General and Comparative Endocrinology

Title: Contributions of water-borne corticosterone as one non-invasive biomarker in assessing nitrate pollution stress in tadpoles of *Rana temporaria*

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Abstract

Among a multitude of stressors to which wildlife is exposed, environmental pollution is a pervasive one that poses a serious threat. The permeable skin of amphibians is likely to increase direct contact of the body with pollutants, making them a group worth studying to access environmental quality. Consequently, finding reliable and complementary biomarkers that will present detectable and predictable changes in response to pollutants is essential to identify pollution sublethal effects on amphibians and to investigate whether these are in part responsible for population declines. The glucocorticoid hormone corticosterone (CORT), involved in many metabolic functions, is often used to measure the physiological stress response to environmental stressors in amphibians. In this study, we evaluated whether water-borne CORT can serve as a non-invasive biomarker for nitrate pollution stress in the European common frog (*Rana temporaria*) by comparing the effect of nitrate exposure on hormone release rates and on other physiological downstream biomarkers, i.e., ultimate physiological effects of the stressor. Specifically, we investigated the effect of different nitrate concentrations (0, 10, 50, and 100 mg/L) on water-borne CORT release rates, age, size, and body condition. Exposure to nitrate pollution significantly increased age at metamorphosis and water-borne CORT release rates, and led to reduced mass and body condition, but only at higher nitrate concentrations (i.e., 50 and 100 mg/L). Considering this similar sensitivity to other acknowledged biomarkers, water-borne CORT was a reliable biomarker of physiological stress in *R. temporaria* exposed to nitrate pollution stress in a controlled single-stressor laboratory approach. Thus, water-borne CORT is a promising method to be included in more holistic approaches. We recommend that such approaches keep testing multiple biomarker combinations, as species are exposed to several stressors likely to interact and produce varied outcomes in different biomarkers in their natural habitats.

Key words: *conservation physiology, environmental stress, European common frog, metamorphosis, biomarker, hormone, amphibians*

1. Introduction

Global change exposes wildlife to multiple chemical, physical, and biological stressors that arise partly from anthropogenic activity (e.g., climate change, pollution), but also from natural sources (rev. in Noyes et al., 2009). The effects of stressors on organisms may be assessed in multiple ways, including alterations in biochemical pathways (e.g., Leite et al., 2010), immune response (e.g., Priyadarshani et al., 2015), number of blood cells (Salinas et al., 2017), morphological responses (e.g., growth, fluctuating asymmetry; Costa et al., 2018; Zhelev et al., 2019), to mention just a few. Physiological biomarkers are commonly used to assess the effect of environmental stressors on the health and performance of species (Cooke et al. 2013; Madlinger et al., 2016). The measurement of the glucocorticoid (GC) hormones, cortisol and/or corticosterone (CORT) is one useful tool to determine physiological stress in vertebrates (Sapolsky et al., 2000; Wikelski & Cooke, 2006; Dantzer et al., 2014). CORT is secreted from the hypothalamic-pituitary-adrenal/interrenal axis in vertebrates and is released in response to environmental stressors but also involved in general metabolic processes, immune functioning, reproduction, growth, and development (Crespi et al. 2013, Kirschman et al., 2017; rev. in Mausbach et al., 2022). An increasing number of studies indicate that CORT levels correlate with survival probability across vertebrate taxa (e.g., Romero & Wikelski, 2001; Cabezas et al., 2007; Rivers et al., 2012; Shewade et al., 2020; Tornabene et al., 2021a) and thus, can predict fitness (rev. in Bonier et al., 2009). Therefore, using CORT as one biomarker of physiological stress is a promising tool in conservation science (rev. in Tornabene et al., 2021b) and eco-evo-physiological studies.

CORT levels can be obtained using invasive (i.e., whole-body or blood plasma/serum samples) or non-invasive (i.e., saliva, urine/feces, and hair/feathers) collection methods (rev. in

Sheriff et al., 2011). In aquatic vertebrates, water-borne hormone sampling is a novel, non-invasive collection technique (fish: rev. in Scott & Ellis, 2007; amphibians: Gabor et al., 2013; Narayan et al., 2019). GCs can pass through gills, mucous membranes, and skin into the surrounding water (rev. in McClelland & Woodley 2021). The water in which the specimen has been contained for 1-2 hours is collected (rev. in Scott & Ellis, 2007; Gabor et al. 2013, 2015), and GCs are extracted from the water and measured using immunoassays (Burraco et al., 2015). However, it remains to be validated whether water-borne CORT is a reliable biomarker for assessing the effect of environmental stress for every species (Davis et al., 2020), life-stage (McClelland & Woodley 2021), and stressor (Tornabene et al. 2021b). Validation methods encompass the technical validation of the assay used (Behringer & Deschner, 2017), a correlation of water-borne CORT levels with endogenous CORT levels from the same individual (McClelland & Woodley 2021), repeatability analyses (Forsburg et al., 2019), and the comparison of stress exposure effects on water-borne CORT level to other downstream biomarkers such as body mass and body condition (Millikin et al., 2019). Experimental conditions can include both external stressors (e.g., confinement, shaking) or manipulated hormone injections (adrenocorticotropic hormone, ACTH) to trigger the CORT stress response (Glennemeier & Denver 2002a, 2002b).

Water-borne CORT assays have been shown to be good surrogates for endogenous CORT measurements at least under laboratory conditions (Gabor et al., 2013, Baugh et al., 2018; McClelland & Woodley, 2021). Thus, water-borne CORT can be considered a good candidate biomarker for measuring pollution stress in freshwater vertebrates inhabiting waters in agricultural landscapes such as (larval) amphibians because agrochemicals can act as environmental stressors and thus, alter CORT levels of amphibians (Hayes et al., 2006; rev. in Mann et al., 2009; McMahon et al., 2011; rev. in Carr & Patino, 2011). For example, exposure to the herbicide atrazine caused a nonlinear increase in whole-body CORT levels in the Cuban treefrog (*Osteopilus septentrionalis*) and high conductivity increased whole-body CORT levels in larvae of the Jefferson salamander (*Ambystoma jeffersonianum*) but not in larvae of the wood frog (*Lithobates sylvaticus*), and the gray treefrog (*Hyla versicolor*; Chambers, 2011). Further, Burraco et al. (2016) demonstrated that exposure to salinity stress and glyphosate increased whole-body CORT levels in spadefoot toad tadpoles (*Pelobates cultripes*). A recent study evaluated whether water-borne CORT could serve as a biomarker of acute salinity stress in three amphibian species and demonstrated an increase of CORT release rates with increasing salinity in the Northern leopard frog (*Lithobates pipiens*; Tornabene et al., 2021b). In contrast, Gavel et al. (2021) could not find an effect of two neonicotinoids (clothianidin and thiamethoxam) on water-borne CORT levels in the Northern leopard frog (*Lithobates pipiens*). However, studies using water-borne CORT as a biomarker for pollution stress are still limited/rare, despite its non-invasive and easier sampling (Gabor et al., 2013).

