

## RESEARCH ARTICLE

# Altered thyroid hormone levels affect body condition at metamorphosis in larvae of *Xenopus laevis*

Katharina Ruthsatz<sup>1</sup> | Kathrin H. Dausmann<sup>1</sup> | Claudia Drees<sup>1</sup> | Laura I. Becker<sup>1</sup> | Lisa Hartmann<sup>1</sup> | Janica Reese<sup>1</sup> | Nikita M. Sabatino<sup>2</sup> | Myron A. Peck<sup>3</sup> | Julian Glos<sup>1</sup>

<sup>1</sup>Institut für Zoologie, University of Hamburg, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany

<sup>2</sup>Department of Life Sciences, Hamburg University of Applied Sciences, Ulmenliet 20, 21033 Hamburg, Germany

<sup>3</sup>Institute for Marine Ecosystem and Fishery Science, University of Hamburg, Olbersweg 24, 22767 Hamburg, Germany

**Correspondence**

Katharina Ruthsatz, Department of Animal Ecology and Conservation, Biocenter Grindel, Universität Hamburg, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany.  
Email: k.ruthsatz@web.de

**Abstract**

Chemical, physical and biological environmental stressors may affect the endocrine system, such as the thyroid hormone (TH) axis in larval amphibians with consequences for energy partitioning among development, growth and metabolism. We studied the effects of two TH level affecting compounds, exogenous L-thyroxine ( $T_4$ ) and sodium perchlorate (SP), on various measures of development and body condition in larvae of the African clawed frog (*Xenopus laevis*). We calculated the scaled mass index, hepatosomatic index and relative tail muscle mass as body condition indices to estimate fitness. Altered TH levels significantly altered the growth, development, survival and body condition in metamorphic larvae in different directions. While exogenous  $T_4$  reduced growth and accelerated development, SP treatment increased growth but slowed down development. Altered TH levels improved body conditions in both treatments and particularly in larvae of the SP treatment but to the detriment of lower survival rates in both TH level altering treatments. The hepatosomatic index was negatively affected by exogenous  $T_4$ , but not by SP treatment indicating a lower lipid reserve in the liver in larvae of  $T_4$  treatment. These altered TH levels as caused by several environmental stressors may have an influence on individual fitness across life, as body condition at the onset of metamorphosis determines metamorphic and juvenile survival. Further research is needed to determine synergetic effects of environmental stressors on TH levels and its effects on physiological traits such as metabolic rate.

**KEYWORDS**

amphibian decline, condition index, developmental plasticity, endocrine disruption, fitness, hepatosomatic index, L-thyroxine, scaled mass index, thyroid hormones, thyrotoxicity

## 1 | INTRODUCTION

Global change exposes wildlife to an array of chemical, physical and biological stressors that arise largely from anthropogenic activity, but also from natural sources (Noyes et al., 2009). A wide range of these stressors has the ability to alter endocrine function in wildlife (Carr & Patiño, 2011). Stressors that alter or disturb endocrine systems are characterized as endocrine disruptors (EDs) (Kloas et al., 2009; Kloas & Lutz, 2006). The impact of EDs in the environment is of special concern in amphibians, which are declining worldwide (Hayes et al., 2006; Stuart et al., 2004). Amphibians have highly permeable skin,

which makes them particularly vulnerable to chemical contaminants (Hayes et al., 2006; Strong, Martin, Jones, Shore, & Halsall, 2017). Furthermore, particularly larval amphibians are limited in their capacity for habitat selection (Sanzo & Hecnar, 2006; Yu, Wages, Cai, Maul, & Cobb, 2013), and are particularly sensitive to EDs due to their critical hormone-regulated development (Hayes, Falso, Gallipeau, & Stice, 2010; Searcy, Snaas, & Shaffer, 2015).

Amphibian metamorphosis is a crucial event in amphibian life history and is driven by several hormones, particularly thyroid hormones (TH) (i.e., triiodothyronine and L-thyroxine [ $T_4$ ]) (Bulaeva, Lanctôt, Reynolds, Trudeau, & Navarro-Martín, 2015; Shi, 2000). Many EDs

target the hypothalamus–pituitary–thyroid axis, which is required for production of THs (Carr & Patiño, 2011). A large number of aquatic contaminants have been shown to disrupt the normal action of THs in amphibians, leading to changes in growth, development and regulation of energy metabolism (Brown & Cai, 2007; Kashiwagi et al., 2009). Pesticides and herbicides, road salt, fertilizers, heavy metals and active pharmaceutical ingredients are known to disrupt TH pathways and production resulting in decreased TH levels (Carr & Patiño, 2011; Fort et al., 2017; Kashiwagi et al., 2009; Ortiz-Santaliestra & Sparling, 2007). Inhibition of TH production pathways results in decreased developmental rates (Bulaeva et al., 2015; Carr et al., 2003) and decelerated energy metabolism (Carr & Patiño, 2011; Fort et al., 2017; Kashiwagi et al., 2009; Ortiz-Santaliestra & Sparling, 2007), with tadpoles metamorphosing at a larger size and older age (Shi, 2000).

