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Article *in* Integrative and Comparative Biology · May 2019

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INVITED PAPER

Endocrine Disruption Alters Developmental Energy Allocation and Performance in *Rana temporaria*

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Synopsis Environmental change exposes wildlife to a wide array of environmental stressors that arise from both anthropogenic and natural sources. Many environmental stressors with the ability to alter endocrine function are known as endocrine disruptors, which may impair the hypothalamus–pituitary–thyroid axis resulting in physiological consequences to wildlife. In this study, we investigated how the alteration of thyroid hormone (TH) levels due to exposure to the environmentally relevant endocrine disruptor sodium perchlorate (SP; inhibitory) and exogenous L-thyroxin (T4; stimulatory) affects metabolic costs and energy allocation during and after metamorphosis in a common amphibian (*Rana temporaria*). We further tested for possible carry-over effects of endocrine disruption during larval stage on juvenile performance. Energy allocated to development was negatively related to metabolic rate and thus, tadpoles exposed to T4 could allocate 24% less energy to development during metamorphic climax than control animals. Therefore, the energy available for metamorphosis was reduced in tadpoles with increased TH level by exposure to T4. We suggest that differences in metabolic rate caused by altered TH levels during metamorphic climax and energy allocation to maintenance costs might have contributed to a reduced energetic efficiency in tadpoles with high TH levels. Differences in size and energetics persisted beyond the metamorphic boundary and impacted on juvenile performance. Performance differences are mainly related to strong size-effects, as altered TH levels by exposure to T4 and SP significantly affected growth and developmental rate. Nevertheless, we assume that juvenile performance is influenced by a size-independent effect of achieved TH. Energetic efficiency varied between treatments due to differences in size allocation of internal macronutrient stores. Altered TH levels as caused by several environmental stressors lead to persisting effects on metamorphic traits and energetics and, thus, caused carry-over effects on performance of froglets. We demonstrate the mechanisms through which alterations in abiotic and biotic environmental factors can alter phenotypes at metamorphosis and reduce lifetime fitness in these and likely other amphibians.

Introduction

Environmental change exposes wildlife to an array of environmental stressors that arise from anthropogenic activities (e.g., climate change, pollution) as well as natural sources (Noyes et al. 2009). Many environmental stressors have the ability to alter endocrine function with physiological consequences to wildlife (Carr and Patiño 2011). In most cases, activation of the neuroendocrine stress axis (Mann et al. 2009; Dantzer et al. 2014) increases stress hormone levels (Denver 1997). These stress hormones also target the hypothalamus–pituitary–thyroid axis, which is responsible for the production of thyroid hormones (THs) (Carr and Patiño 2011), and may synergize with THs resulting in increased TH production (Glennemeier and Denver 2002; Laudet 2011; Kulkarni and Buchholz 2012). Moreover, stress hormones (i.e., glucocorticoids) produced by the neuroendocrine stress axis enhance the sensitivity of cells to THs (Denver 2009).

The impact of environmental stress on the hypothalamus–pituitary–thyroid axis is of special concern for amphibians as their metamorphosis is mainly regulated by THs (i.e., T3 and T4) (Furlow and Neff 2006; Tata 2006), which increase in concentration during this process and determine the

developmental rate (Shi 2000; Brown and Cai 2007). The presence of predators (Relyea 2002; Capellán and Nicieza 2007), crowding (Morey and Reznick 2001), desiccation risk (Gervasi and Foufopoulos 2008), food scarcity (Kupferberg 1997), and temperature (Smith-Gill and Berven 1979; reviewed in Ruthsatz et al. 2018a) are known to increase TH production by activating the neuroendocrine stress axis. Anuran larvae with high TH levels display increased developmental and metabolic rates and decreased growth rates (Rowe et al. 1998; Brown and Cai 2007), which results in shorter larval periods, smaller size at the onset of metamorphosis, and higher energetic maintenance and developmental costs (Denver 1998, 2009; Orlofske and Hopkins 2009). Exposing tadpoles to exogenous THs is an established method to simulate the proximate effects of environmental stressors on the TH system (Denver et al. 2002; Tata 2006; Denver 2009).

Although most environmental stressors lead to increased TH activity or production by the activation of stress hormones, a large number of aquatic contaminants, such as pesticides and herbicides, road salt, fertilizers, heavy metals, and active pharmaceutical ingredients, have been shown to disrupt and inhibit the normal action of THs in amphibians, leading to changes in growth, development, and metabolism (Kashiwagi et al. 2009; Carr and Patiño 2011). Inhibition or a decrease of TH production pathways slows the rate of development (Carr et al. 2003; Bulaeva et al. 2015) and decreases metabolic rates (Carr and Patiño 2011; Ortiz-Santaliestra and Sparling 2007), causing tadpoles to metamorphose at a larger size and older age (Shi 2000). Chemical stressors that alter or disturb endocrine systems are characterized as endocrine disruptors (Kloas and Lutz 2006; Kloas et al. 2009). Larval stages of amphibians are particularly vulnerable to endocrine disruptors due to the reduced ability of this life stage to select or avoid habitats (Sanzo and Hecnar 2006; Yu et al. 2015) and their increased risk of exposure due to their highly permeable skin (Hayes et al. 2006; Strong et al. 2017). An environmentally relevant endocrine disruptor is perchlorate (ClO_4^-), which is a goitrogen that inhibits TH synthesis (Ortiz-Santaliestra and Sparling 2007). Concentrations of perchlorate measured in the field can be high enough to inhibit amphibian metamorphosis (Goleman et al. 2002a, 2002b; Tietge et al. 2005; Ortiz-Santaliestra and Sparling 2007).

Since THs are not only critical for amphibian metamorphosis but also for the regulation of metabolic processes (Frieden 1981; McNabb and King 1993; Sheridan 1994), altered TH levels as caused by environmental stressors may also affect energy budgeting (i.e., metabolic rate and energy stores) at metamorphosis and during metamorphic climax (Sheridan 1994; Orlofske and Hopkins 2009). THs are known to increase the standard metabolic rate (SMR) which represents the energy required to cover basic physiological functions (i.e., maintenance costs) (Rowe et al. 1998; Beck and Congdon 2003, Orlofske and Hopkins 2009). In larval amphibians, increased metabolic rate and maintenance costs constrain the amount of energy that can be stored in the tail and liver tissue to fuel metamorphosis and, hence, less energy can be allocated toward paying costs of development incurred during metamorphic climax (Sheridan and Kao 1998; Orlofske and Hopkins 2009).

During metamorphic climax several larval tissues degenerate (e.g., gills and tail), are rebuilt (e.g., gastro-intestinal tract, brain, and the liver), or are newly generated (e.g., lungs and limbs) (Shi 2000; Tata 2006). Due to the rebuilding of the gastro-intestinal tract accompanied with changes in oral morphology, anuran larvae must rely on stored energy (Beck and Congdon 2003; Orlofske and Hopkins 2009). Tadpoles, which have larger energy reserves and a low metabolic rate at the onset of metamorphosis, are more likely to successfully complete metamorphosis and become juvenile froglets with larger energy stores and higher probabilities of survival (Orlofske and Hopkins 2009). Therefore, energetics (i.e., SMR and size of energy stores) at the onset and after completion of metamorphosis are important fitness variables (Steyermark et al. 2005; Muir et al. 2014; Ruthsatz et al. 2018b).

Numerous studies have characterized metabolism during larval stages, before and during metamorphic climax (Sivula et al. 1972; reviewed in Beck and Congdon 2003; Ruthsatz et al. 2018c), and few studies quantified energetics across life history stages (Pandian and Marian 1985; Beck and Congdon 2003; Orlofske and Hopkins 2009; Orlofske et al. 2017). Only one previous study has quantified the energetic costs of metamorphosis as well as the entirety of larval development using an experimental approach for testing the effect of exogenous stress hormones (Kirschman et al. 2017). However, no study to date has investigated the impact of altered TH levels as caused by environmental stress on growth, development, and energetics (i.e., metabolic rate and body condition) during metamorphosis, after successful completion of metamorphosis, and in early juvenile froglets. Furthermore, there is no knowledge on how environmental stress may affect energy portioning between growth, development,

and metabolism during metamorphic climax. As altered TH levels impact physiological processes, proximate effects of environmental stress may therefore affect energy allocation for development and growth during metamorphic climax resulting in serious effects for later life stages.

The purpose of this study was to investigate the impact of altered TH levels as caused by environmental stressors on energy allocation for growth, development, and energetics at the onset of metamorphosis, during metamorphic climax and after successful completion of metamorphosis in the common frog (*Rana temporaria*). Furthermore, we estimated fitness of juvenile froglets by examining whether an altered TH status experienced during the larval stage affects energetics and performance in later life stages. This study provides a framework for quantifying how environmental stressors impact amphibian metamorphosis allowing more robust projections of how stressful environmental conditions may affect across-life stage survival and fitness in the future.