Given that the breeding season of many amphibian species and arable farming coincide (Ortiz-Santaliestra et al., 2006; Polo-Cavia et al., 2016; Leeb et al., 2021), exposure to pollution stress might be of high ecological relevance in amphibians across life stages (Relyea et al., 2005; Miaud et al., 2011; Goessens et al., 2022). Common agrochemicals found in amphibian habitats include salts, pesticides, and fertilizers (rev. Trudeau et al., 2020). Nitrate (NO_3^-) is the major component of fertilizers and is thus, an ubiquitous pollutant in habitats of (larval) amphibians (e.g., Rouse et al., 1999; De Wijer et al., 2003; Ortiz-Santaliestra and Sparling, 2007). Nitrate can affect growth (Garriga et al., 2017), metamorphosis (Sullivan and Spence, 2003), reduce survival (Watt and Jarvis, 1997), and has also been associated with disruption of the thyroid hormone system (Wang et al., 2015; Poulsen et al., 2018; but not: Edwards et al., 2006). A recent study on Southern leopard frog (*Lithobates sphenocephala*) larvae demonstrated an increase in water-borne CORT release rates when tadpoles were exposed to a combined treatment of atrazine, road-salt, and ammonium nitrate (Adelizzi et al., 2019).

Therefore, water-borne CORT could be a useful additional biomarker of physiological stress for amphibians exposed to nitrate pollution.

In this study, we measured physiological stress in response to pollution stress in tadpoles of the European common frog (*Rana temporaria*). Specifically, we investigated the effect of different nitrate concentrations (0, 10, 50, and 100 mg/L) on water-borne CORT release rates, age, size, and body condition at the onset of metamorphosis (Gosner stage 42; Gosner 1960). We further evaluated whether water-borne CORT can serve as a biomarker for nitrate pollution stress in *R. temporaria* by comparing the effect of nitrate exposure on hormone release rates and on other acknowledged physiological downstream biomarkers in a laboratory set-up. If different biomarkers, including water-borne CORT, respond similarly to nitrate pollution stress, water-borne CORT would qualify as a useful biomarker of physiological stress and a promising tool in amphibian conservation. We tested this hypothesis under laboratory conditions in an attempt to increase the evidence of suitability of this non-invasive method with easier sampling facilitates than traditional CORT assessments. Once successful, the use of water-borne CORT could be included in more holistic approaches with relatively little additional effort to achieve better assessments of environmental stress.

2. Materials and Methods

2.1 Animal collection and oviposition

The anuran *Rana temporaria* was chosen as the model species for this study because it is the most widespread in Europe and occurs in natural as well as anthropogenic habitats: the International Union for Conservation of nature lists *R. temporaria* in the "least concern" category but recognizes pollution as a threat for this species (IUCN, 2021). The fieldwork was carried out at the locality Kleiwiesen (52.328 N, 10.582 E), a site in central Germany near Braunschweig, Lower Saxony. This site comprises a system of ponds surrounded by meadows and mixed deciduous beech forest, sustaining a large population of *R. temporaria*, which breeds in a shallow part of one pond, partly covered with dense reeds (Dittrich et al., 2018). We collected by hand three amplectant pairs at night on 22 March, 2021. We measured on site their snout-vent-length (SVL) using a caliper (in mm, to the closest 0.5 mm), and mass (in g, to the nearest 0.001 g) using an electronic balance (Professional Digital Jewellery Gold Scale Balance, GandG, Kaarst, Germany). Frogs were transported to the laboratory, where each pair was placed in a plastic container filled up to 8 cm with pond water until it spawned. Egg masses were collected within 12 hours of oviposition. Individuals were released at the pond of origin at the locality Kleiwiesen.

2.2 Experimental design and animal care

We used a two-phase experimental design to assess the life stage specific sensitivity to nitrate of embryos (phase 1) and larvae (phase 2). The experiment was conducted in a climate chamber (Kälte-Klimatechnik-Frauenstein GmbH, 38106 Braunschweig, Germany) with a 14:10 h light:dark cycle at 10 ± 0.2 °C (phase 1) and 18 ± 0.1 °C (phase 2), representing average conditions commonly experienced in the field.

Phase 1. – After oviposition, the three clutches were separated carefully. In total, 900 fertilized eggs were allocated to four different nitrate treatments (0, 10, 50, 100 mg NO_3^- x L^{-1}) with three replicates each (3 clutches x 4 treatments x 3 replicates x 25 eggs). Eggs were placed in 36 plastic containers (V= 250 mL), filled with 150 mL aged, filtered, and de-chlorinated water. NO_3^- stock solutions were mixed into the aquaria at the concentrations necessary to reach target values.

Phase 2. – After hatching, larvae were allowed to develop to stage 25 (free-swimming larvae; Gosner, 1960). From each of the four nitrate concentration treatments, 45 (15 per replicate), were moved from their phase 1 plastic containers to 12 standard 12-L aquaria, filled with 9 L of aged de-chlorinated water (4 treatments x 3 replicates x 15 larvae = 180 individuals).

Larvae from the three different clutches were intermixed at this transference. Larval density was 1.66 larvae × L^{-1} in the beginning of the experiment. 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 effectively avoiding any restricted feeding conditions. Any dead or abnormal tadpoles were removed from the aquaria. All surviving tadpoles were kept for further experiments.

2.3 Natal pond and experimental water parameters

Water quality was monitored twice per week during the experiment with the use of the AQUA-Check 2 photometer (Söll GmbH, 95030 Hof, Germany; N=22). Natal pond water parameters were determined at the time of animal collection. Water samples were taken from the edge and the middle of the pond as well as from the spot where the amplectant pairs were caught (N=3). Measurements included nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), pH, phosphate $(PO_4^3)^-$, copper (Cu^{2+}), iron (Fe), and lead (Pb) in mg x L^{-1} (Table S1).

2.4 Nitrate exposures

We added nitrate as sodium nitrate ($NaNO_3$; e.g., Oromí et al., 2009; Wang et al., 2015). Other researchers investigating nitrate toxicity in amphibians have concluded that sodium nitrate is less toxic than ammonium nitrate (NH_4NO_3), which has been used in several studies with amphibians (e.g., Johansson et al., 2001; Garriga et al., 2017). Therefore, it is unlikely that sodium contributed to toxic effects in our study (rev. in Hatch & Blaustein, 2003).

Reagent-grade sodium nitrate (>99% pure; Carl Roth, 76185 Karlsruhe, Germany) was used to prepare stock solutions. Selected concentrations for the experiment were within environmental ranges measured in surface and ground waters in Germany (Sundermann et al., 2020) and consistent with environmental ranges measured in bodies of water in which amphibians breed (e.g., De Wijer et al., 2003; Rouse et al., 1999; Johansson et al., 2001).

The nitrate treatments were prepared in autoclaved water. Each stock solution was electronically pipetted and mixed into the aquaria at the concentrations necessary to reach target values. In phase 2, water was changed every second day and fresh stock solutions were added, which is frequent enough to maintain a constant nitrate level, in accordance with the standard procedure for chemical addition (e.g., Ortiz-Santaliestra & Sparling, 2007).

2.5 Life history variables and ontogenetic staging

After hatching, we measured snout-vent length (SVL), total length (TL), and body mass, and determined ontogenetic stage every other day. Age was measured in days after hatching (dah). The snout-vent length of the larvae and froglets was measured with a caliper to the nearest 0.5 mm. Specimens were dry blotted and weighed to the nearest 0.001 g with an electronic balance (Sartorius A200 S, Göttingen, Germany). Ontogenetic stage was determined by evaluating the status of key morphological features as detailed in Gosner (1960).