An environmental relevant ED is perchlorate ( $\text{ClO}_4^-$ ), which is a goitrogen that inhibits TH synthesis via competitive inhibition of the sodium-iodide symporter (Ortiz-Santaliestra & Sparling, 2007). Because iodide is essential for the production of both  $T_4$  and triiodothyronine, perchlorate may act as a disrupter of amphibian metamorphosis (Ortiz-Santaliestra & Sparling, 2007). Perchlorate salts are strong oxidizers and are widely used as components of fireworks, airbags and currently applied fertilizers (Carr & Patiño, 2011; Schmidt, Schnurr, Wolf, & Braunbeck, 2012; Trumpolt et al., 2005). 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 & Patiño, 2011). Environmental concentrations of perchlorate measured in the field are high enough to inhibit amphibian metamorphosis (Goleman et al., 2002; Goleman, Carr, & Anderson, 2002; Ortiz-Santaliestra & Sparling, 2007; Tietge et al., 2005). In the United States, concentrations from  $3 \mu\text{g}$  to  $30 \text{mg l}^{-1}$  have been found in surface and ground waters (Carr & Patiño, 2011; US Environmental Protection Agency [USEPA], 2004).

Although most environmental contaminants inhibit TH activity or production pathways, some contaminants and other abiotic and biotic environmental stressors appear to enhance TH activity or increase TH levels by the activation of the neuroendocrine stress axis (Dantzer, Fletcher, Boonstra, & Sheriff, 2014; Mann, Hyne, Choung, & Wilson, 2009) and increase of stress hormone levels (Denver, 1997). These stress hormones may synergize with TH resulting in increased TH production (Glennemeier & Denver, 2002; Kulkarni & Buchholz, 2012; Laudet, 2011). Crowding (Ding, Lin, Fan, & Ji, 2015), the presence of predators (Relyea, 2007), food quality and quantity (Courtney Jones, Munn, Penman, & Byrne, 2015), photoperiod (Laurila, Pakkasmaa, & Merilä, 2001), temperature (Vences, Puente, Nieto, & Vieites, 2002) and desiccation risk (Gervasi & Fofopoulos, 2008) may act as environmental stressors that activate the neuroendocrine stress axis resulting in increased TH levels. Therefore, the endocrine system allows for adaptation to stressful conditions resulting in the expression of developmental plasticity. Anuran larvae with high TH levels show increased developmental and metabolic rates and decreased growth rates (Brown & Cai, 2007; Rowe, Kinney, Nagle, & Congdon, 1998; Tata, 2006), which results in shorter larval periods, smaller size at the onset of metamorphosis and higher energetic maintenance costs (Denver, 1998, 2009; Orlofske & Hopkins, 2009). Exposing tadpoles to exogenous THs is an established method to simulate the proximate

effects of environmental stressors on the TH system ultimately inducing developmental plasticity (Denver, 2009; Denver, Glennemeier, & Boorse, 2002; Tata, 2006).

As TH is not only critical for amphibian metamorphosis but also for the regulation of energy metabolism (Frieden, 1981; McNabb & King, 1993; Sheridan, 1994), altered TH levels as caused by several environmental stressors may also affect energetic body condition (i.e., maintenance costs and energy stores) at metamorphosis. Larval anurans fuel the energy required for metamorphosis by resorbing their tail muscle as well as by using stores of fat in the liver, a key organ for lipid storage in ectothermic animals (Bouchard, O'Leary, Wargelin, Charbonnier, & Warkentin, 2016; Jelodar & Fazli, 2012; Sheridan & Kao, 1998). The higher the energy metabolism, and thus, animals' maintenance costs, the less energy can be stored in tail and liver tissue (Orlofske & Hopkins, 2009; Sheridan & Kao, 1998). Anuran larvae with lower tail muscle and liver masses at the onset of metamorphosis have lower energy reserves to cover developmental costs during the metamorphic climax and thus, lower individual fitness in later life stages (Berven, 1990; Bellakhal, Neveu, Fartouna-Bellakhal, Missaoui, & Aleya, 2014; Orlofske & Hopkins, 2009).

Whereas numerous studies demonstrated the effect of endocrine disruption on growth and development, only a few studies investigated the effect of altered TH levels as caused by environmental stressors on body condition and energy stores in larval amphibians to estimate fitness. We investigated age, size, survival and body condition at the onset of metamorphosis in larvae of the African clawed frog (*Xenopus laevis*). Body condition was examined using the scaled mass index (SMI), hepatosomatic index (HIS) (i.e., relative liver mass) and relative mass of the tail muscle (rTMM). This combination of different condition indices provides insight into different aspects of energy mobilization and thus, energy stores during larval development. We assume that the alteration of TH levels by the environmentally relevant ED sodium perchlorate (SP; inhibitory) and by exposure to exogenous L-thyroxine ( $T_4$ ; stimulatory) alters growth, developmental and survival rate and body condition at metamorphosis in *X. laevis*.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental design and study species

