Title:

Bringing the Lab to the Field: Validating Water-Borne Corticosterone as a Conservation Tool in Captive and Wild Amphibian Larvae

Running title: Using Water-Borne Corticosterone in Wild and Laboratory Amphibian Larvae

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Abstract

Assessing physiological stress in wild populations is essential for detecting early signs of environmental deterioration and for informing conservation strategies before population declines become evident. Non-invasive tools such as water-borne corticosterone (WB CORT) sampling offer a promising means to monitor stress responses in free-living amphibians. However, a central concern in conservation physiology is whether methods validated in laboratory settings provide ecologically relevant estimates when applied to wild individuals exposed to complex environments. Here, we tested the utility and ecological relevance of WB CORT sampling in larvae of the European common frog (*Rana temporaria)* by comparing wild-caught and lab-reared larvae. First, we examined whether environmental origin influenced baseline physiological traits by modeling changes in WB CORT and body mass across ontogeny. Wild larvae exhibited significantly higher baseline WB CORT levels and lower body mass than their lab-reared counterparts, suggesting increased allostatic load under field conditions and divergent growth trajectories. We evaluated the sensitivity of these traits to acute (48 h) exposure to nitrate, a widespread agricultural pollutant commonly found in amphibian breeding ponds. While CORT responses were not significantly altered, nitrate exposure led to a significant reduction in body mass in wild but not lab-reared larvae, indicating origin-specific sensitivity. Finally, we evaluated the validity of WB CORT as a proxy for tissue CORT. WB CORT and tissue CORT levels were positively correlated in both origin groups, with no significant difference in slope or strength of correlation, supporting the reliability of WB CORT as a non-invasive measure of physiological state in both wild and lab-reared larvae. Our findings demonstrate that WB CORT sampling captures biologically meaningful variation in amphibian stress physiology and is a valid tool for monitoring across environmental contexts. Integrating such physiological metrics into field assessments can help identify vulnerable populations and guide proactive amphibian conservation strategies.

1. Introduction

Understanding how organisms respond to environmental stress is fundamental for predicting the impacts of global change on biodiversity. Physiological stress responses, mediated by glucocorticoid hormones, help organisms cope with environmental challenges such as habitat degradation, climate change, pollution, and resource fluctuations (Sapolsky et al. 2000; Sapolsky 2002). These hormones, secreted by the hypothalamic–pituitary–adrenal/inter-renal (HPA/HPI) axis, regulate essential processes such as growth, energy allocation, immune function, and behavior to maintain homeostasis (Romero 2004; Crespi et al. 2013; Kirschman et al. 2017; Rollins-Smith 2017). However, chronic or excessive stress can lead to physiological trade-offs, including reduced growth, impaired immune function, and lower reproductive success, ultimately contributing to population declines (Gervasi & Foufopoulos 2008; Burraco & Gomez-Mestre 2016) At the forefront of the global biodiversity crisis (Luedtke et al. 2023), amphibians are highly vulnerable to environmental stress owing to their permeable skin, hormone-regulated metamorphosis, and dependence on both aquatic and terrestrial habitats (rev. in Ruthsatz & Glos 2023). This exceptional sensitivity makes them widely recognized as key bioindicators of ecosystem health (Waddle 2006; Simon et al. 2011). Monitoring their physiological stress responses can therefore serve as an early warning tool (Walls & Gabor 2019), detecting signs of environmental deterioration before population declines become apparent. Despite their importance, physiological stress assessments often rely on invasive techniques like blood collection, which can induce stress, require extensive handling, and may be impractical in field settings (rev. in Ruthsatz & Rico-Millan et al. 2023). Therefore, developing and validating non-invasive approaches for monitoring stress in wild amphibian populations is crucial for conservation efforts.