Material and methods

Study species and experimental design

Rana temporaria was chosen as the model species because it undergoes a habitat transition after metamorphosis associated with complex physiological and morphological changes. Furthermore, it is widely distributed throughout Europe. Five clutches of *R. temporaria* were obtained from Waldpark Marienhöhe in western Hamburg, Germany (53°34'37.4'N 9°46'57.5'E). Larvae were allowed to hatch and develop to Stage 25 (free-swimming larvae; Gosner 1960). From these larvae, 180 individuals originating from different families were intermixed before allocating them randomly to the different treatments (L-thyroxine and sodium perchlorate (SP)) and the control group. Fifteen larvae of *R. temporaria* were kept each in a standard 9.5-L aquarium filled with 8 L of water (i.e., a total of 12 aquaria: 4 × T4, 4 × SP, 4 × Control). The experiment was conducted in a climate chamber (Weiss Umwelttechnik GmbH, 35447 Reiskirchen, Germany) with a 12:12 h light: dark cycle at 22 ± 0.1°C. The experiment was conducted over 5 weeks. Amphibian larvae were fed 50% high-protein flaked fish food (Sera micron breeding feed for fish and amphibians, Sera, 52518 Heinsberg, Germany) and 50% spirulina algae. *Ad libitum* rations were provided twice a day to guarantee that food was available in abundance. The size of the rations was continuously adjusted to account for changes in the size of tadpoles and the number of individuals in each aquarium since Miyata and Ose (2012) indicated that restricted feeding conditions cause an atrophy of thyroid tissue in a similar manner as TH agonists. The flakes were free of perchlorate according to the manufacturer. Each day, any dead or abnormal tadpoles were removed from the aquaria.

T4 and SP exposures

We used a concentration of 250 µg/L SP hydrate (99.99% trace metals basis, 381225 Aldrich, Sigma-Aldrich, St. Louis, MO, USA) to decrease TH levels. This concentration 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 is found in water bodies in which amphibians breed (Smith et al. 2001; Ortiz-Santaliestra and Sparling 2007).

We increased TH levels by exposing tadpoles to 10 µg/L exogenous L-thyroxine (T4, IRMM468 Sigma-Aldrich, Sigma-Aldrich, St. Louis, MO, USA), a concentration which is known to influence amphibian metamorphosis (Lucas and Reynolds 1967; Mann et al. 2009) and is related to increases in T4 observed in tadpoles responding to stress (Denver 1997, 1998). Tadpoles absorb exogenous T4 directly through their permeable skin (Shi 2000; Tata 2006; Coady et al. 2010). T4 and SP treatments were prepared in 0.1 N sodium hydroxide solutions (0.1 N, S2770 SIGMA, Sigma-Aldrich, St. Louis, MO, USA) buffered with 0.1 N muriatic acid solutions as solvents. Solutions were added to the aquaria to achieve a concentration of 10 µg/L T4 and 250 µg/L SP, respectively. A clean solution of 0.1 M sodium hydroxide solution buffered with 0.1 M muriatic acid solution was added to the control aquaria to control for any effect of solvents addition. Water was changed every second day and fresh SP and T4 were added, which is frequent enough to maintain a constant hormone and perchlorate level, in accordance with the standard procedure for chemical and hormonal addition (Goleman et al. 2002a, 2002b; Iwamuro et al. 2003; Rot-Nikcevic and Wassersug 2004).

Processing of specimens

Developmental stage was determined by evaluating the status of key morphological features typical of specific developmental stages, as detailed in Gosner (1960). The age describes the larval duration in days after hatching. Onset of metamorphosis was defined

by the emergence of at least one forelimb (Gosner Stage 42; Gosner 1960). End of metamorphic climax was defined by the complete resorption of the tail (Gosner Stage 46; Gosner 1960).

The snout vent length (SVL) of the 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). At the end of the experiment, metamorphs were euthanized with 200 mg/L of tricaine methanesulfonate ([MS-222], Ethyl 3-aminobenzoate methanesulfonate, E10521 ALDRICH, Sigma-Aldrich, St. Louis, MO, USA) buffered with 200 mg/L of sodium bicarbonate (Sodium bicarbonate, S5761 SIGMA, Sigma-Aldrich, St. Louis, MO, USA) (Stuart et al. 2007) and transferred into ethanol (70%) for further measurements.

Body condition

We estimated body condition (i.e., energy stores) at the onset of metamorphosis and in juvenile froglets by calculating the scaled mass index (SMI) following the procedure outlined by Peig and Green (2009) for each individual. The SMI is a measure of the body condition of an individual as it accounts for the allometric relationship between mass and body structure measures (i.e., SVL) and standardizes each measure so that direct comparisons among individuals can be made (Peig and Green 2009, 2010; MacCracken and Stebbings 2012). The SMI was considered as an accurate condition index in anuran larvae (MacCracken and Stebbings 2012; Dittrich et al. 2016; Ruthsatz et al. 2018b). A high SMI suggests larger energy storages and thus, a good body condition.

SMR

Respiration measurements were made on 8 randomly chosen, late-stage tadpoles (Gosner Stages 39–41) and on 8 post-metamorphic froglets from each aquarium, in total on 192 individuals ($n = 96$, late stage tadpoles; $n = 96$, post-metamorphic froglets) (Orlofske and Hopkins 2009). Animals were not fed 48 h prior to and during the measurement of SMR; thus, tadpoles were in a post-absorptive state (Orlofske and Hopkins 2009). Oxygen consumption was measured by closed respirometry conducted during the natural activity phase between 09:00 and 21:00 h to control for the influence of natural circadian rhythms on respiration (Orlofske and Hopkins 2009). Larvae were placed in respirometers consisting of 30 mL beakers containing 30 mL (minus the volume of the animals) of autoclaved tap water to exclude microbial oxygen consumption with respective T4 and SP concentrations as experienced by individuals during development. Froglets were placed in air-filled respirometers consisting of 30 mL beakers (minus the volume of the animals) due to their transition to lung respiration. Each respirometer was equipped with a fiber optic sensor (Oxygen Dipping Probe DP-PSt7; PreSens Precision Sensing GmbH, Regensburg, Germany) connected to a multichannel oxygen measuring system (Oxy 4 mini; PreSens Precision Sensing GmbH, Regensburg, Germany) and sealed with an air tight rubber plug. O_2 concentration was recorded every 15 s and measured as $O_2 \times L^{-1}$ mL. Prior to each trial, the O_2 fiber optic sensors were calibrated using air-saturated water and a factory-set zero oxygen calibration point at the developmental temperature. Water temperature was controlled by the continuous mixing of the water bath. Oxygen consumption was measured for every animal for 20 min. Empty (control) chambers were run simultaneously in every trial and values were adjusted accordingly. We ensured that $<10\%$ of total O_2 was removed during the measurements to avoid impediment of respiration at low O_2 saturation levels. At the end of the measurements, each animal was removed and its SVL and blotted wet body mass were determined. Tadpoles were placed back into rearing containers.

Energetic costs during metamorphic climax

After reaching the onset of metamorphosis at Stage 42 (Gosner 1960), two metamorphosing individuals were collected from each tank for respirometry using the same respirometer equipment and software parameters described above (Oxy 4 mini; PreSens Precision Sensing GmbH, Regensburg, Germany). In total, 24 individuals (3 treatments $\times$ 4 aquaria $\times$ 2 individuals) were measured during metamorphic climax. Unlike the previous SMR measurements, 12 h respirometry trials were repeated until the completion of metamorphosis when the tail was fully resorbed at Stage 46 (Gosner 1960; Orlofske and Hopkins 2009). No fasting prior to the O_2 mL $\times$ h^{-1} measurements was needed because tadpoles stop feeding due to the remodeling of mouthparts and digestive tract during metamorphosis (Hourdry et al. 1996). Prior to the measurements, tadpoles were rinsed and drained to remove excess moisture and wet mass was recorded to the nearest $\pm$ 0.001 g. Respirometer chambers consisted of 1000 mL sealed glass culture bottles filled with 950 mL (minus the volume of the animals) well aerated, autoclaved, dechlorinated tap water with respective T4 and SP

concentrations. To provide a ramp for the tadpoles to emerge from the water to facilitate air breathing, a 3.0 cm x 6.5 cm piece of glass was placed against the inner side of the chamber. After each 12 h respirometry trial, tadpole developmental stage and mass, SVL, and TL were recorded. Before the start of the next 12 h trial, the water in each chamber was replaced. Each trial was started at approximately the same time (09:00–10:00 h and 21:00–22:00 h). When individuals completed metamorphosis at Gosner Stage 46 (complete resorption of the tail; froglets sit on the ramp), they were removed from the chamber and SVL was measured (i.e., total length since the tail was resorbed). After removing the tadpoles from the chambers, they were rinsed and drained to remove excess moisture and finally weighed to the nearest ± 0.001 g for body condition calculations. The total duration of metamorphic climax was recorded in hours.

Post-metamorphic performance

After completing metamorphosis at Gosner Stage 46, all surviving tadpoles were transferred into separate aquaria containing a small amount of water to avoid desiccation and placed in a climate chamber maintained at 22 ± 0.1°C, representing an average temperature commonly experienced in the field. Froglets were fed *ad libitum* with adult *Drosophila melanogaster* for 7 days prior to being subjected to the performance trials. Exposure to T4 and SP was stopped after completion of metamorphosis. Prior to the performance tests, the metamorphs were measured for SVL to the nearest 0.5 mm and blotted wet body mass to the nearest ± 0.001 g for body condition calculations.

We measured jumping ability and sprint speed of newly metamorphosed frogs to test if the alteration of TH levels experienced during the larval stage would affect juvenile locomotory performance. Both traits can influence escape from predators and foraging success in this species, and hence can provide a functional link between selection of diet and thermal environment and fitness in juvenile and adult stage (Àlvarez and Nicieza 2002). We placed froglets on a clean, flat surface (0.06 m x 3.00 m) bordered by 30 cm high walls and chased them with a probe by lightly prodding at the urostyl (posterior part) to induce an escape response (Beck and Congdon 2000; Àlvarez and Nicieza 2002). Measurements of jumping ability were conducted according to the procedure of Àlvarez and Nicieza (2002): to assess jumping ability, we recorded a total of five jumps per individual using two response variables: (1) maximum jump distance, defined as the length of the longest leap and (2) mean jump distance, defined as the mean length of the five leaps. Measurements of sprint speed were conducted according to the procedure of Beck and Congdon (2000): The sprint speed, that is, the distance covered in the first 30 s of a trial, was measured twice on every individual. The mean of the two measurements was used in the analysis. After the experiments, froglets were euthanized with MS-222 and mass and SVL were measured. Froglets were transferred into ethanol (70%) for further analyses.