Three clutches of *X. laevis* were obtained from the captive breeding facility of the Universitätsklinikum Hamburg Eppendorf (UKE, Martinistr. 52, 20246 Hamburg, Germany). Larvae were allowed to hatch and develop to developmental stage 25 (free-swimming larvae; Gosner, 1960). From these larvae, 135 individuals originating from different families were intermixed before allocating them randomly to the different treatments ( $T_4$  and SP) and the control group. Fifteen larvae of *X. laevis* were kept each in a standard 9.5 liter aquarium filled with 8 liters of water (i.e., a total of nine aquaria:  $3 \times T_4$ ,  $3 \times \text{SP}$ ,  $3 \times \text{control}$ ). The experiment was conducted in a climate chamber (Weiss Umwelttechnik GmbH, Reiskirchen, Germany) with a 12:12 hours light/dark cycle at  $19 \pm 0.5^\circ\text{C}$ . The experiments ran for 3 weeks. All surviving larvae had reached the onset of metamorphosis at that time (Gosner, 1960). Amphibian larvae were fed high-protein flaked fish

food (Sera micron breeding feed for fish and amphibians; Sera, Heinsberg, Germany) and spirulina algae ad libitum. This food was provided twice a day to guarantee that it was available in abundance. Furthermore, the amount of provided food was continuously adjusted during the entire experiment to control for differences in tadpole size and density between the aquaria, as Miyata and Ose (2012) indicated that a restricted feeding condition causes atrophy of thyroid tissue similar 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).

*X. laevis* was chosen as the study species (Figure 1), because it is the best investigated amphibian species in terms of TH system and development (Buchholz, 2017), providing physiological background knowledge for the patterns investigated in this study. Although *X. laevis* is unusual among frogs because the adults remain primarily aquatic, results from the aquatic tadpole-stage are transferable to more typical frog species in which adults are terrestrial (Sullivan & Spence, 2003).

## 2.2 | Thyroxine and sodium perchlorate exposures

We used the environmentally relevant concentration of  $250 \mu\text{g l}^{-1}$  SP (sodium perchlorate hydrate 99.99% trace metals basis; Sigma-Aldrich, St. Louis, MO, USA) to achieve a decrease in TH levels in tadpoles. This selected concentration of SP is within environmental ranges measured in surface and ground waters of many industrial nations (Carr & Theodorakis, 2006; Motzer, 2001; Mukhi & Patiño, 2007; Tietge et al., 2005) and in bodies of water in which amphibians breed (Ortiz-Santaliestra & Sparling, 2007; Smith, Theodorakis, Anderson, & Kendall, 2001).

We achieved increased TH levels by exposing tadpoles to  $10 \mu\text{g l}^{-1}$  exogenous  $T_4$  (Sigma-Aldrich), a concentration that is known to influence amphibian metamorphosis (Lucas & Reynolds, 1967; Mann et al., 2009) and is related to increases in  $T_4$  observed in tadpoles responding to stress (Denver, 1997, 1998). Tadpoles absorb exogenous  $T_4$  directly through their permeable skin (Coady et al., 2010; Shi, 2000; Tata, 2006).

$T_4$  and SP treatments were prepared in 0.1 N sodium hydroxide solutions (sodium hydroxide solution 0.1 N, S2770 SIGMA; Sigma-Aldrich) buffered with 0.1 N muriatic acid solutions as solvents.



**FIGURE 1** Tadpole of the African clawed frog (*X. laevis*) with characteristic barbels lateral to the mouthpart [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Solutions were added to the aquaria. 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 the addition of solvents. Each treatment and the control set-up was replicated three times (i.e., 45 larvae, 15 larvae per aquarium, per treatment and control in total). Water was changed every second day and fresh SP and  $T_4$  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 (Bulaeva et al., 2015; Goleman et al., 2002, 2002; Iwamuro et al., 2003; Miwa & Inui, 1987; Ortiz-Santaliestra & Sparling, 2007; Rot-Nikcevic & Wassersug, 2004; Tietge et al., 2005).