2.6 Body condition

Body condition was determined at the onset of metamorphosis (Gosner stage 42; Gosner 1960), using the scaled mass index (SMI) following Peig & Green (2009). The SMI accounts for the allometric relationship between mass and body length and is a standardized measure of the body condition that can be directly compared among individuals (Peig & Green, 2009; 2010). The SMI has been previously employed as a condition index in anuran larvae (MacCracken & Stebbings, 2012; Dittrich et al., 2016; Ruthsatz et al., 2018, 2019, 2020a, b). A high SMI suggests greater energy storages and, thus, a good body condition. The SMI slope is calculated from the regression of log transformed SVL and log transformed mass.

$$\text{SMI} = \left[individual\ Mass \times \left(\frac{mean\ SVL\ of\ population}{individual\ SVL}\right)^{slope\ of\ regression\ logMass\ \sim logSVL}\right]$$

2.7 CORT assays

At the onset of metamorphosis (Gosner stage 42; Gosner 1960), we measured CORT levels in 60 tadpoles (4 treatments x 3 replicates x 5 tadpoles) using the established waterborne assay protocol by Gabor et al. (2013) integrating Mausbachs et al. pers. observations as explained below. Briefly, each tadpole was placed in a freshly cleaned (EtOH) glass bowl containing 50 mL of aged and filtered tap water for 1 h. For each sampling batch a control water sample was run to control for potential background hormonal traces in the sampling water (Mausbach et al. pers observation). After the hour-long sample collection period, we determined body mass and SVL of each tadpole, which was then returned to the respective aquarium. Samples were taken between 1800 and 2100 h in the night and between 3 and 12 tadpoles were sampled simultaneously.

All water hormone samples were stored at − 20 °C. Samples were further processed after 28 days, in order of sampling. The extraction was conducted as described below following Mausbach et. al. in prep. protocol, which uses the extraction scheme described in Fürtbauer et al. (2015) previously used in fish with minor adaptations..tThawed samples were first filtered with Q8 Whatman filter paper to remove suspended particles and then filtered through C18 solid-phase extraction columns (Oasis Vac Cartridge HLB 3 cc/ 60 mg, 30 µm; Waters, Inc., Switzerland) with a vacuum manifold (Visiprep Vacuum Manifold; Sigma-Aldrich, Germany). These cartridges do not require priming, can be run dry and can be frozen following the instructions from Waters, Inc. We cleaned the manifold before each use using 4 ml of HPLC-grade methanol and 4 ml of nanopure water. The columns were returned to the -20 °C freezer until hormones were eluted with 4 ml of HPLC-grade methanol with a vacuum manifold (Visiprep Vacuum Manifold; Sigma-Aldrich, Germany), which was cleaned with Methanol and nanopure water between batches again. During this process samples were transferred into 5ml Eppendorf tubes. Afterwards the methanol was evaporated using a sample concentrator (Stuart sample concentrator, SBHCONC/1; Cole-Parmer, United Kingdom) under a fine N_2 stream at 45 °C using a block heater (Stuart block heater, SBH130D/3; Cole-Parmer, United Kingdom). Dried samples were stored at - 20°C until Enzyme- Immunoassay analysis, which took place in July, 2021.

The hormonal levels were measured using DetectX Corticosterone ELISA (Enzyme Immunoassay) kits purchased from Arbor Assays (K014-H5, Ann Arbor, MI, USA; assay has a range of 19.53–5000 pg Corticosterone/ml). This assay has been previously validated for wood frogs (*Lithobates sylvaticus*; Gavel et al., 2019) and has also been successfully used for *Rana arvalis* (Mausbach et al. 2022). We re-suspended the dried sample in a total volume of 125 µl consisting of 5% ethanol (95% lab grade) and 95% enzyme-immunoassay (EIA) buffer. After re-suspension, samples were frozen at -20 °C until measurement of hormonal levels via EIA. Samples and kit reagents were brought to room temperature and vortexed before plating. We measured corticosterone concentration in duplicates for all samples on 96-well plates according to the kit's instructions. The plates were read with a Tecan Spark® Microplate Reader at 450 nm (Tecan, Switzerland). In total, we ran twelve plates.

Control samples and negative controls did not show CORT levels at detectable ranges. We used MyAssays online tools to calculate the hormonal concentration of samples (https://www.myassays.com/arbor-assays-corticosterone-enzyme-immunoassay-kit-improved-sensitivity.assay). Standards (high and low level concentration groups) that were run on each plate were used to calculate intra- and interplate coefficient of variation. Intraplate variation was overall 38.22% (high: 23.96, low: 52.48) and interplate variation was on average 33.68 (high: 19.25, low: 45.22). These values are rather high which means that run duplicates sometimes varied a lot (first time this laboratory conducted hormonal analyses). As the results

are still conclusive, we considered the values as suited for analysis (see Discussion for details). The coefficient of variation of duplicates for all samples was 13.6 %.

2.8 Statistics

For all statistical tests Cran R (Version 4.1.1, R Development Core Team 2021) for Windows was used unless otherwise noted. All plots were constructed using ggplot2 (Wickham, 2011) and Adobe Illustrator CS6. A raw data table in xlsx format, including all original measurements, will be deposited in Figshare under DOI:XXX after acceptance.
Following Gabor et al. (2013), we multiplied CORT release rates (pg/ml) by the volume of the re-suspension solution (0.125 ml) and standardized values by dividing by the body mass of each individual, resulting in CORT release rates units being pg/g/h.

Before statistical analyses, all dependent variables (CORT, SVL, body mass, age, and body condition) were log-transformed. Data were analyzed using linear mixed-effect models [*lmer* function, lme4 package, Type III model, covariance type: variance components, REML (restricted maximum likelihood) method for parameter estimation, 100 iterations (Bates et al., 2007)], entering "Nitrate" (0, 10, 50, and 100 mg/L) as fixed factor. Log-transformed "CORT release rates", "size at metamorphosis" (as measured by SVL and body mass), "age", and "body condition" (as measured by SMI) were used as dependent variables in five separate models (Table 1). P values were obtained from likelihood-ratio tests, which compared the models with the respective null-model. 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 analyzed individual animals, and n is the total number of tested aquaria. Linear mixed-effect models were followed by post hoc comparisons (Tukey's test; Tukey HSD function, multcomp package) with Bonferroni correction to compare all possible pairwise combinations of treatments when overall tests were significant (Table 1). For all tests and models statistical significance was accepted for $\alpha < 0.05$.

Even though we corrected for body mass in CORT release rate values, Millikin et al. (2019) suggested that size is still correlated with CORT measures. Therefore, we performed linear regressions between body mass and size-corrected CORT release rates (Fig. 2A). We further tested whether body condition is correlated with CORT release rates (Fig. 2B).

3. Results

The experiment was conducted for five weeks. All animals had reached the onset of metamorphosis at that time (Gosner stage 42; Gosner, 1960). Exposure to different nitrate concentrations affected CORT release rates, age, body mass, and body condition, whereas SVL at the onset of metamorphosis was not affected by nitrate concentration (Table 1; Fig. 1).