Data preparations

SMR

Prior to statistical analysis, we plotted the O_2 consumption rate of each tadpole over time and visually assessed activity peaks to exclude them for the determination of SMR (Orlofske and Hopkins 2009). The SMR was expressed in mL as $O_2 \times h^{-1} \times mg^{-1}$ (blotted wet body mass) and was determined from the slope of linear least squares regressions of O_2 concentration versus time (Hasting and Burggren 1995; Rowe and Funck 2017). The relationship between O_2 consumption rate ($O_2 \times h^{-1}$ mL) and blotted wet body mass (mg) was described using a simple linear regression according to Orlofske and Hopkins (2009): $\ln(O_2\text{rate}) = a + b \ln(\text{mass})$, where O_2 rate was given in $O_2 \times h^{-1}$ mL, mass in mg and a and b are regression coefficients.

Oxygen consumption rate during metamorphic climax

The first 10 min of each trial (i.e., after starting and after every water change) were excluded from the analyses because tadpoles may have still been recovering from handling. Oxygen consumption rates were interpolated between consecutive respiratory trials for each individual to generate a continuous respiration profile that covered the entire metamorphic period. The mean (±SD) O_2 consumption rate of all tadpoles was 5.136 (±0.271) $O_2 \times h^{-1}$ mL in SP treatment, 4.323 (±0.385) $O_2 \times h^{-1}$ mL in the control group, and 2.719 (±0.493) $O_2 \times h^{-1}$ mL in T4 treatment. To estimate the total O_2 consumed (mL) throughout metamorphic climax, the O_2 consumption rate was calculated by multiplying the O_2 consumption rate (mL $\times h^{-1}$) by the corresponding time interval (h). Total oxygen consumption was converted to Joule (J) using a conversion factor of 18.8 J × mL^{-1} O_2, due to the combustion of mainly protein during metamorphosis(Schmidt-Nielsen 1990) to determine total energy expenditure.

Energy expenditure and allocation during metamorphic climax

Total energy expenditure was divided into maintenance costs and developmental costs following the procedure according to Beck and Congdon (2003): The slope and intercept of the regression of the O_2 consumption and blotted wet body mass of late stage tadpoles (Gosner Stage 39-41) provided the values for the constants used in an integration to calculate maintenance costs over time. The integration assumed a linear decrease in mass over the course of metamorphosis and an exponential relationship between mass and SMR (Orlofske and Hopkins 2009). Energy allocated to development was calculated by subtracting maintenance costs from total energy expenditure during metamorphic climax.

Statistical analyses

For all statistical tests R 3.4.1 (R Development Core Team 2007), Windows was used. Before the analysis, all independent variables in the models were tested for possible correlations using Spearman's rank correlation (cor.test function). Consequentially, variables were included in statistical analysis when the correlation was significant but well below the suggested threshold of 0.7 for eliminating variables (Fielding and Haworth 1995; Chin 1998) (Supplementary Table S1–S3).

Data were analyzed using linear mixed-effect models [lme, Type III model, covariance type: variance components, REML (restricted maximum likelihood) method for parameter estimation, 100 iterations (Bates and Sarkar 2007)], entering "Treatment" (T4, SP, and Control) as fixed factor. "Growth, development, and energetics at onset of metamorphosis" (as measured by age, body mass, SVL, SMR, and SMI), "Energetics during metamorphic climax" (as measured by % change in wet mass, duration, metabolic rate, total energy used, maintenance costs, and % developmental costs), "Post-metamorphic performance and body condition" (as measured by maximum jump distance, average jump distance, mean sprint speed, SMI, SMR, body mass, and SVL) were used as dependent variables in separate models (Appendix Table A1). *P*-values were obtained from likelihood-ratio tests, which compared the models with the respective null-model (Crawley 2007). To address dependencies in the data, the variable "aquarium" was included as a random factor. Residuals of each model were visually checked for normal distribution (QQ-plots). *N* refers to the total number of analyzed tadpoles, *n* refers to the total number of analyzed aquaria linear mixed-effect models followed by *post hoc* comparisons with Bonferroni correction (Tukey's test; Tukey Honestly Significant Difference function, multcomp package, version 1.2-13) to compare all possible pairwise combinations of treatments when overall tests were significant (Appendix Table A1). We performed linear regressions on variables of metamorphic climax according to Orlofske and Hopkins (2009) and on variables of post-metamorphic stage (Appendix Fig. A2).

Results

O_2 consumption rate

Tadpoles in late stages (Gosner Stages 39-41) ranging in mass from 375 to 425 mg (Control), 467 to 588 mg (SP), and 172 to 251 mg (T4) were used for O_2 consumption rate measurements. Mass and O_2 consumption rate were negatively correlated in all treatments. The equation approximating the relationship between mass and O_2 consumption rate was: $\ln(O_2)=7.82 - 1.04\ln(\text{mass})$ in SP treatment ($R^2 = 0.435$, $P \leq 0.001$, $n=8$), $\ln(O_2) = 1.81–0.07\ln(\text{mass})$ in the control group ($R^2 = 0.404$, $P=0.005$, $n = 8$), and $\ln(O_2) = 0.99–0.02\ln(\text{mass})$ in T4 treatment ($R^2 = 0.491$, $P= <0.001$, $n=8$).

Metamorphic and physiological traits at the onset of metamorphosis

Body mass, SVL, and age were correlated but were included in the analysis for better comparisons with related studies (Supplementary Table S1).

There were significant treatment effects on age, SVL, body mass, SMI, and variance of SMR, but no effects on (mean) SMR (Appendix Table A1). Thus, altered TH levels have an effect on metamorphic traits and body condition at the onset of metamorphosis. Larvae exposed to SP were significantly the largest, heaviest, and oldest animals at the onset of metamorphosis compared to Control and T4 animals (Appendix Table A1, Fig. 1A–E). SMI was significantly highest in control animals compared to T4 and SP animals. Consequently, altered TH levels reduced body condition and thus, the energy storage in the larvae at the onset of metamorphosis to different extent (Appendix Table A1, Fig. 1D).

Survival (±SD) from the start of the experiments (Gosner Stage 25) to the onset of metamorphosis (Gosner Stage 42) in the treatment groups was: Control: 91.66 ± 2.49%; SP: 98.32 ± 3.33%, and T4: 81.67 ± 6.67%. Therefore, only high TH levels led to significantly reduced survival as compared to control and SP treatments (Appendix Table A1, Fig. 1F).

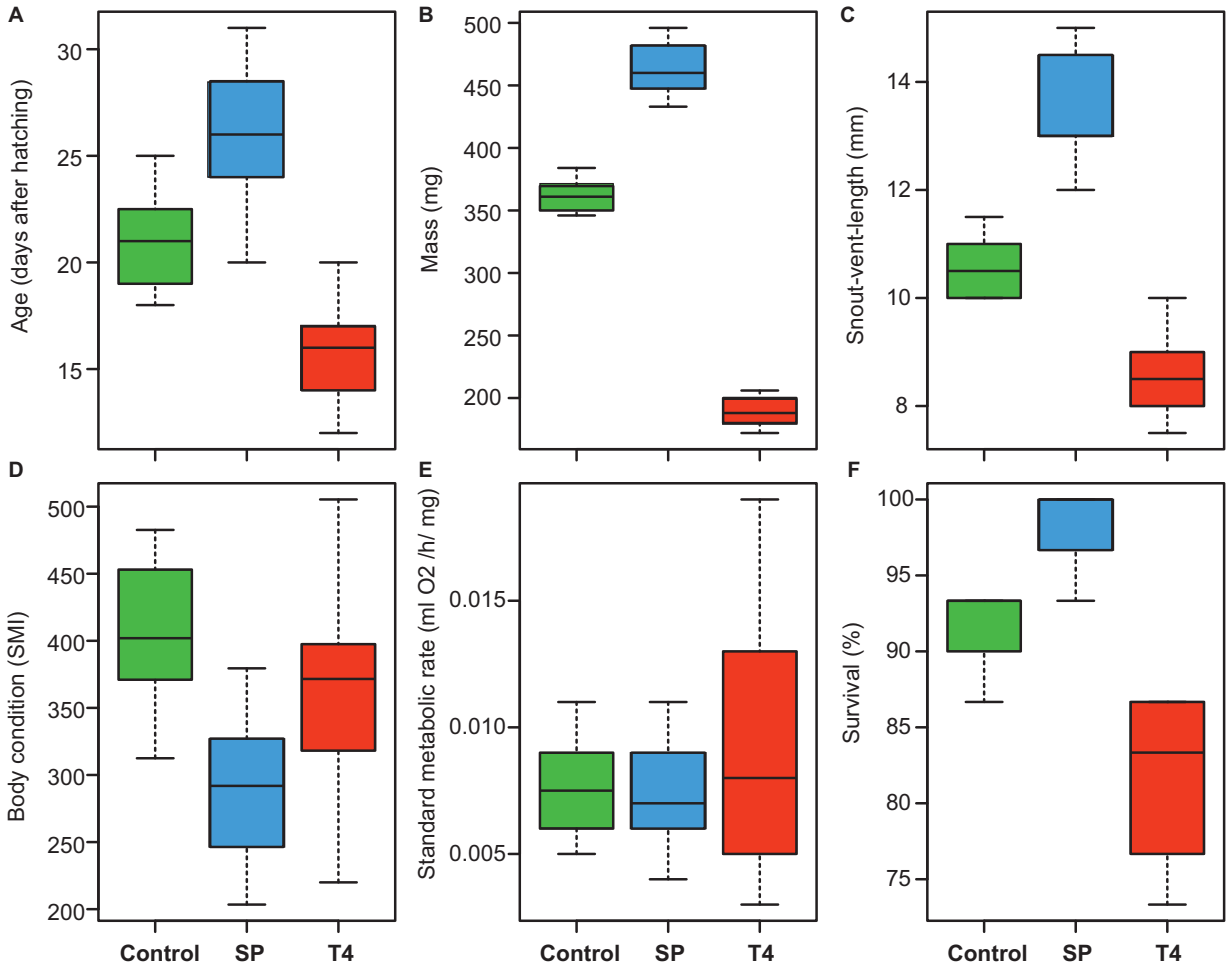

Fig. 1 Effects of altered TH levels on **(A–C)** metamorphic traits $N(n)$=163(12), **(D)** body condition $N(n)$=163(12), **(E)** SMR $N(n)$=96(12), and **(F)** survival ($n = 12$) in tadpoles of the common frog (*R. temporaria*) at the onset of metamorphosis. Centered bars: SP, low TH levels. Bars to the left: Control group, Bars to the right: T4, high TH levels, Boxplot characteristics: Bar = median, Box = interquartile range, $N(n)$ = total number of studied individuals (total number of aquaria).

