by the loss of plasticity in the size (length and mass) of *X. laevis* at 16 °C, thus not following the TSR (Walczyńska et al., 2016). Although temperatures >28 °C were not tested, *X. laevis* has a substantially narrower developmental thermal window compared to temperate *R. temporaria* (Janzen, 1967; Ruthsatz et al., 2018). The high thermal sensitivity of growth and developmental rate in larval *R. temporaria* was not surprising as selection may favor a high sensitivity of both rates due to temperature variation resulting in a high capacity for a plastic response in both rates in temperate species (Janzen, 1967; Seebacher et al., 2015).

The capacity for temperature-induced developmental plasticity is known to be related to local adaptations in anuran larvae (Laugen et al., 2002; Muir et al., 2014; Drakulić et al., 2016) and thus, is population-specific. Usually, the capacity for temperature-induced plasticity is highest in populations from heterogeneous environments and/or high latitudes within species (Ståhlberg et al., 2001). In this study, we tested individuals of a single population in both species. Local adaptation might cause population-specific differences in the capacity for temperature-induced developmental plasticity and different impacts of altered TH levels on this capacity. In *R. temporaria*, numerous studies could demonstrate that the capacity for temperature-induced developmental plasticity is population-specific and arises from local adaptations due to geographic differences in mean and/or variance in temperature (Laugen et al., 2002, 2003; Drakulić et al., 2016; Groezinger et al., 2018). Such differences might be especially important in the light of ongoing (and projected) environmental change (Drakulić et al., 2016). Plasticity may play a key role in the initial steps of the adaptation to rapid environmental change since genetic adaptation is typically a slower process that may span many generations. Not accounting for variation in plasticity within a species can lead to inaccurate predictions about the vulnerability of populations to environmental change (Orizaola and Laurila, 2016). Therefore, we stress the importance of conducting studies to examine geographic variation in the capacity for temperature-induced plasticity and how environmental stressors affect this capacity-y in different populations to make more robust predictions of the impacts of global (climate) change across anuran species.

5. Conclusions

Although several studies on environmental stressors on anuran larvae have demonstrated that altered TH levels impact growth, development and survival, few studies have investigated whether these proximate effects of global (climate) change influence the capacity for temperature-induced developmental plasticity in tadpoles. In this study, high levels of TH impaired the ability of the larvae of both a tropical and temperate anuran species to display temperature-induced developmental plasticity. Therefore, environmental stressors leading to high levels of TH via the activation of stress-hormones are likely to make anuran larvae less able to cope with warmer developmental temperatures. Although some larval habitats may be contaminated with TH inhibiting substances, the majority of environmental factors activate stress-hormones and thus, cause high TH levels in anuran larvae. The present findings emphasize that the larvae of both species may suffer from the interactive effect of higher temperatures and a disrupted TH system caused by global change. However, the strength of impacts of environmental stressors on the capacity for temperature-induced plasticity may differ between populations in both species studied here and anurans in general. As THs also manage energy expenditure, future studies should focus on investigating if altered TH levels also impair physiological plasticity, as a balanced energy budget obviously is crucial for survival. Species that cannot compensate for long-term (e.g. average warming) or short-term (e.g. increased variability) changes in abiotic factors by buffering metamorphic and physiological traits will be most affected by global change.

Conflicts of interest

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CRediT authorship contribution statement

Katharina Ruthsatz: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Kathrin H. Dausmann:** Conceptualization, Supervision, Validation, Writing - review & editing. **Claudia Drees:** Formal analysis, Writing - review & editing. **Laura I. Becker:** Investigation, Writing - review & editing. **Lisa Hartmann:** Investigation, Writing - review & editing. **Janica Reese:** Investigation, Writing - review & editing. **Steffen Reinhardt:** Investigation, Writing - review & editing. **Tom Robinson:** Investigation, Writing - review & editing. **Nikita M. Sabatino:** Investigation, Visualization, Writing - review & editing. **Myron A. Peck:** Conceptualization, Supervision, Validation, Writing - review & editing. **Julian Glos:** Conceptualization, Data curation, Formal analysis, Supervision, Validation, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2020.102599.

Appendix

Table A1

Effects of altered TH levels and temperature during development on developmental rate (Gosner stages per day after hatching; DR), snout-vent length (SVL), total length (TL), mass, and growth rate (mg per day after hatching; GR) in tadpoles of the common frog (*Rana temporaria*) and the African clawed frog (*Xenopus laevis*) at the onset of metamorphosis (Gosner stage 42) (Gosner, 1960). P for generalized (GR, DR, mass) linear mixed-effects models, using the covariates 'Treatment' (T4, SP, and Control), 'T_{dev}' and the interactions of 'Treatment*T_{dev}' as fixed factors; 'aquarium' as random factor. SP = low TH levels. T4 = high TH levels. Significance was set at $P < 0.05$.N is the total number of analyzed individual animals, and n is the total number of tested aquaria.