## 2.3 | Processing of specimens

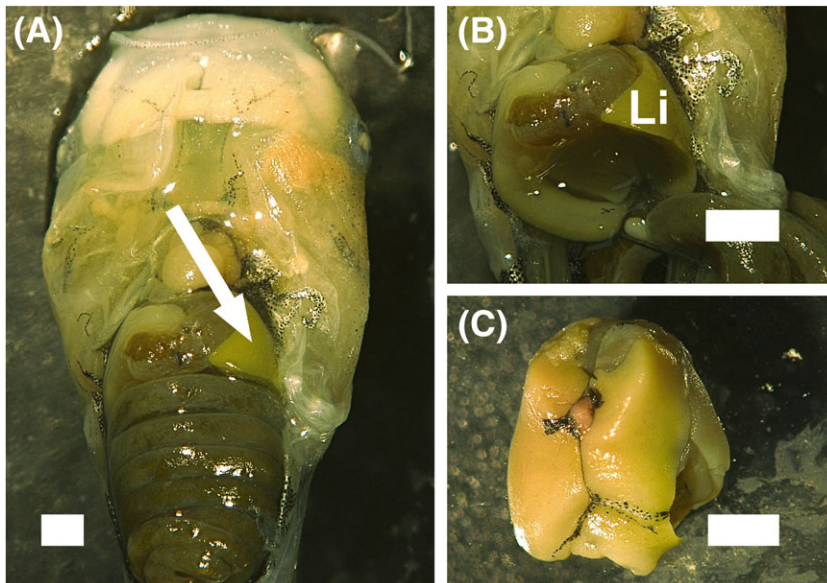
Developmental stage was determined by evaluating the status of key morphological features typical of specific developmental stages, as detailed in Gosner (1960). The developmental stage of each tadpole was recorded according to the procedure of Ortiz-Santaliestra and Sparling (2007). The age describes the larval duration in days after hatching to the onset of metamorphosis. Onset of metamorphosis was defined by the emergence of at least one forelimb (Gosner stage 42; Gosner, 1960). The snout-vent length (SVL) and total length of the larvae were 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 tadpoles were killed with  $200 \text{ mg l}^{-1}$  of tricaine methanesulfonate ([MS-222], Ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich) buffered with  $200 \text{ mg l}^{-1}$  of sodium bicarbonate (Sigma-Aldrich) (Stuart, Chanson, Cox, Young, & Rodrigues, 2007) and transferred into ethanol (70%) for liver and tail muscle dissections. The ethanol-preserved specimens were rehydrated in a decreasing ethanol series (70%, 50%, 30% and water) to achieve their original wet weight.

Liver and tail muscle dissections were made using a digital stereomicroscope (Keyence VHX-500F) (Figure 2). Livers were dabbed and weighed with an electronic balance (digital gold scale; Smart Weigh) and dried for further analysis. After tail muscle dissection, fins were removed and tail muscle was dabbed and weighed.

## 2.4 | Condition indices

We estimated the body condition (i.e., energy stores) at the onset of metamorphosis by calculating a combination of three different condition indices. The SMI is a measure of the entire body condition of an individual as it accounts for the allometric relationship between mass and a body structure measure and standardizes each measure so that direct comparisons among individuals can be made (MacCracken & Stebbings, 2012; Peig & Green, 2009, 2010). The SMI was considered as an accurate condition index in anuran larvae (Dittrich, Drakulić, Schellenberg, Thein, & Rödel, 2016; MacCracken & Stebbings, 2012). A high SMI suggests larger energy stores and thus, a good body condition. We followed the procedure outlined by Peig and Green (2009) to calculate the SMI for each individual.

The HSI describes the status of energy stored in the liver of animals, which is a good indicator of recent fat storage in the animal (Bolger & Connolly, 1989; Htun-Han, 1978). A decrease in HSI



**FIGURE 2** Liver dissection of a *X. laevis* larvae from sodium perchlorate treatment at Gosner stage 42 (forelimb emergence) using the digital binocular Keyence VHX-500F. White square length: 1.00 mm. (A) Abdominal cavity with 20× magnification. Arrow: liver. (B) Abdominal cavity with dissected spiral gut. Li, liver with 30× magnification. (C) Dissected liver with 30× magnification [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

suggests the mobilization of the liver reserves toward the metabolic requirements (Jelodar & Fazli, 2012). The HSI was calculated according to the method of Htun-Han (1978):

$$HSI = \frac{\text{liver wet weight}}{\text{whole body wet weight}} \times 100$$

(Htun-Han, 1978; Jelodar & Fazli, 2012).

The rTMM was used as an index for energy condition in anuran larvae as tails are major sites of fat storage in many species (Scott, Casey, Donovan, & Lynch, 2007; Sheridan & Kao, 1998) and probably provide a source of protein energy for tadpoles during metamorphosis (Hourdry, L'Hermite, & Ferrand, 1996; MacCracken & Stebbings, 2012). rTMM was calculated from absolute wet tail muscle mass as a percentage of total wet body mass.

## 2.5 | Statistical analyses

For all statistical tests, R 3.4.1 (R Development Core Team, 2007) for Windows was used. Before the analysis, all dependent variables in the models were tested for possible correlations using Spearman's rank correlation (*cor.test* function). Subsequently, variables were included in statistical analysis when the correlation was significant but well below the suggested threshold of 0.7 for eliminating variables (Chin, 1998; Fielding & Haworth, 1995) or not significant (Supporting information, Table S1).

Data were analyzed using linear mixed-effect models [lmer function, lme4 package, vers. 1.1–16, Type III model, covariance type: variance components, REML (restricted maximum likelihood) method for parameter estimation, 100 iterations (Bates & Sarkar, 2007)], entering "Treatment" ( $T_4$ , SP and control) as fixed factor. "Size at metamorphosis" (as measured by SVL and body mass), "age at metamorphosis" (as measured by age in days after hatching), "body condition" (as measured by SMI, rTMM and HSI), and "survival" were used as dependent variables in separate models (Table 1). *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. *N* refers to the total number of individual analyzed tadpoles. Linear mixed-effect models were followed by post hoc comparisons (Tukey's test; Tukey HSD function, multcomp package, vers. 1.2–13) to compare all possible pairwise combinations of treatments when overall tests were significant (Table 1).