CORT release rates – CORT release rate increased with nitrate concentration and was the lowest and the highest in larvae exposed to 0mg/L and 100mg/L, respectively (Table S2; Fig. 1A). Pairwise-comparisons between nitrate concentrations revealed significantly higher CORT release rates in larvae exposed to 100mg/L compared to the control group (Table 1; Fig. 1A). However, there were no significant differences between larvae exposed to 10mg/L or 50mg/L and the control group (Table 1; Fig. 1A). As measured duplicates partially varied a lot in this study the non- significance also could be biased by this inaccuracy. CORT release rate is negatively correlated with body mass (Fig. 2A). The water control samples did not show CORT levels in a detectable range.

Age at metamorphosis – Age increased with nitrate concentration and was the lowest and the highest in larvae exposed to 0mg/L and 100mg/L, respectively (Table S2; Fig. 1B). Pairwise-comparisons between nitrate concentrations revealed that larvae exposed to 100mg/L were significantly older compared to the control group (Table 1; Fig. 1B). However, there were no significant differences between larvae exposed to 10mg/L or 50mg/L and the control group (Table 1; Fig. 1B).

Size at metamorphosis – Body mass was significantly affected by nitrate concentration (Table 1). Larvae revealed the highest and the lowest body mass when raised under 0mg/L and 50mg/L, respectively (Table S2). Pairwise-comparisons between nitrate concentrations demonstrated that body mass was significantly lower in larvae exposed to 50 mg/L and 100mg/L compared to the control group (i.e., 0mg/L; Table 1; Fig. 1C). Larvae raised under a nitrate concentration of 10mg/L did not significantly differ in body mass compared to the control group (Table 1; Fig. 1C). Nitrate exposure did not lead to an effect on SVL (Table 1; Fig. S1).

Body condition – SMI was significantly affected by nitrate concentration (Table 1). Larvae revealed the highest and the lowest body condition when raised under 0mg/L and 50mg/L, respectively (Table S1). Body condition was significantly lower at all nitrate concentrations compared to the control group (Table 1; Fig. 1D). Linear regressions revealed that body condition decreased significantly with CORT release rates (Fig. 2B).

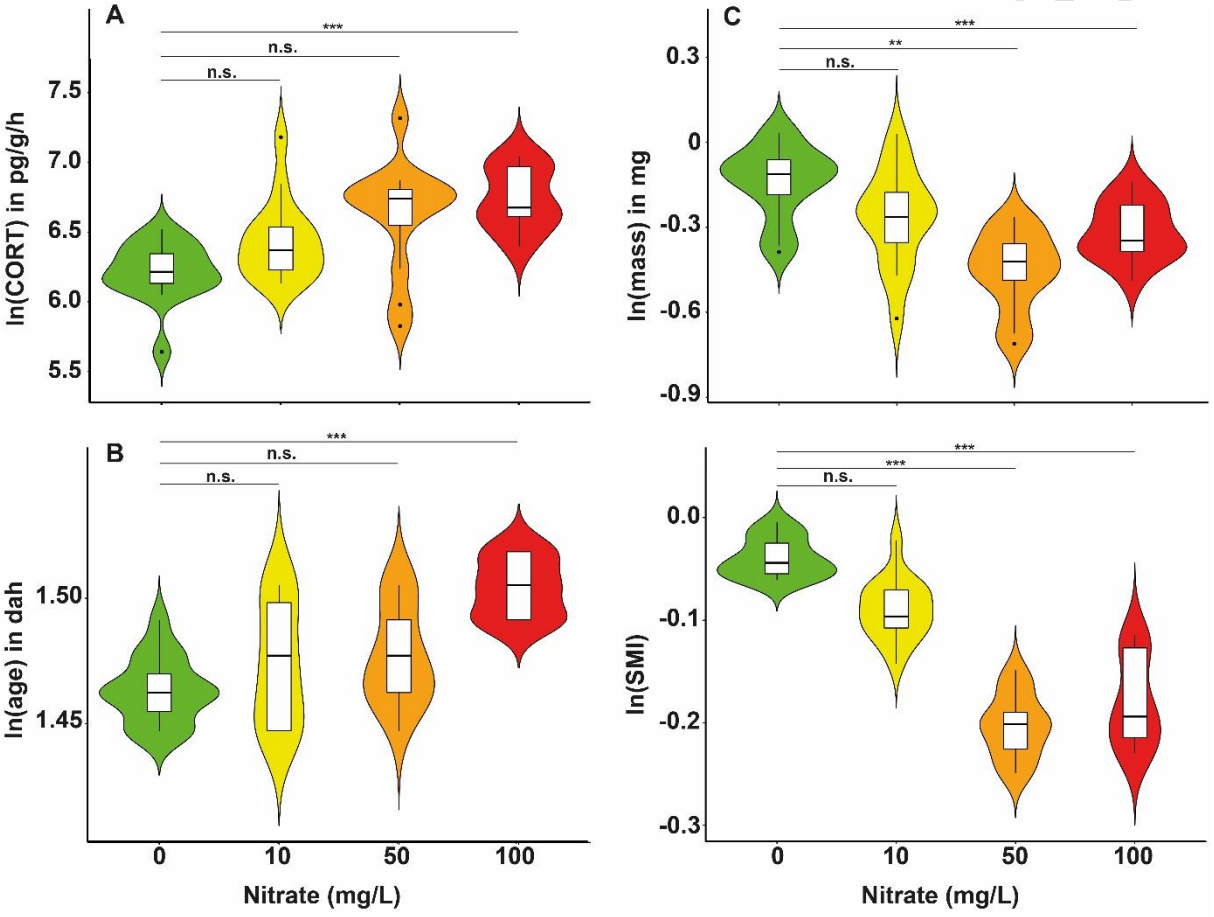
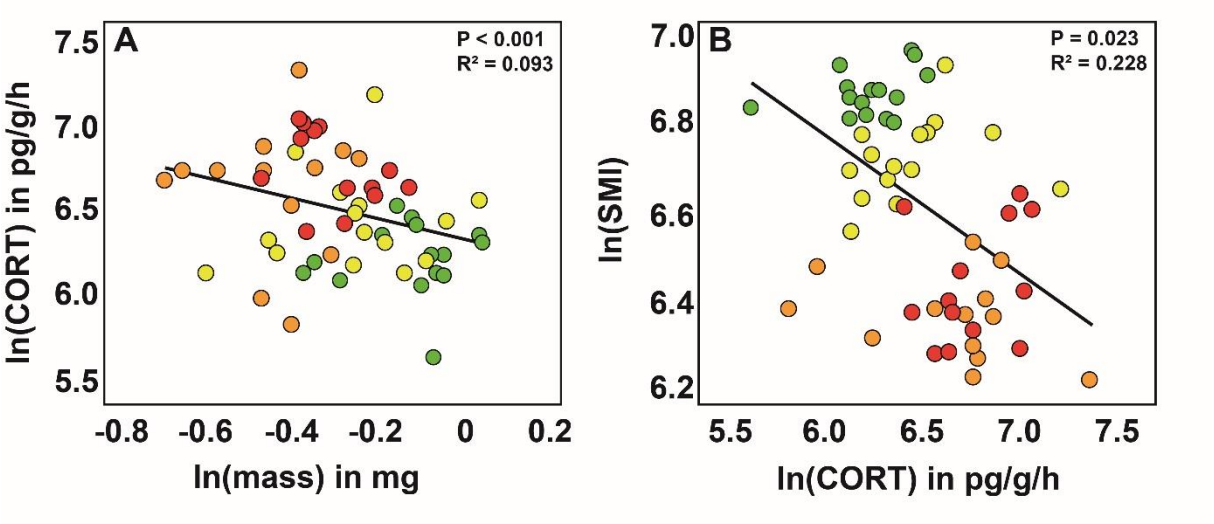