Model type	Dependent variable	Fixed effects	*Rana temporaria*				*Xenopus laevis*			
			Coefficient	SE	P	N (n)	Coefficient	SE	P	N (n)
GLMM	Developmental rate (GS/d) (DR)	Treatment [Control]	−0.46	0.08	< **0.001**	627 (48)	−0.42	0.02	**0.046**	475 (45)
		Treatment [SP]	−0.06	0.11	0.522	627 (48)	0.39	0.03	**0.008**	475 (45)
		Treatment [T4]	0.18	0.11	0.093	627 (48)	0.02	0.03	< **0.001**	475 (45)
		T_{dev}	0.06	0.00	< **0.001**	627 (48)	0.62	0.00	**0.016**	475 (45)
		T_{dev}* Treatment [SP]	−0.02	0.00	**0.002**	627 (48)	−0.03	0.00	**0.003**	475 (45)
		T_{dev}* Treatment [T4]	−0.02	0.00	< **0.001**	627 (48)	0.01	0.00	**0.009**	475 (45)
LMM	SVL (mm)	Treatment [Control]	26.6	0.95	< **0.001**	627 (48)	14.21	4.99	**0.007**	475 (45)
		Treatment [SP]	−0.22	1.35	0.871	627 (48)	11.3	7.06	**0.001**	475 (45)
		Treatment [T4]	−9.81	1.35	< **0.001**	627 (48)	−7.4	1.32	**0.001**	475 (45)
		T_{dev}	−0.70	0.04	< **0.001**	627 (48)	−0.21	0.22	**0.010**	475 (45)
		T_{dev}* Treatment [SP]	0.10	0.06	0.264	627 (48)	0.21	0.31	0.51	475 (45)
		T_{dev}* Treatment [T4]	0.35	0.06	< **0.001**	627 (48)	−0.06	0.31	0.84	475 (45)
LMM	TL (mm)	Treatment [Control]	72.6	3.22	< **0.001**	627 (48)	49.14	3.05	**0.004**	475 (45)
		Treatment [SP]	−2.19	4.56	0.634	627 (48)	39.8	4.32	**0.01**	475 (45)
		Treatment [T4]	−17.08	4.57	**0.001**	627 (48)	−6.31	4.32	**0.01**	475 (45)
		T_{dev}	−1.94	0.14	< **0.001**	627 (48)	−0.65	0.72	**0.013**	475 (45)
		T_{dev}* Treatment [SP]	0.31	0.21	0.141	627 (48)	0.31	1.02	0.76	475 (45)
		T_{dev}* Treatment [T4]	0.48	0.21	**0.023**	627 (48)	−0.14	1.02	0.89	475 (45)
GLMM	Mass (mg)	Treatment [Control]	1185.5	44.12	< **0.001**	627 (48)	433.42	22.96	**0.066**	475 (45)
		Treatment [SP]	64.31	62.40	**0.31**	627 (48)	147.02	32.47	< **0.001**	475 (45)
		Treatment [T4]	−432.43	62.40	< **0.001**	627 (48)	−182.64	32.47	< **0.001**	475 (45)
		T_{dev}	−39.67	2.01	< **0.001**	627 (48)	−10.92	1.02	**0.029**	475 (45)
		T_{dev}* Treatment [SP]	0.22	2.84	**0.938**	627 (48)	−1.46	1.44	0.920	475 (45)
		T_{dev}* Treatment [T4]	14.41	2.84	< **0.001**	627 (48)	4.24	1.44	0.771	475 (45)
GLMM	Growth rate (mg/d)	Treatment [Control]	32.13	3.48	< **0.001**	627 (48)	14.10	1.85	**0.020**	475 (45)
		Treatment [SP]	−13.08	4.92	**0.011**	627 (48)	1.83	2.62	< **0.001**	475 (45)
		Treatment [T4]	−8.29	4.92	0.099	627 (48)	0.43	2.62	**0.020**	475 (45)
		T_{dev}	−0.97	0.15	< **0.001**	627 (48)	−0.17	0.44	**0.034**	475 (45)
		T_{dev}* Treatment [SP]	0.55	0.22	**0.017**	627 (48)	−0.00	0.63	0.996	475 (45)
		T_{dev}* Treatment [T4]	0.23	0.22	0.304	627 (48)	−0.15	0.63	0.817	475 (45)