## 3 | RESULTS

We only calculated a model for one size measure (SVL) as the variables SVL and total length were highly correlated (Supporting information, Table S1). Body mass and SVL were correlated as well, but were included in the analysis for better comparisons with related studies.

Survival from the start of the experiments (Gosner stage 25) to onset of metamorphosis (Gosner stage 42) in the treatment groups were: control  $86.7 \pm 6.7\%$ ; SP  $60.0 \pm 6.7\%$ ; and  $T_4$   $68.9 \pm 4.4\%$ . Therefore, altered TH levels in both treatments led to significantly reduced survival as compared to control treatments (Table 1). There was no variability in the age at metamorphosis and thus, length of the larval period. All tadpoles of one treatment group entered metamorphosis on the same day, 25 days after hatching in the control, 30 days in the SP treatment and 16 days in the  $T_4$  treatment. Thus, the larval period of the larvae in the  $T_4$  treatment was only half as long as that of the SP treatment and two-thirds of that of the  $T_4$  treatment (Figure 3). Development was delayed in the SP individuals compared to controls during developmental period.  $T_4$  individuals showed a faster development compared to controls.

There were significant treatment effects on SVL, body mass, SMI and HSI, but not on rTMM (Table 1). Thus, altered TH levels have an effect on metamorphic traits, entire body condition and particularly on lipid reserves in the liver. Larvae exposed to SP were significantly the largest, heaviest and oldest animals at the onset of metamorphosis compared to control group and  $T_4$  animals (Table 1) (Figure 4). SMI was significantly highest in SP animals compared to control animals. The  $T_4$  treated larvae differed significantly in HSI as compared to the SP-treated and control animals. None of the larvae showed any

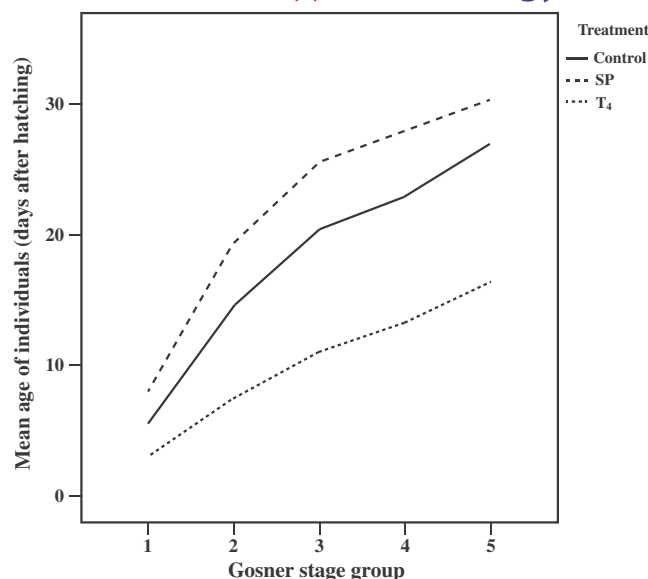
**TABLE 1** Effects of altered thyroid hormone levels on metamorphic traits, survival and body condition in larvae of the African clawed frog (*X. laevis*) at the onset of metamorphosis (at least one forelimb present, Gosner stage 42) (Gosner, 1960)

Dependent variable	LMM																		
	Estimate					Tukey's test (pairwise comparisons)													
	Control	SP	T <sub>4</sub>	χ <sup>2</sup>	df	P	N (n)	Control-SP	SP-T <sub>4</sub>	Control-T <sub>4</sub>									
	Estimate	Estimate	Estimate	Estimate	Estimate	Estimate	Estimate	Estimate	Estimate	Estimate									
Snout-vent length (mm)	15.30	16.8	12.55	32.8	2	<.001	97 (9)	1.50	4.23	4.23	<.001	-4.26	-11.39	-11.39	<.001	-2.75	-8.06	-8.06	<.001
Body mass (mg)	442.60	696.06	267.86	42.79	2	<.001	97 (9)	2.35	15.88	15.88	<.001	-428.20	-26.04	-26.04	<.001	-174.74	-11.21	-11.21	<.001
Survival (%)	86.66	60	68.89	16.61	2	<.001	(9)	-26.67	-5.55	-5.55	<.001	8.89	1.85	1.85	.15	-17.78	-3.70	-3.70	<.001
Scaled mass index	417.34	477.02	441.86	12.45	2	.002	97 (9)	59.68	3.77	3.77	<.001	24.52	1.61	1.61	.24	-35.16	-2.11	-2.11	.08
Hepatosomatic index	3.00	3.19	1.90	24.15	2	<.001	95 (9)	0.19	1.07	1.07	.52	-1.29	-6.82	-6.82	<.001	-1.10	-6.34	-6.34	<.001
Relative tail muscle mass	0.22	0.25	0.23	5.01	2	.08	97 (9)	-	-	-	-	-	-	-	-	-	-	-	-

LMM, linear mixed-effects model; SP, sodium perchlorate; T<sub>4</sub>, thyroxine.

χ<sup>2</sup> and P for LMM, using "treatment" (control, SP, T<sub>4</sub>) 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.

Significance was set at P < .05.