Energetic costs and energy allocation during metamorphic climax

Duration of metamorphosis and total energy expenditure were correlated (Supplementary Table S2), but were included in the analysis for better comparisons with related studies (Orlofske and Hopkins 2009).

The duration (h) of metamorphic climax differed significantly between both treatments and control groups (Appendix Table A1, Fig. 2A) with animals from the SP treatments having the longest metamorphic climax (mean duration: 203.8 ± 9.6 h) and those from T4 the shortest (mean duration: 62.5 ± 6.6 h). SMR ($O_2 \times h^{-1} \times mg^{-1}$ mL) during climax was significantly higher in animals in the T4 treatment and revealed a higher variance compared to the SP treatment and the control animals (Appendix Table A1, Fig. 2B). Mass loss during metamorphic climax was highest in T4 and control animals and was significantly lower in SP treatment (Fig. 2C, Appendix Fig. A1).

The O_2 consumption rate of individual tadpoles and treatments varied throughout climax, but showed no definitive pattern with respect to the developmental stage or body size (Fig. 3). In contrast, total O_2 consumption increased linearly throughout climax for all treatments and in the control group (Fig. 3). However, the slopes of the relationships varied among treatments and control group (Fig. 3). As a result, the total amount of energy used to complete metamorphic climax also varied considerably (Appendix Table A1, Fig. 2D). Animals from SP treatment used six times more energy during metamorphic climax than animals from T4 treatment. Compared to control animals, tadpoles exposed to T4 could allocate 24% less energy to development during metamorphic climax.

When total energy used during metamorphic climax was portioned into maintenance costs and development, we found that, on average, the majority

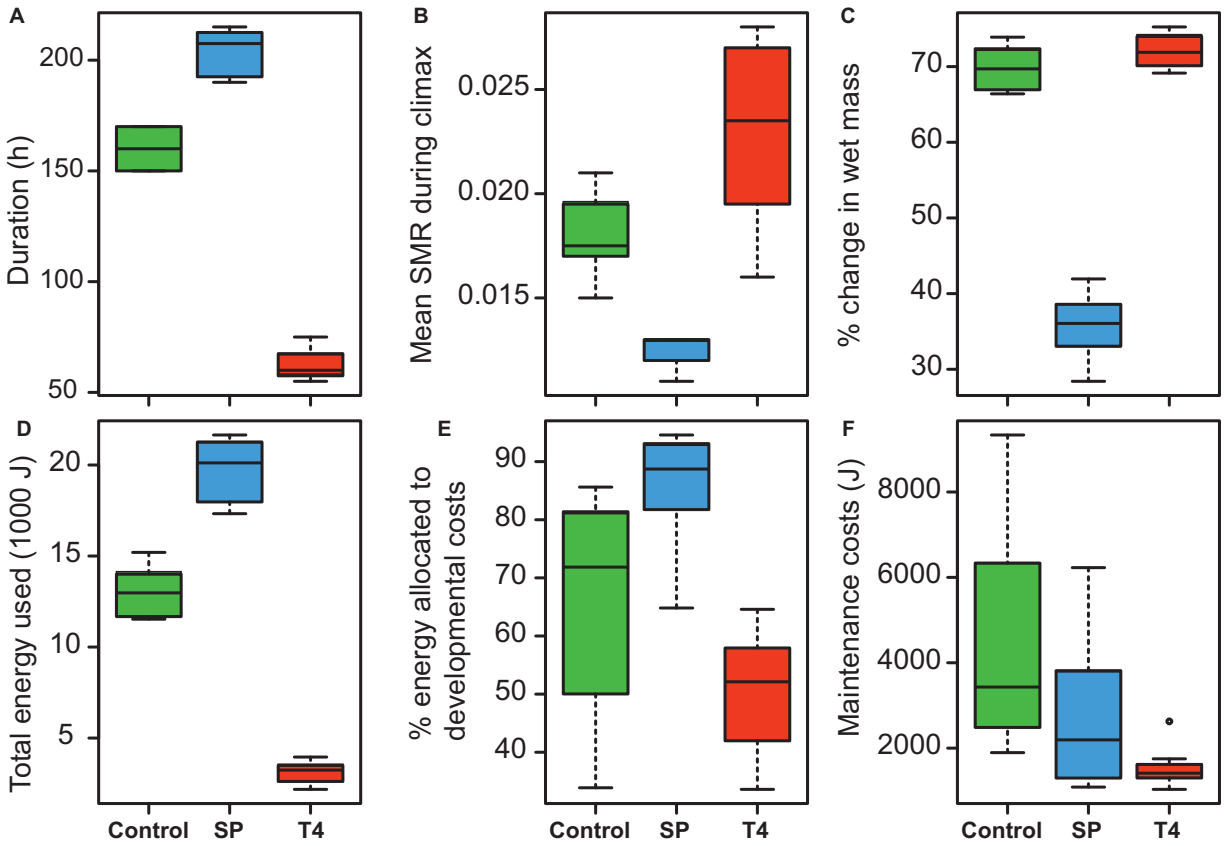

Fig. 2 Effects of altered TH levels on energetics during metamorphic climax in tadpoles of the common frog (*R. temporaria*). **(A)** duration of metamorphic climax (h), **(B)** mean SMR during metamorphic climax (O_2/h/mg mL), **(C)** % change in wet mass, **(D)** total energy used during metamorphic climax (kJ), **(E)** % of total energy used during metamorphic climax allocated to developmental costs, and **(F)** maintenance costs (J) during metamorphic climax. Centered bars: SP, low TH levels. Bars to the left: Control group, Bars to the right: T4, high TH levels, Boxplot characteristics: Bar = median, Box = interquartile range, *N*(*n*) =24(12), where *N*(*n*) = total number of studied individuals (total number of aquaria).

of energy used during metamorphic climax was for development in all groups. Whereby individuals from SP treatment allocated significantly the largest percentage and those from T4 treatment allocated the smallest percentage to developmental costs (Appendix Table A1, Fig. 2F, Fig. 4). Animals from SP treatment allocated 40% more energy to development than those from the T4 treatment. Compared to control animals, tadpoles exposed to T4 used 76% less energy during metamorphic climax due to the 61% reduction in the duration of metamorphosis.

In the SP treatment, the total energy used during metamorphic climax increased linearly with increasing duration of metamorphic climax but this was not the case in T4 animals. Mass at the onset of metamorphosis was negatively related to percent of energy allocated to development in T4 treatment, but not in SP animals nor in the control group (Appendix Fig. A2). Mass at the onset of metamorphosis was not related to total energy used during climax in any group (Appendix Fig. A2).

Post-metamorphic performance and body condition

Body mass, SVL, and age were correlated but were included in the analysis for better comparisons with related studies (Supplementary Table S3).

Analogous to the onset of metamorphosis, there were significant differences between the treatments relating to age, SVL, body mass, and SMI in post-metamorphic froglets (Appendix Table A1, Fig. 4). Compared with SMR at the onset of metamorphosis, SMR of post-metamorphic froglets was significantly reduced in SP compared to Control and T4 animals (Appendix Table A1, Fig. 5E). Froglets that experienced an altered TH status as larvae had a significantly reduced body condition compared to control froglets (Appendix Table A1, Fig. 5D). Therefore, altered TH levels reduced body condition and thus, the energy stores in both larvae at the onset of metamorphosis and post-metamorphic froglets, indicating a carry-over effect in relation to size of energy stores. The mean (±SD) survival from the start of the experiment