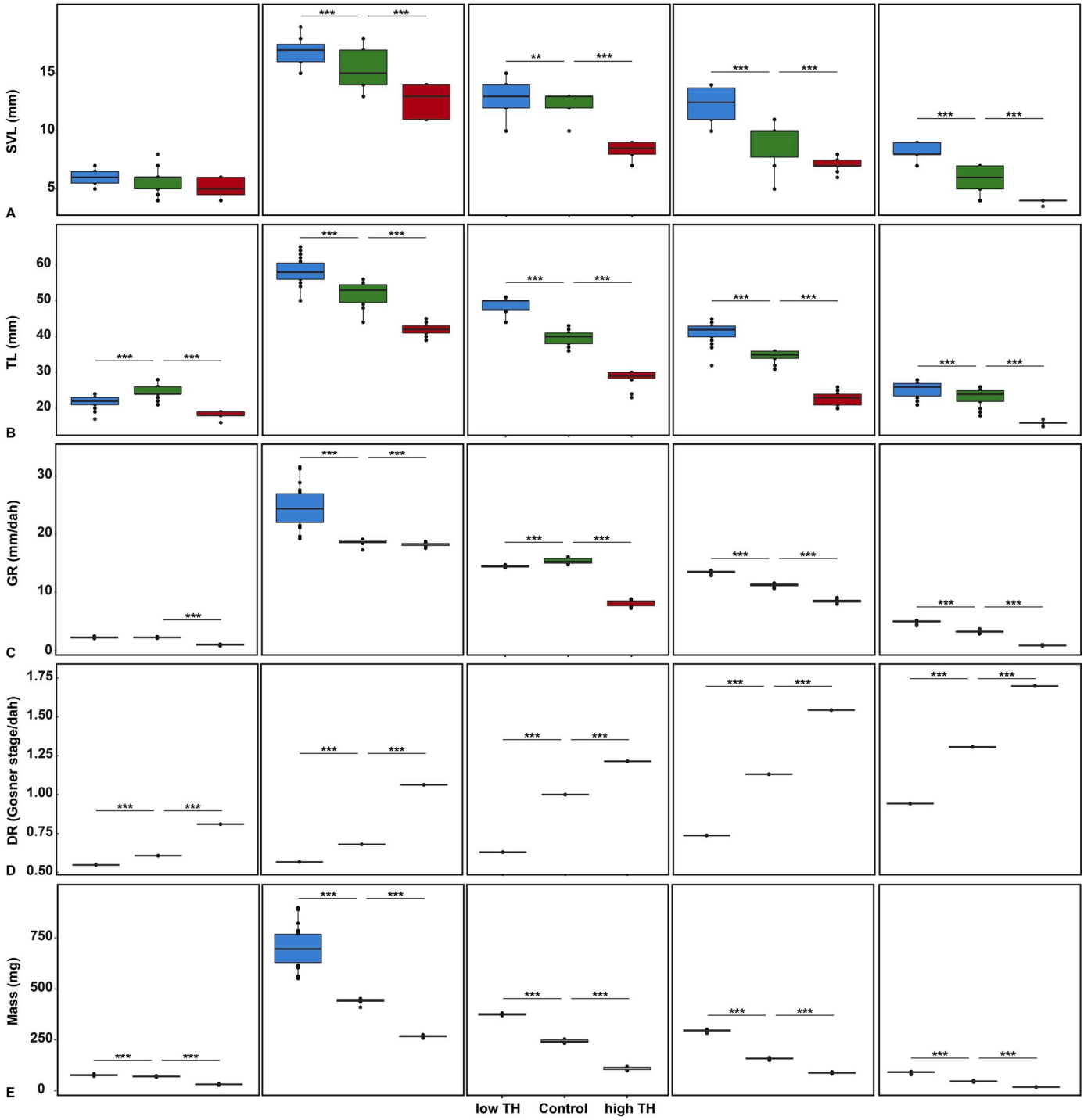

Fig. A1. Effects of altered TH levels on (A) snout-vent length (mm; SVL), (B) total length (mm; TL), (C) growth rate (mg/day; GR), (D) developmental rate (Gosner stage/day after hatching; DR), and (E) mass (mg) at the onset of metamorphosis (Gosner stage 42; Gosner, 1960) in *Xenopus laevis*. F. l. t. r.: Blue: Low TH levels = SP treatment. Green: Control group. Red: High TH levels = T4 treatement. Pairwise multiple comparisons were made using Mann-Whitney-U-Test as post hoc test with Bonferroni correction. Asteriscs between two groups demonstrate significant difference. Significance was set at $P < 0.05$.