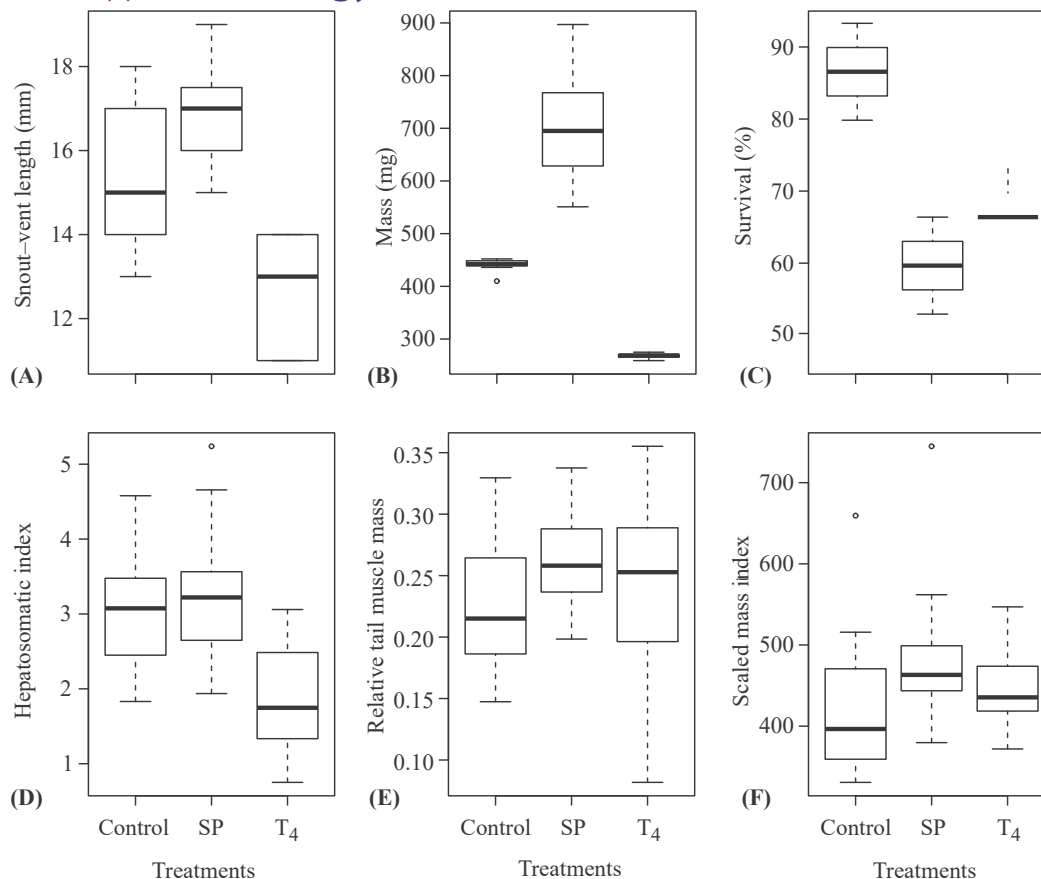


**FIGURE 3** Treatment effects ("Control", "SP" and "T<sub>4</sub>") on mean age in tadpoles of the African clawed frog (*X. laevis*) at Gosner stage group 1-5: (1) pre-limb (absence of hind limbs, Gosner stages 24-26); (2) limb bud (hind limb visible, but no clear joint formed, Gosner stages 27-34); (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) (Gosner, 1960; Ortiz-Santaliestra & Sparling, 2007). Control = solid line; SP = dashed line; T<sub>4</sub> = dotted line. Mean age at Gosner stage group was calculated from mean of summed up mean age at every Gosner stage within one Gosner group. SP, sodium perchlorate; T<sub>4</sub>, thyroxine

significant difference related to rTMM. Consequently, altered TH levels as caused by environmental stressors affected body condition and thus, energy storage in the larvae at the onset of metamorphosis to a different extent (Table 1) (Figure 4).

## 4 | DISCUSSION

Amphibians play an important role in both aquatic and terrestrial ecosystems, and are currently the most threatened group among all vertebrates (Stuart et al., 2004). Therefore, it is essential to understand how different environmental stressors affect amphibian metamorphosis and body condition by altering TH levels (Cary Coyle & Karasov, 2010). In this study, we demonstrate that increased or decreased TH levels as caused by different environmental stressors affect metamorphic traits and body condition in tadpoles of *X. laevis* in different ways. When tadpoles were exposed to the environmentally relevant ED SP, they revealed the best body condition and the largest size at but also the longest larval duration until the onset of metamorphosis. In contrast, tadpoles exposed to exogenous T<sub>4</sub> (i.e., simulating the proximate effect of environmental stressors inducing the expression of developmental plasticity) revealed smaller energy reserves in the liver and the smallest size at metamorphosis, which they reached after the shortest larval duration. In both instances of altered TH levels, survival rate was substantially reduced.