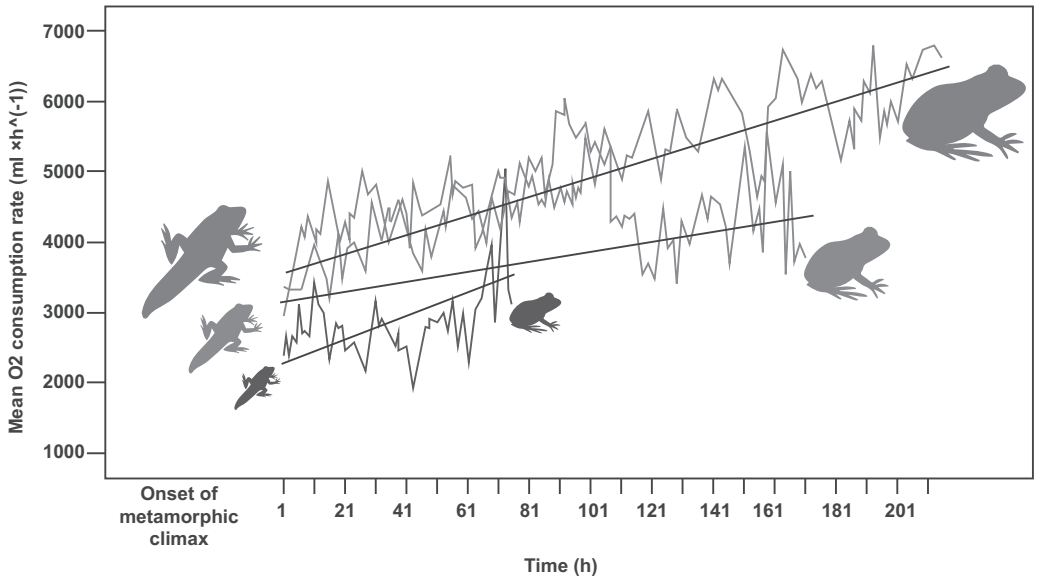

Fig. 3 Mean energy metabolism during metamorphic climax in tadpoles of the common frog (*R. temporaria*) and comparative body size at the onset and after completion of metamorphic climax at different TH levels (dotted: SP treatment (low TH levels); black: Control group; gray: T4 treatment (high TH levels)). Mean O_2 consumption rate (mL $\times h^{-1}$). Dotted black line: SP. Continuous black line: Control group. Gray line: T4. $N(n)$=24(12). Regression lines for linear increase of total O_2 consumption rate (mL $\times h^{-1}$) during metamorphic climax: SP: Total O_2 = 4.44 × time − 14.56, R^2= 0.989, $P < 0.001$. Control: Total O_2 = 5.23 × time − 53.41, R^2= 0.987, $P < 0.001$. T4: Total O_2 = 2.67 × time + 0.382, R^2 = 0.897, $P < 0.001$. $N(n)$=total number of studied individuals (total number of aquaria).

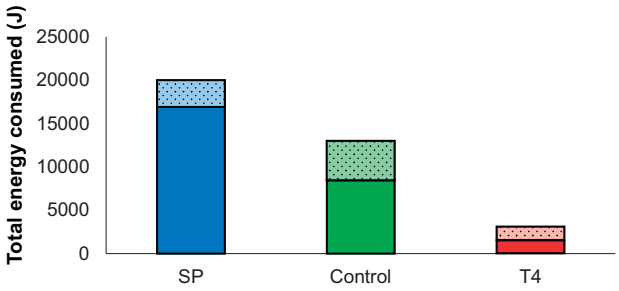

Fig. 4 Mean total energy consumed during metamorphic climax in tadpoles of the common frog (*R. temporaria*) at altered TH levels. Bar to the left: SP treatment = low TH levels. Centered bar: = control group. Bar to the right: T4 treatment = high TH levels. Filled segments for energy allocated to developmental costs (DC). Dotted segments for energy allocated to maintenance costs. Energy for DC: SP = 85.8%, Control = 65.8%, and T4 = 50.3%.

(Gosner Stage 25) to 7 days after completion of metamorphosis was: SP: 76.7 ± 3.3%, Control: 63.3 ± 3.3%, and T4: 56.7 ± 3.3%. Analogous to the onset of metamorphosis, only increased TH levels led to significantly reduced survival as compared to Control and SP treatments (Appendix Table A1, Fig. 5F).

Mass and SVL were significantly correlated (Supplementary Table S3). Therefore, we used mass for linear regressions on performance traits according to Àlvarez and Nicieza (2002). Post-metamorphic performance was significantly affected by treatment (Appendix Table A1). Animals exposed to T4 had decreased jumping ability (i.e., average and maximum jump distance) and a reduced sprint speed compared to Control and SP animals (Appendix Table A1, Fig. 5A–C). Performance traits were not correlated to mass within the treatments (Appendix Fig. A3A–C) but across all tested individuals (Appendix Fig. A4A–C). This suggests that differences in performance may have resulted from size differences caused by altered TH levels during the larval stage. Besides this strong size effect, there might be size independent effects which we were not able to evaluate pick up because of the highly diverse body sizes within treatment groups. Froglets which experienced decreased TH levels as larvae performed better than those within Control and T4 groups. We found the poorest performance in T4 animals. Consequently, altered TH levels caused persistent effects on metamorphic and energetic traits and, thus, led to carry-over effects on performance of juvenile froglets.

Discussion

Amphibians are the sentinels of global change because of their sensitivity and their need to use both aquatic and terrestrial environments to complete their life cycle (James and Semlitsch 2011). Environmental stressors have been shown to alter TH levels in amphibian larvae with consequences for energy portioning among

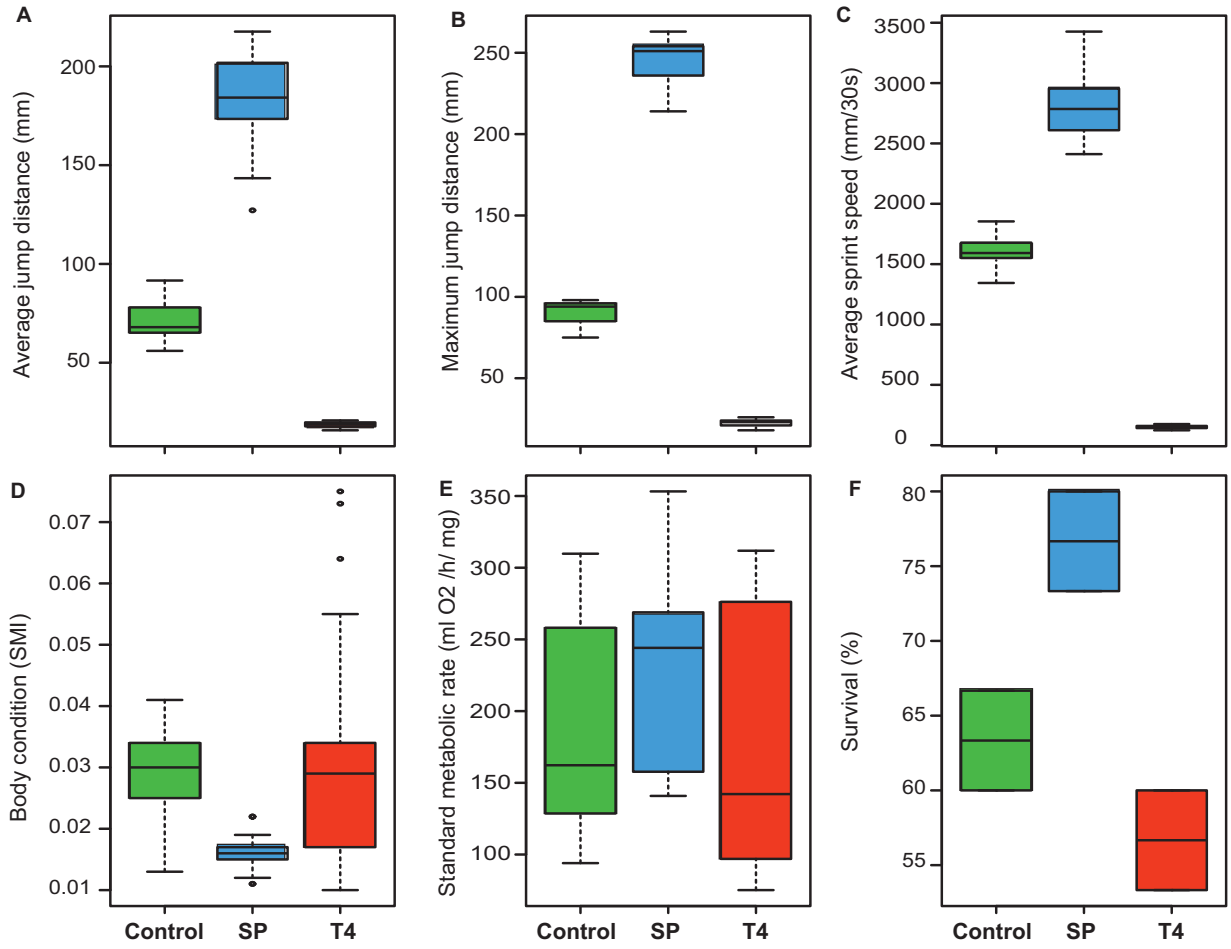

Fig. 5 Effects of altered TH levels on (**A–C**) post-metamorphic performance $N(n)$= 119(12), (**D**) body condition $N(n)$=119(12), (**E**) standard metabolic rate $N(n)$=96(12), and (**F**) survival $n = 12$ in froglets of the common frog (*R. temporaria*) 7 days after completion of metamorphosis. Bars to the left: SP, low TH levels. Centered bars: Control group, Bars to the right: T4, high TH levels, Boxplot characteristics: Error bar = median, Box = interquartile range, Dots = outliers, minimum and maximum values.

development, growth, and metabolism (Ruthsatz et al. 2018b). Differences in energy allocation to development during metamorphosis may reduce juvenile performance and body condition which, in turn, impact survival and thus, fitness. Therefore, it is essential to understand how different environmental stressors affect energetics during and after amphibian metamorphosis and juvenile fitness by altered TH levels (Cary Coyle and Karasov 2010). This study demonstrates how changes in energy allocation caused by altered TH levels (associated with poor conditions in a larval habitat), can carry over across metamorphosis and alter juvenile performance, with significant influence on fitness.