Fig. 1. Effects of nitrate pollution stress on **A** size corrected ln(CORT release rate in pg/g/h), **B** ln(age in dah), **C** ln(body mass in mg), and **D** ln(SMI) as a measure for body condition in larvae of the European common frog (*Rana temporaria*) at the onset of metamorphosis (at least one forelimb present, Gosner stage 42) (Gosner, 1960). Boxes and whiskers show 25th to 75th and 10th to 90th percentiles, respectively; black lines indicate the median. Dots = outliers. Violin plot colors indicate nitrate concentration: green = 0 mg/L, yellow = 10 mg/L, orange = 50 mg/L, and red = 100 mg/L. Asterisks indicate significant differences between nitrate treatments (*p<0.05; **p < 0.01; ***p < 0.001). n.s. = non-significant differences between nitrate treatments.



339 **Fig. 2** Linear regressions of **A** ln(body mass in mg) and ln(CORT release rates in pg/g/h) and
340 **B** ln(CORT release rates in pg/g/h) and ln(SMI) as a measure for body condition. Regression
341 lines show the significant relationship between two variables, respectively. Dot colors indicate
342 nitrate concentration: green = 0 mg/L, yellow = 10 mg/L, orange = 50 mg/L, and red = 100
343 mg/L.

344 **Table 1.** Effects of nitrate pollution stress on size corrected CORT release rate (pg/g/h), SVL
345 (mm), body mass (mg), and body condition (SMI) in larvae of the European common frog
346 (*Rana temporaria*) at the onset of metamorphosis (at least one forelimb present, Gosner stage
347 42) (Gosner, 1960). LMM, linear mixed-effects model, using "Nitrate" (0, 10, 50, and 100
348 mg/L) as the fixed factor; 'aquarium' as the random factor. N is the total number of analyzed
349 individual animals, and n is the total number of tested aquaria. Pairwise multiple comparisons
350 were made using Tukey's test as post hoc test with Bonferroni correction. Significance was set
351 at P < .05.

LMM						**Tukey's Test (pairwise comparisons)**						
Dependent variable	**Fixed effects**	**Estimate (SE)**	**t-value**	**P**	**N (n)**		**0-10**	**0-50**	**0-100**	**10-50**	**10-100**	**50-100**
CORT release rates (pg/g/h)	Intercept	6.21 (0.11)	59.12	<0.001	56 (12)	**Estimate (SE)**	0.23 (0.14)	0.41 (0.15)	0.52 (0.15)	0.17 (0.15)	0.29 (0.15)	0.11 (0.15)
	10 mg/L	0.23 (0.14)	1.55	0.165		**z-value**	1.55	2.71	3.50	1.18	1.97	0.78
	50 mg/L	0.41 (0.15)	2.71	**0.029**		**P**	0.406	0.198	**0.012**	0.637	0.198	0.864
	100 mg/L	0.52 (0.15)	3.50	**0.009**		**N**	30	28	28	28	28	28
Snout-vent length (mm)	Intercept	2.65 (0.01)	175.46	**<0.001**		**Estimate (SE)**	-0.00 (0.02)	0.01 (0.02)	0.03 (0.02)	0.01 (0.02)	0.03 (0.02)	0.02 (0.02)
	10 mg/L	-0.00 (0.02)	-0.09	0.928		**z-value**	-0.09	0.72	1.65	0.81	1.74	0.92
	50 mg/L	0.01 (0.02)	0.72	0.494		**P**	1.000	0.889	0.348	0.849	0.299	0.794

	100 mg/L	0.03 (0.02)	1.65	0.139	**N**	30	28	28	28	28	28
Body mass (mg)	Intercept	-0.13 (0.03)	-3.68	**0.009**	**Estimate (SE)**	-0.12 (0.05)	-0.31 (0.06)	-0.17 (0.06)	-0.18 (0.06)	-0.05 (0.06)	0.13 (0.06)
	10 mg/L	-0.12 (0.05)	-2.34	0.055	**z-value**	-2.34	-5.72	-3.22	-3.45	-0.95	2.42
	50 mg/L	-0.31 (0.06)	-5.72	**<0.001**	**P**	0.087	**<0.001**	**0.007**	**0.003**	0.778	0.071
	100 mg/L	-0.17 (0.06)	-3.22	**0.013**	**N**	30	28	28	28	28	28
Age (dah)	Intercept	1.46	0.00	**<0.001**	**Estimate (SE)**	0.01 (0.01)	0.01 (0.01)	0.04 (0.01)	0.00 (0.01)	0.03 (0.01)	0.02 (0.01)
	10 mg/L	0.01	0.01	0.434	**z-value**	0.82	1.21	3.74	0.39	2.92	2.50
	50 mg/L	0.01	0.01	0.260	**P**	0.842	0.617	**<0.001**	0.979	0.108	0.059
	100 mg/L	0.04	0.01	**0.006**	**N**	30	28	28	28	28	28
Body condition (SMI)	Intercept	6.81 (0.02)	246.26	**<0.001**	**Estimate (SE)**	-0.11 (0.03)	-0.37 (0.03)	-0.32 (0.03)	-0.26 (0.03)	-0.20 (0.03)	0.05 (0.04)
	10 mg/L	-0.11 (0.03)	-2.86	**0.024**	**z-value**	-2.86	-9.47	-8.07	-6.64	-5.24	1.37
	50 mg/L	-0.37 (0.03)	-9.47	**<0.001**	**P**	0.126	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.512
	100 mg/L	-0.32 (0.03)	-8.07	**<0.001**	**N**	30	28	28	28	28	28

4. Discussion

Here, we evaluated whether water-borne CORT could be added to the needed toolkit of biomarkers of physiological stress in amphibian larvae exposed to nitrate pollution. We compared the effect of nitrate exposure on hormone release rates and on other physiological downstream biomarkers. Exposure to nitrate pollution significantly increased age at metamorphosis and water-borne CORT release rates, and led to reduced mass and body condition, but only at higher nitrate concentrations (i.e., 50 and 100 mg/L). Considering this similar sensitivity of different biomarkers in response to nitrate pollution, water-borne CORT might be a reliable complementary biomarker of physiological stress in amphibians exposed to nitrate pollution stress. Environmental pollution is a pervasive stressor that poses a serious threat to amphibians (Hayes et al., 2006; 2010; Rohr et al., 2011; Rohr and Palmer, 2013) and CORT levels have become a prevalent endpoint for assessing stress levels in amphibians in response to pollution stress (McMahon et al. 2011; Davis et al., 2020; Gavel et al., 2021).

4.1 Water-borne CORT can serve as a biomarker for nitrate pollution stress - but with some limitations

A useful biomarker would show a reliable signal with increasing pollution and would show a similar sensitivity to the pollutant as other downstream biomarkers (Tornabene et al., 2021b). We found that water-borne CORT release rates increased in response to increasing nitrate pollution and were significantly higher at the highest nitrate concentrations in comparison to the control group. Other physiological downstream biomarkers such as age,

mass, and body condition at the onset of metamorphosis revealed the same signal with increasing nitrate pollution. Our results indicate that water-borne CORT is a reliable biomarker for nitrate pollution stress at high concentrations but is less sensitive than biomarkers such as mass and body condition. However, its non-invasive and relatively easy use makes it a useful tool with broad applicability. We therefore reinforce that combining several biomarkers in conservation studies will help to assess the effects of environmental stress more holistically and probably better represent complex physiological processes that compose the response to pollution.