**FIGURE 4** Effects of altered thyroid hormone levels on metamorphic traits (A,B), survival rate (C) and body condition (D-F) in tadpoles of the African clawed frog (*X. laevis*) at the onset of metamorphosis. Boxplot characteristics: Error bar = median. Box = 1. and 3. quartiles. Dots = outliers, minimum and maximum values. Whiskers = 1.5-fold interquartile range. SP, sodium perchlorate; T<sub>4</sub>, exogenous L-thyroxine

#### 4.1 | Alteration of thyroid hormone level affects metamorphic traits and reduces survival

Altered TH levels affected growth and developmental rates in various ways. Because the metamorphic process in amphibians is controlled primarily by the thyroid system, any alteration of growth and developmental rates suggests an impact on the thyroid system homeostasis (Sowers, Mills, & Klaine, 2009). However, the tested compounds may assert their effects via several pathways also influencing growth, development, body condition and particularly survival rate. Nevertheless, the thyroid axis is arguably the most crucial one influencing energetics and the expression of developmental plasticity. Differences in growth and developmental rate between the treatments occurred already early in larval development indicating that alteration of TH levels proceeds with immediate effect from the beginning of the exposure to SP and T<sub>4</sub>.

When aquatic contaminants such as SP affect the TH system, endocrine disruption is usually inhibitory as also confirmed in the present study (Calow, 1991; Fleeger, Carman, & Nisbet, 2003; Mann et al., 2009). Beside SP as the ED, Karaoglu, Kutrup, and Ogut (2010) could show the effect of environmentally relevant concentrations of ammonium nitrate fertilizer on metamorphic traits in tadpoles of the marsh frog (*Pelophylax ridibundus*). Cary Coyle and Karasov (2010) showed the inhibiting effect of polybrominated diphenyl ethers on anuran metamorphosis. Consequently, contaminated breeding ponds may lead to

large but old metamorphs. Although a large size at the onset of metamorphosis is associated with higher individual fitness in later life stages (Berven, 1990), longer larval durations are concomitant with increased pressures by predators in the aquatic habitat and desiccation risk (Kloas & Lutz, 2006; Lefcort, Meguire, Wilson, & Ettinger, 1998).

Besides aquatic contamination, changes in abiotic and biotic factors are known to accelerate developmental rate through increased TH levels mediated by stress hormones (Dantzer et al., 2014; Denver, 1997; Gervasi & Foufopoulos, 2008). We simulated the stimulatory effect of such environmental stressors on the TH system by exposing tadpoles to exogenous T<sub>4</sub> (Coady et al., 2010; Mann et al., 2009). Smaller metamorphs are known to have lower fitness since they are older at first reproduction and have smaller clutch sizes (Berven, 1990; Edwards et al., 2006; Semlitsch, Scott, & Pechmann, 1988). Therefore, increased TH levels as caused by environmental stressors may lead to early metamorphosed juveniles, which are likely to be undersized compared to non-stressed conspecifics (Cauble & Wagner, 2005; Mann et al., 2009). However, for species that stay aquatic both as juveniles and adults, such as *X. laevis*, metamorphosing earlier at a smaller size may be favorable in terms of avoiding predators specialized on tadpoles.

Larvae exposed to T<sub>4</sub> and SP exhibited reduced survival rates until the onset of metamorphosis indicating that any alteration of the TH level negatively affects survival. However, alterations in survival rate could also be indicative of general toxicity of both chemicals. Given the lower survival of tadpoles exposed to T<sub>4</sub> and SP relative to the

controls, it seems that the results obtained could have reflected a general toxicity, rather than an alteration in the thyroid system. However, the OECD (2007) reported results of the interlaboratory survey among five international laboratories in which exposure studies for 3 weeks were conducted for SP at 62.5, 125, 250 and 500  $\mu\text{g l}^{-1}$  as nominal. At 500  $\mu\text{g l}^{-1}$  SP (measured concentrations were ranged within 440–600  $\mu\text{g l}^{-1}$ ), no mortality was observed in four studies and mortality of 4% was reported from one laboratory. These results suggest that a concentration of 250  $\mu\text{g l}^{-1}$  might be too low for causing 40% mortality in the present study. Therefore, the cause of the mortalities at 250  $\mu\text{g l}^{-1}$  was not only the direct toxicity of SP. Measures of TH, thyroid histology or TH-related gene expression would help confirm that thyroid disruption did indeed occur in the exposed organisms.

## 4.2 | Energy storages and body condition

As THs are the major triggers of energy metabolism and are positively correlated with metabolic rate (Burraco & Gomez-Mestre, 2016; McNabb & King, 1993; Rowe et al., 1998) and thus, maintenance costs (Orlofske & Hopkins, 2009). Larvae with altered TH levels as caused by environmental stressors may differ in energy storage and thus, body condition at the onset of metamorphosis. In this study, we used a combination of three different condition indices to provide a complex insight into different energy storage organs (i.e., liver, tail muscle and entire energy stores) and thus, to generate an overall pattern of how altered TH levels influence body condition in larval amphibians. Body condition as measured by SMI was higher in larvae exposed to SP and  $T_4$  suggesting that length growth and mass increase seem to be equally affected by the metamorphic and metabolic effects of both TH level altering compounds. However, higher body conditions do not generally indicate that altered TH levels may not affect the ability to fuel energy storage. Individuals in bad condition may be missing due to a possible toxic effect of both compounds.