Getting off to a bad start: altered TH status affects metamorphic traits, survival, and energy stores at the onset of metamorphosis

Our results confirm those from previous studies demonstrating that high (low) TH levels increase (decrease) developmental rate in amphibians resulting in younger (older) but smaller (larger) individuals at the onset of metamorphosis (Shi 2000; Bulaeva et al. 2015). Very few studies, however, have investigated the mechanistic underpinnings of altered physiological mechanisms (i.e., SMR and energy stores) associated with altered TH levels as caused by environmental stressors. Since THs are the major triggers of energy metabolism and are positively correlated with metabolic rate (McNabb and King 1993; Rowe et al. 1998; Burraco and Gomez-Mestre 2016) including maintenance costs (Orlofske and Hopkins 2009), larvae experiencing environmental stress may show a different capacity to store energy, which in turn impacts their body condition at the onset of metamorphosis. Surprisingly, our results show that differences in age and size at the onset of metamorphosis are not related to SMR but may result from direct impacts of TH status on rates of growth and development. Moreover, altered TH status generally reduced the ability of larvae to fuel

energy stores before the onset of metamorphosis. Several studies showed that slower developing larvae had higher lipid reserves (Àlvarez and Nicieza 2002; Scott et al. 2007; Kirschman et al. 2017) and thus, better body condition. In the present study, slow developmental rates in tadpoles exposed to SP did not increase body condition. Changes in body condition may be indicative of alterations in hepatic lipid mobilization, which can occur in response to chemical exposures, or general toxicity associated with both compounds (Kirschman et al. 2017). As in both treatments altered TH levels affected growth, development, and body condition, environmental stressors causing alteration of TH status may lead to disadvantaged starting conditions into metamorphic climax.

Energy allocation during metamorphic climax

Metamorphic climax is a period of profound change in the morphology and physiology of anuran larvae that is entirely supported by stored energy (Crump 1981, Beck and Congdon 2003; Orlofske and Hopkins 2009; Wright et al. 2011; Kirschman et al. 2017) as tadpoles fast due to a reorganization of the digestive system and mouthparts (Pandian and Marian 1985; Hourdry et al. 1996; Beck and Congdon 2003; Orlofske and Hopkins 2009). We suggest that exposure to environmental stressors (causing an alteration in TH status) can alter the allocation of stored energy to development during metamorphic climax and thus, may alter energetic efficiency of metamorphosis.

As developmental rate determines the total energy needed for physiological and morphological reorganization and maintenance energy expenditure during metamorphic climax, tadpoles with high TH levels in total spend least energy whereas tadpoles with low TH status spend most. However, depending on the TH status the percentage allocated to development differs. For instance, tadpoles with low TH status could allocate about 60% more energy to development than those with high TH status and roughly one-third more than tadpoles in the control group. Tadpoles with high TH levels need more energy to cover their maintenance costs even though as much energy as possible should be allocated to development during this stage of reorganization. Differences in energetic costs may be related to the significant differences in sizes between animals in the different treatments. Beck and Congdon (2003) and Orlofske and Hopkins (2009) found similar, negative relationships between developmental costs and size at the initiation of metamorphic climax in the southern toad (*Bufo terrestris)* and in the pickerel frog (*Lithobathes palustris*). Therefore, individuals with a large body size at the onset of metamorphosis as found in tadpoles exposed to SP have a significant physiological advantage over smaller tadpoles during the metamorphic climax because they complete metamorphosis more efficiently (i.e., in total use proportionally less energy for metamorphic climax and have a lower SMR) than their smaller conspecifics exposed to T4 (Pandian and Marian 1985, Orlofske and Hopkins 2009). This prolonged larval development and more efficient use of energy stores comes at the ecological cost of increased risks of mortality due to higher predation in their aquatic habitat as well as increased risks of desiccation (Lefcort et al. 1998; Kloas and Lutz 2006).

Differences in energetic costs and efficiency of metamorphosis may also be caused by differing status of energy reserves since metamorphosis is fueled by internal macronutrient stores (Beck and Congdon 2003). To meet the energetic demands of these complex reorganizations, larvae oxidize glycogen stores first (Sawant and Varute 1973), followed by lipid stores (from fat body, liver, and tail), and protein stores (primarily from tail; Wright et al. 2011). Scott et al. (2007) emphasized that energy stores acquired in the larval stage are an important contributor to post-metamorphic success and, ultimately, fitness. Therefore, accumulating large lipid stores before the onset of metamorphic climax should be advantageous. In this study, we determined body energy stores by calculating the SMI for body condition, indicating that altered TH levels decrease internal energy stores. However, measurements of the size of fat body or the liver may be more precise in terms of lipid store size. In a previous study on *Xenopus laevis*, we found that high TH levels reduced the amount of fat stored in the liver (Ruthsatz et al. 2018b). We suggest that differences in SMR caused by altered TH levels during metamorphic climax and thus, energy allocation to maintenance costs might have contributed to a reduced energetic efficiency in tadpoles with high TH levels. In particular, internal lipid stores may be reduced in larvae exposed to unfavorable conditions as stress (i.e., high stress hormone and TH levels), necessitating mobilization of macronutrients from energy stores to meet increased energy demands before the onset of metamorphosis (Crump 1981; Sapolsky et al. 2000; Kirschman et al. 2017). Moreover, Kirschman et al. (2017) showed that stressed larvae oxidized greater amounts of protein stores early in metamorphic climax, as they may need to shift to protein stores to fuel metabolism earlier when lipids stores were exhausted due to an

increased lipid catabolism by T4 (Picon and Bouhnik 1968; Sawant and Varute 1973), which is unusal for amphibians (Scott et al. 2007).

Post-metamorphic performance

Pechenik et al. (1998) emphasized that metamorphosis is not "a new beginning." In organisms with complex life cycles such as anurans, factors in the larval environment have strong carry-over effects on juveniles and adults (Smith 1987; Berven 1990; Goater 1994; Altwegg and Reyer 2003; Pechenik 2006; Scott et al. 2007, Morey and Reznick 2001) and thus, important fitness consequences and population-level impacts (Bouchard et al. 2016). One of the most important traits affecting success (survival and reproduction) in terrestrial habitats for anurans is body size (reviewed in Chelgren et al. 2006). Juvenile size is positively linked to both juvenile survivorship and adult fitness. Larger size at emergence has been correlated with increased dispersal and survival and earlier or larger size at first breeding (reviewed in van Allen et al. 2010; Chelgren et al. 2006). The consequences of size at metamorphosis for subsequent growth and survival can be mediated, at least in part, by locomotor performance and storage of energy reserves (Àlvarez and Nicieza 2002). Moreover, the consequences of variation in these two traits are most likely interdependent.

In our experiment, carry-over effects on juvenile performance were mainly mediated by variation in body size at metamorphic climax. Juvenile body mass was positively related to jumping ability and sprint speed. Our findings are in accordance with those of previous studies indicating that size at metamorphosis determines locomotor performance in juvenile frogs: John-Alder and Morin (1990) found that size at metamorphosis was positively correlated with jumping ability in the Fowler's toad (*Anaxyrus woodhousii*, formerly *Bufo woodhousii)*. Furthermore, Goater et al. (1993) found a positive relationship of size and burst speed in the common toad (*Bufo bufo*). Beck and Congdon (1999) demonstrated a positive relationship of size and sprint speed, and endurance in the southern toad (*B. terrestris*). As discussed above, energetic efficiency differed between treatments due to differences in size but probably also due to differences in allocation of internal macronutrient stores. Nevertheless, we assume that juvenile performance may also be influenced by a size-independent effect of achieved TH status. Under increased maintenance costs due to high TH levels, larvae reduce tissue production during climax (i.e., energy allocated to developmental costs) and may instead allocate energy to maintenance. Furthermore, Kirschman et al. (2017) demonstrated that stressed larvae deplete their lipid stores very fast and proceed by depleting protein stores for meeting energy demand during climax. Sustained protein catabolism may present challenges for anuran larvae, because protein stores available for oxidation include muscle and newly formed adult structures crucial for terrestrial survival and juvenile locomotor performance (Pandian and Marian 1985; Beck and Congdon 2003; Orlofske and Hopkins 2009; Wright et al. 2011; Kirschman et al. 2017). Differences in TH status on energy allocation of macronutrients may act synergistically with a strong size effect to alter juvenile performance but, due to differences in body size within and among treatments, this remains untested here.

Locomotor performance can have a positive influence on dispersal from natal ponds, food acquisition, and predator avoidance (Beck and Congdon 2000; reviewed in Àlvarez and Nicieza 2002). Therefore, tadpoles exposed to environmental stressors which increase TH levels may suffer from difficulties in food acquisition and predator avoidance arising from their poor locomotor performance. Those difficulties may inhibit compensatory growth and storage of energy reserves. Furthermore, Morey and Reznick (2001) found that smaller metamorphs suffered higher mortality rates due to their increased foraging activity to compensate for growth differences and, thus, a reduction of poor larval environment as in froglets exposed to exogenous T4 during the larval stage. In contrast, froglets which experienced low TH levels during the larval stage due to aquatic contamination may indeed reveal a strong juvenile performance and thus, may display an advantage in food acquisition and predator avoidance. Nevertheless, the number of froglets which emerge from the natal pond may be extremely reduced under natural conditions due to their substantially longer duration of metamorphosis.