As the use of CORT as stress biomarker in amphibian conservation studies is increasing, we suggest considering the following contexts that might affect the reliability of water-borne CORT in laboratory approaches: First, *life stage* might affect CORT release rates through the skin as the amphibian skin changes dramatically during development and metamorphosis (Shi, 2000; Tata, 2006). In a recent study on the Northern leopard frogs (*Lithobates pipiens*), McClelland and Woodley (2021) validated water-borne CORT as a method for prometamorphic tadpoles but not for premetamorphic tadpoles, tadpoles undergoing metamorphosis, or metamorphs. However, these correlations might be species-specific and thus, should be validated accordingly. Second, CORT samples tend to reveal a high variability (personal communication I. Gomez-Mestre) that limit the ability to detect relationships between stressors and water-borne CORT if these exist (Tornabene et al., 2021b). Using a large *sample size* can avoid inconclusive results. Third, *rearing conditions* might affect CORT release rates as tadpoles are exposed to released CORT of their conspecifics in a same aquarium which they can take up through their skin and gills (Wack et al., 2010; Gabor et al., 2018, 2019). This ambient CORT might impact the HPI axis affecting CORT release rates and lead to confounding effects on CORT release rates (Tornabene et al., 2021 b). So, individual housing of the tadpoles would be advantageous but is often not feasible in common garden experiments. Fourth, baseline and stress induced CORT levels are known to be *phylogenetically divergent* (Kulkarni et al., 2017) and thus, need to be validated for each species. For example, Millikin et al. (2019) could not validate the method for spotted salamanders (*Ambystoma maculatum*) and Tornabene et al. (2022) found that water-borne CORT served as a biomarker of salt stress only in *L. pipiens* but not in boreal chorus frogs (*Pseudacris maculata*) or barred tiger salamanders (*Ambystoma mavortium*). Fifth, Mausbach et al. (2022) found clear differences in CORT profiles among different populations of the moor frog (*Rana arvalis*) due to genetic divergences and local adaptation. Therefore, water-borne CORT release rates in response to a specific stressor might be highly *population-specific* and drawing of general conclusions should be avoided.

4.2 Pollution stress may alter metabolic demands as a result of increased CORT levels with possible ramifications for later life stages

In contrast to CORT release rates and age, significant changes in mass and body condition were already detectable at a nitrate concentration of 50 mg/L. Glucocorticoids such as CORT are metabolic active hormones that promote the availability and allocation of energy, and are known to increase metabolic rate and thus, energy demands in vertebrates such as amphibians (e.g., Sapolsky et al., 2000; Kirschman et al., 2017; Preest and Cree, 2008; but see: Francis et al., 2018). For example, Wack et al. (2012) demonstrated that metabolic rate increased with elevated plasma CORT levels in the red-legged salamander (*Plethodon shermani*). If CORT is chronically elevated due to long-term stress exposure, also growth during development is reduced, likely due to changes in metabolism and lipid storage (Dahl et al., 2012; Glennemeier and Denver, 2002 a,b; Bryant et al., 2022).

We suggest that already small increments of endogenous CORT, even if not detectable in water-borne samples, might alter metabolic pathways and led to decreased mass and body condition at metamorphosis found in the present study. Consequently, nitrate pollution stress

may alter metabolic demands as a result of increased CORT levels with possible ramifications for later life stages since the size of larvae at metamorphosis is an effective predictor of (future) fitness in amphibians (Smith, 1987; Beck and Congdon, 2000; Boone et al., 2007; Ruthsatz et al., 2019; but not: Semlitsch et al., 1988; Earl and Whiteman, 2015). Further, chronically elevated CORT levels during larval development are known to lead to adverse effects in the terrestrial stage such as a reduced immune capacity (Gervasi and Foufopoulos, 2008) and locomotory performance (Wack et al., 2013). These results clearly highlight the limits of water-borne CORT as a biomarker for (nitrate) pollution stress. Nevertheless, CORT release rates turned out to be a reliable biomarker at high levels of pollution and thus, can be added to the conservation physiology toolbox.

An additional advantage for the use of multiple biomarkers is that conservation purposes require stress level assessments *in situ*, where tadpoles are exposed to variable environmental conditions that are otherwise standardized in laboratory experiments. For instance, tadpole growth and development are influenced by diet composition (Kupferberg, 1997), local food availability is likely to influence consumption by tadpoles (Kloh et al., 2019) and, on the other hand, tadpoles may actively change their diet preferences in response to changing temperatures (Carreira et al., 2016). Thus, human caused sources of stress are likely to interact with many other factors that could be influencing any particular biomarker (e.g., growth and time to metamorphosis), justifying the simultaneous employment of different ones.

4.3 Nitrate pollution might enable the synergy of CORT and thyroid hormones through endocrine disruption

In amphibians, the HPI axis is the primary endocrine system controlling the physiological response to stressors via the regulation of CORT (Denver, 1997; Gomez-Mestre et al., 2013; Rollins-Smith, 2017). In turn, CORT is known to target the hypothalamus–pituitary–thyroid axis, which is responsible for the production of thyroid hormones (TH) (Carr and Patiño, 2011), the major drivers of amphibian metamorphosis (Furlow and Neff, 2006; Tata, 2006). This endocrine crosstalk induces an increase in developmental rate through the effect of CORT on tissue sensitivity and responsivity to TH (Glennemeier and Denver, 2002a,b; Kulkarni and Buchholz, 2012; Sterner and Buchholz, 2022). In contrast, we found that age at metamorphosis was the highest and thus, developmental rate was the lowest in larvae with the highest CORT release rates indicating the lack of a CORT/TH synergy.

Many pollutants have been shown to disrupt and inhibit the normal action of THs in amphibians, leading to changes in metabolism, growth, and development (rev. in Mann et al., 2009; Carr and Patiño, 2011). Also, nitrate has been suggested to inhibit TH production through competition with iodine uptake in thyroid follicles (rev. in Trudeau et al., 2020), resulting in delayed metamorphosis. Reduced TH levels as a result of nitrate exposure might explain the slower developmental rate at high nitrate concentrations found in the present study.

However, the effects and sensitivity of tadpoles to nitrate seem to be species-specific (Lenuweit, 2009) as Wang et al. (2015) demonstrated that a delayed metamorphosis was related to reduced TH levels in tadpoles of the Chinese toad (*Bufo gargarizans*), whereas Xie et al. (2019) found an increase in developmental rate. Edwards et al. (2006), however, could not find any effect of nitrate exposure on TH levels in tadpoles of the Southern toad (*Bufo terrestris*). Alternatively, a slower developmental rate in animals exposed to high nitrate concentrations might also be explained by additional energetic costs due to detoxification mechanisms (rev. in Sokolova, 2021).