As high levels of TH increase energetic maintenance costs, larvae may require more energy to fuel their increased metabolism and less energy remains to be accumulated in energy storage until metamorphosis (Dupré, Just, & Ritchart, 1986; Schmidt et al., 2012). Contrary to these expectations, body condition of larvae exposed to  $T_4$  was not reduced but larvae revealed smaller fat stores in the liver, probably due to the increasing effect of TH on maintenance costs. Otherwise, changes in HSI can be indicative of alterations in hepatic lipid mobilization, which can also be indicative of alterations in the metabolic activity (specifically associated with xenobiotic metabolism) of the liver or liver pathologies, both of which can occur in response to chemical exposures, or general toxicity associated with both compounds. Zaya, Amini, Whitaker, Kohler, and Ide (2011) demonstrated that endocrine disruption induced by atrazine led to a reduced liver weight in tadpoles of *X. laevis* but did not affect lipid stores in liver and fat body. Further studies are needed to investigate liver metabolism in tadpoles exposed to different environmental stressors.

Despite the liver as a storage organ for fat, the tail muscle is known as the main fat and protein storage, as it is resorbed during metamorphosis when anuran larvae stop eating due to tissue reorganization (Hourdry et al., 1996; Shi, 2000). In this study, altered TH levels did not affect the tail muscle mass and thus, the size of fat and protein

storages in *X. laevis*. In contrast, Yu et al. (2013) demonstrated the effect of the endocrine disrupter chlorothalonil (a broad-spectrum agricultural fungicide) on tail length (tail degeneration) in larvae of the African clawed frog (*X. laevis*) and the New Mexico spadefoot toad (*Spea multiplicata*). Our results are not in agreement with those of Yu et al. (2013), which may be explained in two different ways: either, only the lipid storages are affected by the calorogenic effect of TH or xenobiotic metabolism of the liver and not the storage of lipids and amino acids in muscle tissue, which comprise most of the tail tissue in anuran larvae. Alternatively, the energy requirement from tail tissue starts to differ between treatments at later developmental stages, after metamorphosis has set in. At these later developmental stages (Gosner stage >42) aquatic contaminants, such as chlorothalonil and perchlorate, may possibly interfere with pathways that trigger tail resorption by altering expression of genes involved in tail cell death as occurs at tail absorption during metamorphosis (Yu et al., 2013).

## 4.3 | Ecological significance

In this study, we demonstrated that altered TH levels affect body condition, but impairs survival rate and results in either large but old or young but small metamorphs, which are both known to be disadvantaged in comparison with conspecifics developing in undisturbed larval habitats. Both a prolonged duration in larval development and a small size at metamorphosis are known to reduce fitness and may outweigh the advantage of an increased body condition. As environmental stressors in a larval habitat may influence the reproductive outcome of a whole population, altered TH levels as caused by environmental stressors may subsequently lead to declining amphibian populations.

## 5 | CONCLUSION

In the near future, the combination of naturally and anthropogenically induced environmental stressors will occur in natural habitats of amphibian populations more frequently and all environmental stressors will affect larval anurans by their effect on the TH system and other pathways. In case of chemical stressors, tadpoles may additionally suffer from possible toxic effects. Despite the impact on metamorphic traits, we could demonstrate that altered TH levels as caused by aquatic contaminants and environmental stressors reduces survival and affects entire body condition and energy storage probably due to the metabolic function of THs. Future studies should focus on the combined effects that different environmental stressors have on energy store and body condition. Moreover, stress experienced during early life stages may have long-lasting effects in later life stages and thus, on the survival of amphibian populations. Therefore, long-term studies are needed to understand completely the consequences of altered TH levels as caused by environmental stressors during the larval stages on the phenotype and fitness of the adults.

## ACKNOWLEDGMENTS

The authors would particularly like to thank J. Bank for his experimental advice and technical assistance and C. Dittrich for her methodical

advice. We would also like to thank A. Haas for his experimental advice and technical support.

## FUNDING INFORMATION

Not funded.

## CONFLICT OF INTEREST

The authors did not report any conflict of interest.

## AUTHOR CONTRIBUTIONS

KR, KD, MP and JG conceived and designed the study. KR, NS, LB, JR and LH conducted the experiments. KR and CD performed the statistical analysis. All authors participated in manuscript editing and final approval.

## ETHICAL APPROVAL

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. The experiments were conducted under permission from the *Amt für Verbraucherschutz, Lebensmittelsicherheit und Veterinärwesen* in Hamburg, Germany (Billstraße 80, D-20539 Hamburg; Gz. V1305/591-00.33, no. 03/16).

## ORCID

Katharina Ruthsatz  <http://orcid.org/0000-0002-3273-2826>

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Ruthsatz K, Dausmann KH, Drees C, et al. Altered thyroid hormone levels affect body condition at metamorphosis in larvae of *Xenopus laevis*. *J Appl Toxicol*. 2018;1–10. <https://doi.org/10.1002/jat.3663>