Conclusion

The present findings for *R. temporaria* emphasize that impact of environmental stressors on the energy allocation to development, growth, and metabolism during and after metamorphosis results in altered costs associated with metamorphic climax and lead to carry-over effects on juvenile performance. Therefore, ongoing global environmental change and anthropogenic disturbances of larval habitats will

result in altered phenotypes at the onset of metamorphosis and relatively higher energetic costs during climax with deleterious consequences for juvenile froglets. Any changes in metamorphic traits and energy allocation caused by exposure of larvae to stress that affect post-metamorphic performance and therefore, survival and growth or delay time to maturity could have important impacts on fitness and population persistence (James and Semlitsch 2011). In addition, in temperate anurans such as the common frog (*R. temporaria*), a strong locomotor performance is also extremely beneficial for accumulating energy reserves which are used for gonad development and for survival during hibernation (Reading and Clarke 1995). Energy reserves are built up after emergence before the onset of the next winter (reviewed in Reading and Clarke 1995; Chen et al. 2011). Therefore, froglets which experienced environmental stress during the larval stage may suffer in two different ways: a small body size reduces locomotor performance which in turn impedes foraging and thus, storage of energy reserves essential for successful hibernation. Our study highlights necessity to investigate how different environmental stressors impact energy allocation to development, growth, and metabolism across life stages and result in carry-over effects. Our results suggest that alteration of the TH levels, especially during the critical developmental window of metamorphic climax, may have direct fitness consequences by impairing juvenile locomotor performance. Indeed, these results provide mechanistic evidence for the often-cited fitness cost of increased developmental rates. Since very little is known about the behavior or mortality of juvenile anurans in the wild (Tarvin et al. 2015), more long-term studies are needed to understand the consequences of altered TH levels as caused by environmental stressors during the larval and early juvenile stages on the phenotype and fitness of the adults. A subsequent physiological-based understanding of how environmental stressors affect the efficiency of anuran metamorphosis and survival in terrestrial stage will help to make better projections of anthropogenic impacts and to develop conservation strategies.

Acknowledgment

The authors thank J. Bank for his experimental advice and technical assistance.

Supplementary data

Supplementary data available at *ICB* online.

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Appendix

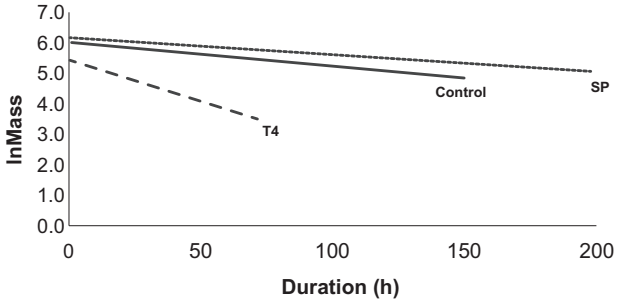

Fig. A1 Mass loss during the duration (h) of metamorphic climax in tadpoles of the common frog (*R. temporaria*) at altered TH levels. Regression lines for linear decrease of mass (mg) during metamorphic climax. Dotted: SP treatment = low TH levels (Slope: −0.002, R^2 = 0.89, $N = 24$). Continuous = Control group (Slope: −0.007, R^2 = 0.93, $N = 24$). Dashed: T4 treatment = high TH levels (Slope: −0.024, R^2= 0.98, $N = 24$).

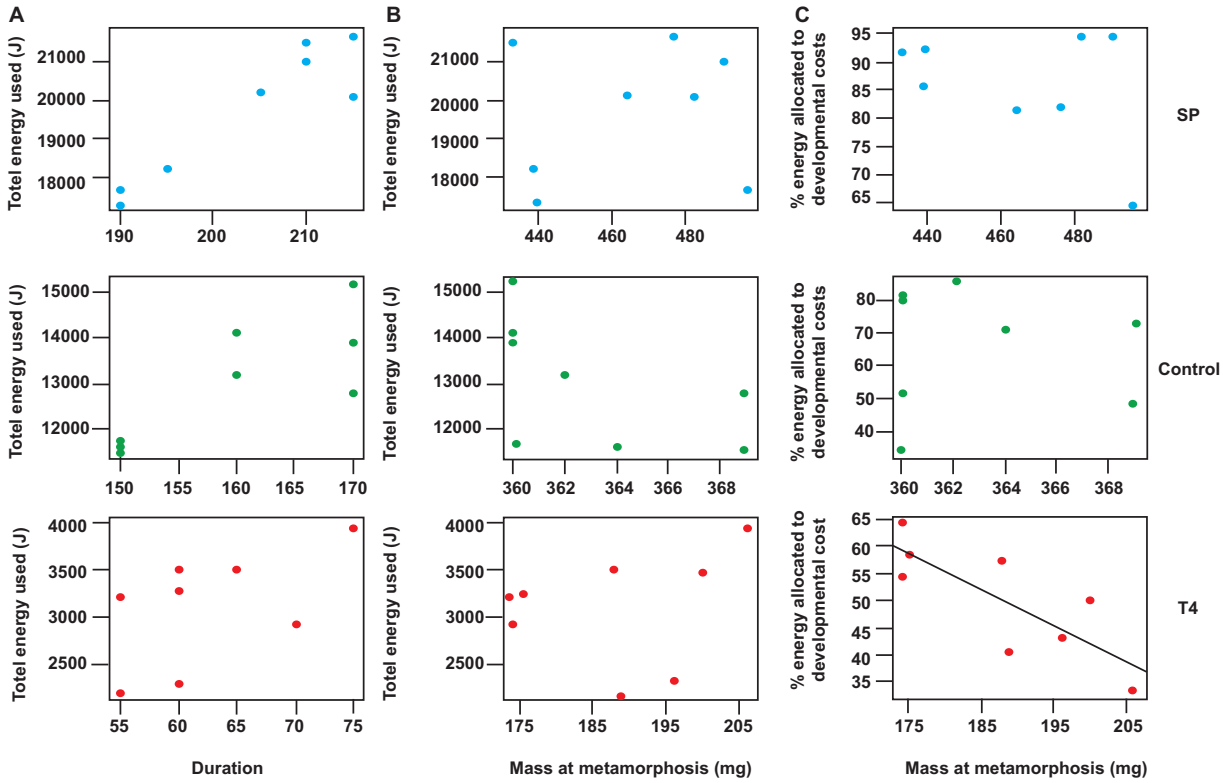

Fig. A2 Linear regressions of variables of metamorphic climax in tadpoles of the common frog (*R. temporaria*) at the onset of metamorphosis (at least one forelimb present, Gosner Stage 42) (Gosner 1960). **(A)** Duration (h) of metamorphic climax and total energy used during metamorphic climax (J). **(B)** Mass at the onset of metamorphosis (mg) and total energy used during metamorphic climax (J). **(C)** Mass at the onset of metamorphosis (mg) and % of total energy used allocated to developmental costs during metamorphic climax. Black dots: SP, low TH levels. Gray dots: T4, high TH levels. White dots: Control group. Significance was set at $P < 0.05$. Regression lines show the significant relationship between two variables. $N = 24$.

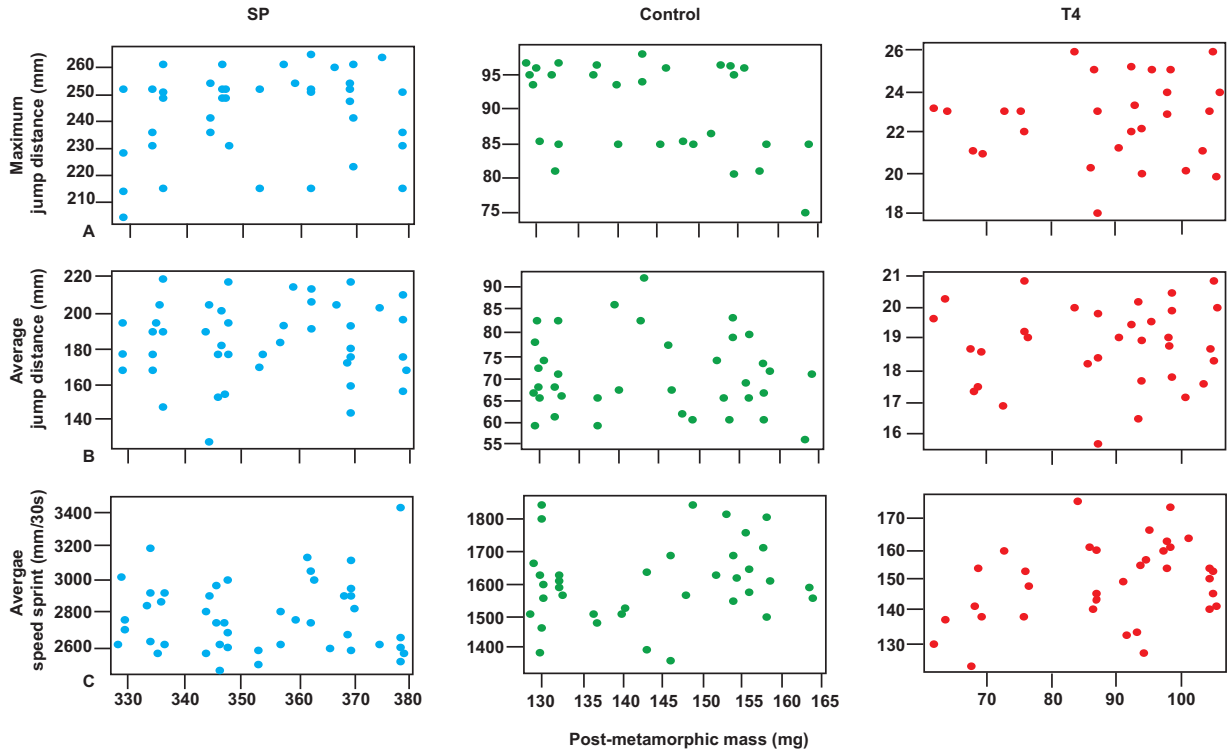

Fig. A3 Linear regressions of variables of post-metamorphic mass (mg) and performance (i.e., (**A**) maximum jump distance (mm), (**B**) average jump distance (mm), and (**C**) average sprint speed (mm/30 s)) in froglets of the common frog (*R. temporaria*) 7 days after completion of metamorphosis. Black dots: SP, low TH levels, $N = 46$. Gray dots: T4, high TH levels, $N = 34$. White dots: Control group, $N = 39$. Significance was set at $P < 0.05$. Regression lines show the significant relationship between two variables.