5. Conclusion

Conservation of amphibians is crucial as they face a major global decline, with exposure to aquatic pollution as one of the major contributing factors (Wake and Vredenburg 2008; Alford, 2010; rev. in Trudeau et al., 2020; Hill et al., 2021). Consequently, identifying reliable

biomarkers is essential to detect sublethal effects of pollutants and to investigate whether these are in part responsible for enigmatic amphibian population declines. Here, we could qualify water-borne CORT release rates as a reliable non-invasive biomarker for pollution stress in tadpoles of *R. temporaria* in a controlled single-stressor laboratory approach. Our results also corroborate that the simultaneous use of several biomarkers in conservation studies should help to assess the effects of environmental stress more holistically, as different biomarkers may vary in sensitivity and possibly under different environmental circumstances.

Under natural conditions, amphibian larvae are exposed to multiple simultaneously occurring stressors (Boone et al., 2007; Buck et al., 2012; Gabor et al., 2019) as well as ambient CORT that can alter baseline CORT levels resulting in adaptions to chronical stress exposure and reduced release rates if larvae are exposed to acute stressors (Bryant et al., 2022; Mausbach et al., 2022). Future studies should therefore assess the reliability of water borne CORT levels *in situ* using several populations. It is always important to test whether the laboratory sensitivity of a biomarker is representative of field sensitivity for a given population to aid conservation strategies.

6. Acknowledgements

We thank Ashwini V. Mohan, Robin Schmidt, and Sangeeta Kumari for field assistance. We are grateful to Caitlin Gabor for providing helpful information on the CORT assays and to Ivan Gomez-Mestre and Pablo Burraco for their advice while conceiving the study. We thank Karsten Hiller and Kerstin Schmidt-Hohagen for providing the possibility for carrying out the CORT assays using the microplate reader in the laboratory of the BRICS (Braunschweig Integrated Centre of Systems Biology) and Michelle-Amirah Khalil and Antonia Henne for their help in the laboratory. We thank Meike Kondermann and Gabriele Keunecke for their help in the laboratory in Braunschweig. The study was financed by the DFG (RU 2541/1-1) and the Technical University of Braunschweig.

Supplementary material

Table S1. Natal pond and experimental water parameters (mean ± SD). Natal pond water parameters were determined at the time of animal collection. Quality of tap water used in experiments was monitored twice per week during the experiment. Measurements included nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), pH, phosphate (PO_4^{3+}), copper (Cu^{2+}), iron (Fe), and lead (Pb) in mg x L^{-1}.

Type	NO_3^-	NO_2^-	NH_4^+	PO_4^{3+}	Cu^{2+}	Fe	Pb	pH	N
Pond water	< 6	0.07 ± 0.01	0	0.35 ± 0.11	<0.02	0.14 ± 0.02	0	6.9 ± 0.1	3
Tap water used in experiments	< 6	0	0	0.08 ± 0.01	<0.02	<0.1	0	7.1 ± 0.1	22

Table S2. Effects of nitrate pollution stress on mean (± SD) CORT release rates (pg/g/h), snout-vent length (SVL, mm), body mass (mg), age (dah), and body condition (SMI) in larvae of the European common frog (*Rana temporaria*) at the onset of metamorphosis (Gosner stage 42, Gosner 1960). N is the total number of analysed individual animals, and n is the total number of tested aquaria. See text for further details.

Nitrate exposure (mg/L)	CORT (pg/g/h)	SVL (mm)	Mass (mg)	Age (dah)	SMI	N(n)
0	510,16 (±98,77)	14,20 (±0,49)	877,27 (±107,48)	29.07 (±0,88)	913,71 (±40,71)	15(3)
10	651,65 (±223,13)	14,16 (±0,67)	777,87 (±129,67)	29,80 (±1,65)	815,67 (±56,19)	15(3)
50	804,39 (±293,85)	14,42 (±0,57)	640,85 (±85,90)	30,00 (±1,35)	627,59 (±45,57)	13(3)
100	864,61 (±189,68)	14,73 (±0,66)	732,77 (±74,47)	31,92 (±0,86)	664,04 (±67,54)	13(3)

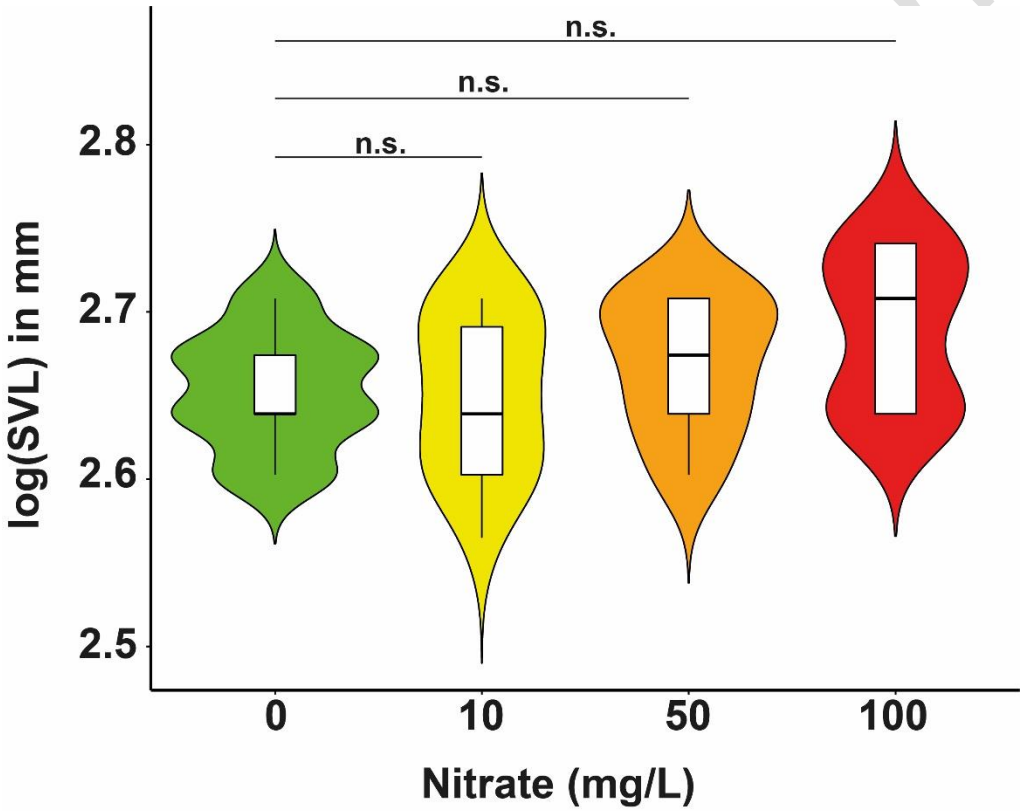


Fig. S1 Effects of nitrate pollution stress on ln(SVL in mm) in larvae of the European common frog (*Rana temporaria*) at the onset of metamorphosis (at least one forelimb present, Gosner stage 42) (Gosner, 1960). Boxes and whiskers show 25th to 75th and 10th to 90th percentiles, respectively; black lines indicate the median. Dots = outliers. Violin plot colors indicate nitrate concentration: green = 0 mg/L, yellow = 10 mg/L, orange = 50 mg/L, and red = 100 mg/L. n.s. = non-significant differences between nitrate treatments

520

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Author contributions

KR conceived and designed the study. KR, FB, and MV collected animals from the field. KR and FB conducted the experiments. KR, and FB carried out the CORT assays. JM supervised the CORT assays. JM contributed, led, and instructed the hormonal analysis and supervised the conduction of extraction and EIA digitally. KR performed the statistical analysis and led the writing of the manuscript. All authors participated in manuscript editing and final approval. KR supervised and administrated the project. KR raised the funding.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Statement of Ethics

The authors have no ethical conflicts to disclose. The experiments were conducted under permission from the *Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit*, Germany (Gz. 33.19-42502-04-20/3590). Fieldwork in Lower Saxony was carried out with permits of Stadt Braunschweig (Stadt Braunschweig - Fachbereich Umwelt und Naturschutz, Richard-Wagner-Straße 1, 38106 Braunschweig; Gz. 68.11-11.8-3.3).