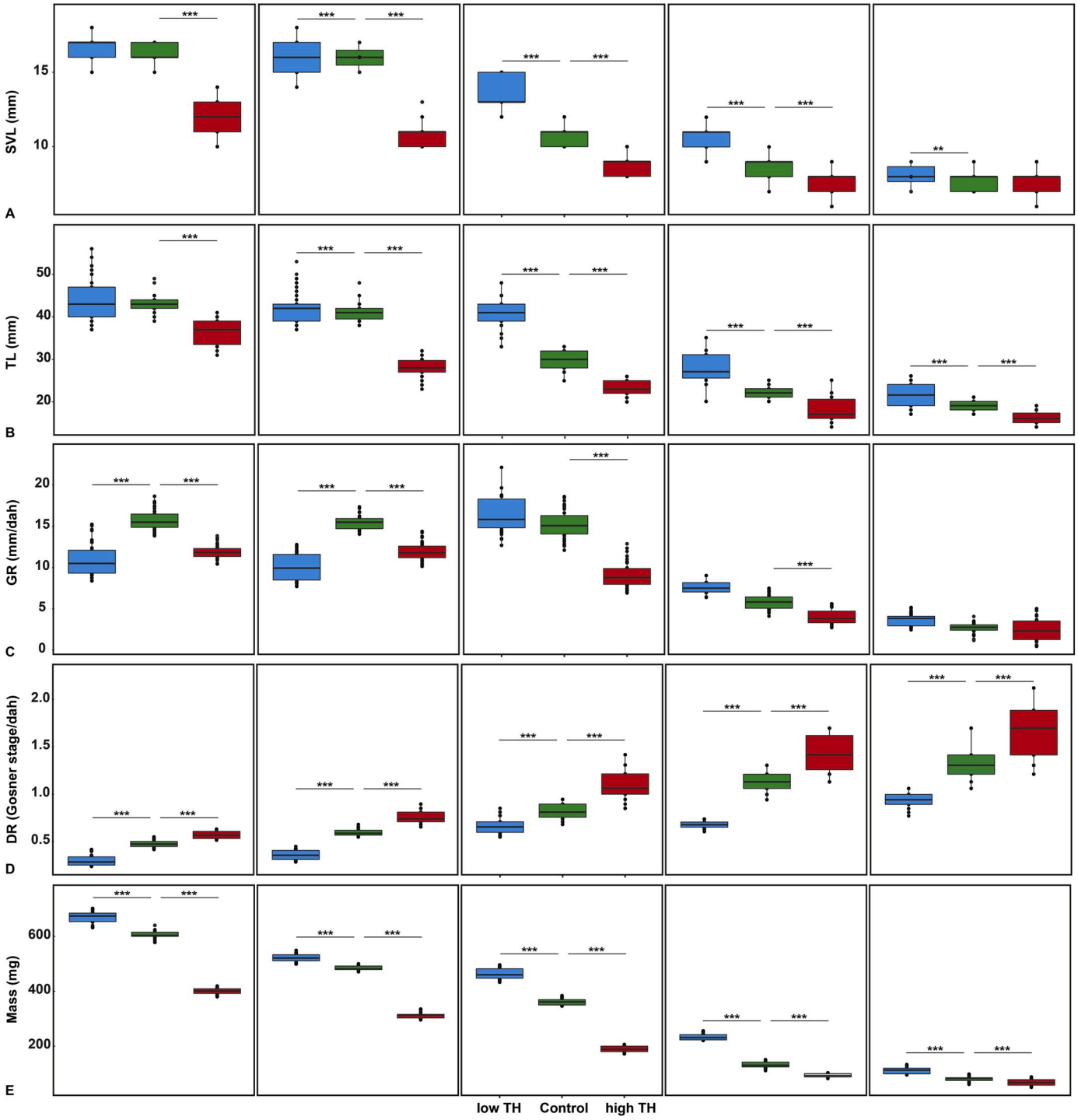

Fig. A2. Effects of altered TH levels on (A) snout-vent length (mm; SVL), (B) total length (mm; TL), (C) growth rate (mg/day; GR), (D) developmental rate (Gosner stage/day after hatching; DR), and (E) mass (mg) at the onset of metamorphosis (Gosner stage 42; Gosner, 1960) in *Rana temporaria*. F. l. t. r.: Blue: Low TH levels = SP treatment. Green: Control group. Red: High TH levels = T4 treatement. Pairwise multiple comparisons were made using Mann-Whitney-U-Test as post hoc test with Bonferroni correction. Asteriscs between two groups demonstrate significant difference. Significance was set at $P < 0.05$.

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