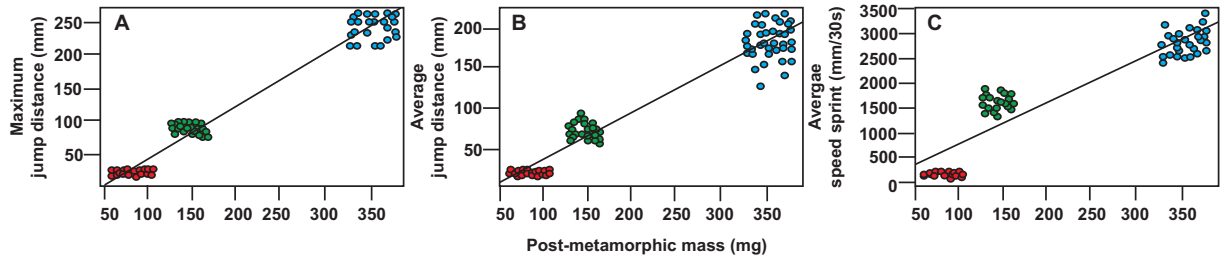

Fig. A4 Linear regressions of variables of post-metamorphic mass (mg) and performance (i.e., (**A**) maximum jump distance (mm), (**B**) average jump distance (mm), and (**C**) average sprint speed (mm/30 s)) stage in froglets of the common frog (*R. temporaria*) 7 days after completion of metamorphosis across treatments. Black dots: SP, low TH levels, $N = 46$. Gray dots: T4, high TH levels, $N = 34$. White dots: Control group, $N = 39$. Significance was set at $P < 0.05$. Regression lines show the significant relationship between two variables.

Table A1 Effects of altered TH levels on metamorphic traits, energetics, survival, and performance in larvae and froglets of the common frog (*R. temporaria*)

		LMM							Tukey's Test (pairwise comparisons)								
		Estimate (SE)							Control: SP			SP: T4			Control: T4		
Developmental stage	Dependent variable	Control	SP	T4	χ^2	df	*P*	*N* (*n*)	Estimate (SE)	z	*P*	Estimate (SE)	z	*P*	Estimate (SE)	z	*P*
Onset of metamorphosis	Mass (mg)	362.00 (1.99)	100.45 (2.76)	−173.20 (2.90)	90.311	2	**<0.001**	163 (12)	100.45 (2.767)	36.31	**<0.001**	−273.66 (2.85)	−95.92	**<0.001**	−173.20 (2.9)	−59.73	**<0.001**
	Age (dah)	20.96 (0.34)	5.12 (0.48)	−4.90 (0.50)	53.611	2	**<0.001**	163 (12)	5.12 (0.48)	10.53	**<0.001**	−10.02 (0.50)	−20.00	**<0.001**	−4.90 (0.50)	−9.62	**<0.001**
	SVL (mm)	10.5 (0.10)	2.99 (0.14)	−1.97 (0.14)	64.63	2	**<0.001**	163 (12)	2.99 (0.14)	21.08	**<0.001**	−4.97 (0.14)	−33.97	**<0.001**	−1.97 (0.14)	−13.31	**<0.001**
	SMI	408.13 (7.34)	−121.39 (10.21)	−50.17 (10.70)	39.44	2	**<0.001**	163 (12)	−121.39 (10.22)	−11.88	**<0.001**	71.22 (10.53)	6.76	**<0.001**	−50.17 (10.71)	−4.68	**<0.001**
	SMR (mL O_2/h/mg)	0.007 (0.001)	−0.0004 (0.001)	0.001 (0.001)	5.14	2	**0.076**	96 (12)	–	–	–	–	–	–	–	–	–
	Survival (%)	91.66 (2.29)	6.66 (3.2)	−9.99 (3.24)	16.57	2	**<0.001**	(12)	6.66 (3.24)	2.05	0.98	−16.66 (3.24)	−5.14	**<0.001**	−9.997 (3.24)	−3.08	**0.006**
Metamorphic climax	% change in wet mass	69.78 (1.29)	−34.07 (1.83)	2.25 (1.83)	48.30	2	**<0.001**	24 (12)	−34.07 (1.83)	−18.62	**<0.001**	36.33 (1.83)	19.85	**<0.001**	2.25 (1.83)	1.23	0.433
	Duration of metamorphosis (h)	160.00 (3.21)	43.75 (4.54)	−97.50 (4.54)	59.70	2	**<0.001**	24 (12)	43.75 (4.54)	9.62	**<0.001**	−141.25 (4.54)	−31.06	**<0.001**	−97.5 (4.54)	−21.44	**<0.001**
	Mean oxygen consumption rate (mL O_2/h) during metamorphosis	0.018 (0.001)	−0.005 (0.001)	0.005 (0.001)	20.66	2	**<0.001**	24 (12)	−0.005 (0.001)	−3.36	**0.002**	0.01 (0.001)	6.43	**<0.001**	0.005 (0.001)	3.06	**0.006**
	Total energy used (J)	13004.1 (463.4)	6701.9 (655.3)	−9898.2 (655.3)	55.06	2	**<0.001**	24 (12)	6701.9 (655.3)	10.23	**<0.001**	−16600.1 (655.3)	−25.33	**<0.001**	−9898.2 (655.3)	−15.1	**<0.001**
	Maintenance costs (J)	4466.9 (653.2)	−1725.5 (923.8)	−2925.2 (923.8)	8.98	2	**0.011**	24 (12)	−1725.5 (923.8)	−1.86	**0.148**	−1199.7 (923.8)	−1.299	0.395	−2925.2 (923.8)	−3.16	**0.004**
	% developmental costs	65.75 (4.834)	20.06 (6.83)	−15.46 (6.83)	17.58	2	**<0.001**	24 (12)	20.06 (6.83)	2.93	**0.009**	−35.53 (6.83)	−5.19	**<0.001**	−15.46 (6.83)	−2.26	0.06
Post−metamorphosis (7 days after completion of metamorphosis)	Average jump distance (mm)	70.90 (5.07)	112.80 (7.14)	−52.11 (7.20)	49.71	2	**<0.001**	119 (12)	112.803 (7.13)	15.80	**<0.001**	−164.91 (7.17)	−22.99	**<0.001**	−52.11 (7.20)	−7.23	**<0.001**
	Maximum jump distance (mm)	90.15 (1.51)	156.08 (2.05)	−67.59 (2.21)	89.31	2	**<0.001**	119 (12)	156.08 (2.05)	75.89	**<0.001**	−223.68 (2.13)	−104.66	**<0.001**	−67.59 (2.21)	−30.49	**<0.001**
	Mean sprint speed (mm/30s)	1610.09 (25.44)	1186.17 (34.58)	−1460.51 (37.57)	81.595	2	**<0.001**	119 (12)	1186.17 (34.58)	34.30	**<0.001**	−2646.69 (36.24)	−73.03	**<0.001**	−1460.51 (37.57)	−38.87	**<0.001**
	Age (dah)	35.82 (0.37)	5.092 (0.514)	−10.64 (0.55)	61.23	2	**<0.001**	119 (12)	5.092 (0.514)	9.89	**<0.001**	−15.73 (0.53)	−29.43	**<0.001**	−10.64 (0.55)	−19.18	**<0.001**
	SVL (mm)	11.41 (0.18)	2.28 (0.24)	−1.14 (0.26)	43.34	2	**<0.001**	119 (12)	2.28 (0.24)	9.23	**<0.001**	−3.43 (0.25)	−13.35	**<0.001**	−1.14 (0.26).	−4.29	**<0.001**
	Mass (mg)	143.12 (2.22)	209.91 (3.03)	−55.30 (3.26)	88.88	2	**<0.001**	119 (12)	209.91 (3.03)	69.26	**<0.001**	−265.22 (3.14)	−84.22	**<0.001**	−55.31 (3.26)	−16.93	**<0.001**
	SMI	187.29 (11.65)	34.52 (15.88)	−7.519 (17.12)	7.79	2	**0.02**	119 (12)	34.528 (15.88)	2.174	0.075	−50.17 (16.50)	−2.548	**0.029**	−7.519 (17.12)	−0.439	0.899
	SMR (ml O2/h/mg)	0.028 (0.002)	−0.012 (0.003)	0.002 (0.003)	23.66	2	**<0.001**	96 (12)	−0.01 (0.002)	−4.62	**<0.001**	0.01 (0.003)	5.40	**<0.001**	0.002 (0.003)	0.77	0.718
	Survival (%)	63.33 (1.92)	13.33 (2.22)	−6.67 (2.22)	24.91	2	**<0.001**	(12)	13.33 (2.22)	5.99	**<0.001**	−20.00 (2.22)	−8.99	**<0.001**	−6.67 (2.22)	−3.00	**0.007**

Notes: Effects measured at the onset of metamorphosis (at least one forelimb present, Gosner Stage 42) (Gosner, 1960), during metamorphic climax (until complete resorption of the tail, Gosner Stage 46), and 7 days after completion of metamorphosis. χ^2 and P for linear mixed-effects models (LMM), using "treatment" (Control, SP, T4) as the fixed factor; "aquarium" as the random factor. N is the total number of analyzed individual animals, and n is the total number of tested aquaria. Pairwise multiple comparisons were made using Tukey's test as post hoc test with Bonferroni correction. **Bold** indicates significance was set at $P < 0.05$. SP: decreased TH levels, T4: increased TH levels. dah = days after hatching.