Funding

The study was financed by the DFG (RU 2541/1-1) and the Technical University of Braunschweig.

Figure captions

Fig. 1. Effects of nitrate pollution stress on **A** size corrected ln(CORT release rate in pg/g/h), **B** ln(age in dah), **C** ln(body mass in mg), and **D** ln(SMI) as a measure for body condition in larvae of the European common frog (*Rana temporaria*) at the onset of metamorphosis (at least one forelimb present, Gosner stage 42) (Gosner, 1960). Boxes and whiskers show 25^{th} to 75^{th} and 10^{th} to 90^{th} percentiles, respectively; black lines indicate the median. Dots = outliers. Violin plot colors indicate nitrate concentration: green = 0 mg/L, yellow = 10 mg/L, orange = 50 mg/L, and red = 100 mg/L. Asterisks indicate significant differences between nitrate treatments (*$p<0.05$; **$p < 0.01$; ***$p < 0.001$). n.s. = non-significant differences between nitrate treatments.

Fig. 2 Linear regressions of **A** ln(body mass in mg) and ln(CORT release rates in pg/g/h) and **B** ln(CORT release rates in pg/g/h) and ln(SMI) as a measure for body condition. Regression lines show the significant relationship between two variables, respectively. Dot colors indicate nitrate concentration: green = 0 mg/L, yellow = 10 mg/L, orange = 50 mg/L, and red = 100 mg/L.

Supplementary material

Table S1. Natal pond and experimental water parameters (mean ± SD). Natal pond water parameters were determined at the time of animal collection. Quality of tap water used in experiments was monitored twice per week during the experiment. Measurements included nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), pH, phosphate (PO_4^{3+}), copper (Cu^{2+}), iron (Fe), and lead (Pb) in mg x L^{-1}.

Type	NO_3^-	NO_2^-	NH_4^+	PO_4^{3+}	Cu^{2+}	Fe	Pb	pH	N
Pond water	< 6	0.07 ± 0.01	0	0.35 ± 0.11	<0.02	0.14 ± 0.02	0	6.9 ± 0.1	3
Tap water used in experiments	< 6	0	0	0.08 ± 0.01	<0.02	<0.1	0	7.1 ± 0.1	22

Table S2. Effects of nitrate pollution stress on mean (± SD) CORT release rates (pg/g/h), snout-vent length (SVL, mm), body mass (mg), age (dah), and body condition (SMI) in larvae of the European common frog (*Rana temporaria*) at the onset of metamorphosis (Gosner stage 42, Gosner 1960). N is the total number of analysed individual animals, and n is the total number of tested aquaria. See text for further details.

Nitrate exposure (mg/L)	CORT (pg/g/h)	SVL (mm)	Mass (mg)	Age (dah)	SMI	N(n)
0	510,16 (±98,77)	14,20 (±0,49)	877,27 (±107,48)	29.07 (±0,88)	913,71 (±40,71)	15(3)
10	651,65 (±223,13)	14,16 (±0,67)	777,87 (±129,67)	29,80 (±1,65)	815,67 (±56,19)	15(3)
50	804,39 (±293,85)	14,42 (±0,57)	640,85 (±85,90)	30,00 (±1,35)	627,59 (±45,57)	13(3)
100	864,61 (±189,68)	14,73 (±0,66)	732,77 (±74,47)	31,92 (±0,86)	664,04 (±67,54)	13(3)

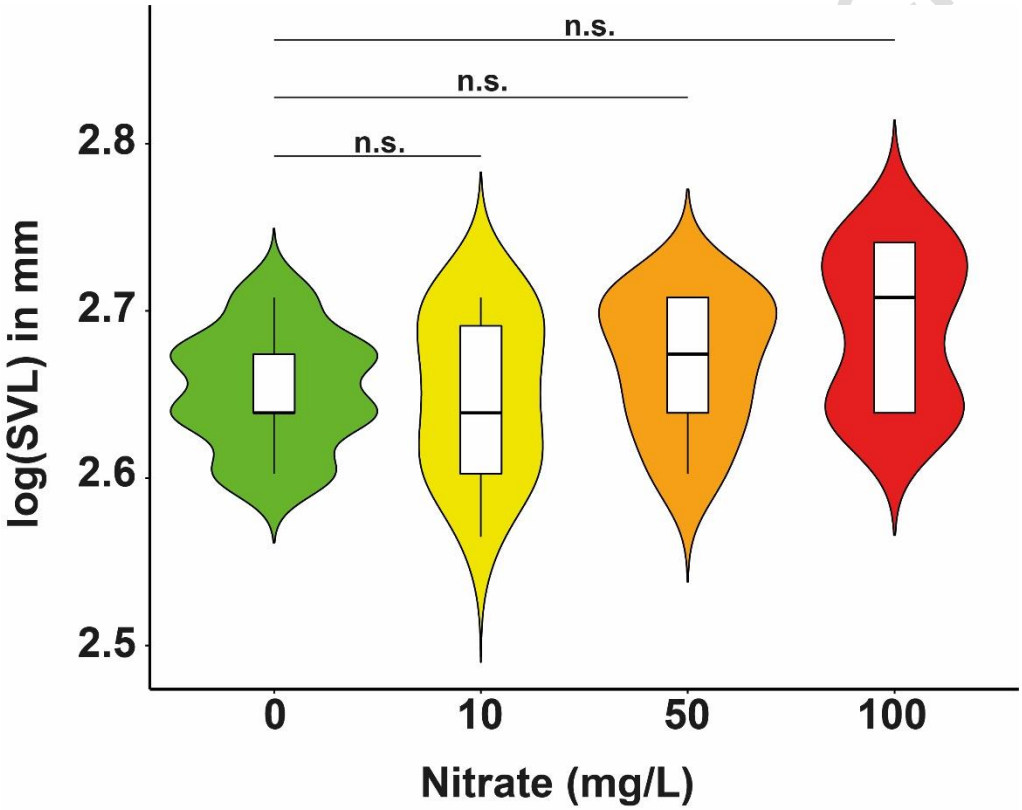

Fig. S1 Effects of nitrate pollution stress on ln(SVL in mm) in larvae of the European common frog (*Rana temporaria*) at the onset of metamorphosis (at least one forelimb present, Gosner stage 42) (Gosner, 1960). Boxes and whiskers show 25th to 75th and 10th to 90th percentiles, respectively; black lines indicate the median. Dots = outliers. Violin plot colors indicate nitrate concentration: green = 0 mg/L, yellow = 10 mg/L, orange = 50 mg/L, and red = 100 mg/L. n.s. = non-significant differences between nitrate treatments.