Assessing physiological stress in wild populations requires minimally invasive methods that reliably reflect endocrine status. While cortisol is the predominant glucocorticoid (GC) in primates, ungulates, and teleost fish (Schreck & Tort 2016; Suarez-Bregua et al. 2018), corticosterone (CORT) is the major GC in sauropsids (Angelier & Chastel 2009) and amphibians (Glennemeier & Denver 2002). GC levels can be measured from a range of sample media with varying invasiveness (Madliger et al. 2018), from highly invasive (e.g., whole-body homogenates) to mildly invasive (e.g., blood, saliva, or skin swabbing), to non-invasive methods (e.g., passive urine and feces collection) (rev. in Sheriff et al. 2011). Water-borne corticosterone (WB CORT) sampling has emerged as a promising non-invasive technique for assessing stress in aquatic vertebrates, including amphibians (rev. in Scott & Ellis 2007; Gabor et al. 2013; Ruthsatz et al. 2023). This method quantifies CORT released into water via gills, mucous membranes, and skin, eliminating the need for blood sampling or prolonged handling (rev. in McClelland & Woodley 2021). The water in which the specimen has been contained for a standardized time (e.g., 1–2 hours) is then collected and GCs are extracted from the water, followed by measurements through immunoassays (Burraco et al. 2015). WB CORT allows repeated measurements across life stages while minimizing sampling stress, making it an ethically sound tool for physiological monitoring (Narayan et al. 2019; Forsburg et al. 2019).

While WB CORT has been validated for amphibians in laboratory settings (e.g., Forsburg et al. 2019; McClelland & Woodley 2021; Ruthsatz & Rico-Millan 2023), its application in wild populations remains largely untested (but see Gabor et al. 2013; Millikin et al. 2019; Tornabene et al. 2021). Amphibians in natural environments face multiple simultaneous stressors, including predation risk, food limitation, fluctuating temperatures, and chemical pollutants, all of which can influence baseline CORT levels and stress sensitivity (rev. in Ruthsatz et al. 2023; Sinai et al. 2024). Both intrinsic biological factors and external environmental conditions might

further affect the reliability of WB CORT measurements (rev. in Ruthsatz et al. 2023) and must be considered when interpreting data, particularly in wild populations where additional stressors may complicate hormone regulation. Intrinsic factors such as life stage, individual variability, and rearing conditions can influence WB CORT levels, potentially making it a less sensitive biomarker for environmental stress than other sampling methods (rev. in Tornabene et al. 2021). In amphibian larvae, CORT levels fluctuate throughout ontogeny (Glennemeier & Denver 2002; Chambers et al. 2011; Ruthsatz & Rico-Millan et al. 2023), while physiological and morphological changes such as gill degeneration (Burggren & West 1982) and increasing keratinization (rev. in Schreiber & Brown 2003) may alter CORT release dynamics. External factors such as CORT uptake from conspecifics, ambient temperature, skin barrier-affecting pathogens, and environmental pollution can further complicate WB CORT sampling in wild amphibians (rev. in Ruthsatz & Rico-Millan et al. 2023). In particular, chemical contaminants prevalent in breeding habitats may disrupt physiological processes and interfere with CORT excretion through the skin, potentially compromising WB CORT reliability as a stress indicator (McClelland & Woodley 2021). Given the widespread occurrence of pollution in aquatic environments, determining whether WB CORT sampling remains a reliable tool under such conditions is essential.

Among the various environmental stressors affecting amphibian populations, pollution is of particular concern because of its pervasive nature and potential physiological impacts (Luedtke et al. 2023; rev. in Martin et al. 2024). Many amphibians breed in water bodies associated with agricultural landscapes, where exposure to agrochemicals, including pesticides and fertilizers, is common (Brühl et al. 2013; Goessens et al. 2022). Both pesticides and fertilizers have been extensively studied for their detrimental effects on amphibian stress physiology and survival (Van Meter et al. 2019; Trudeau et al. 2020), particularly in combination with other environmental stressors (rev. in Ruthsatz & Glos 2023; Sinai et al. 2024). As a primary component of fertilizers, nitrate (NO_3^-) is one of the most widespread contaminants in amphibian breeding habitats (Rouse et al. 1999; Ortiz-Santaliestra & Sparling 2007), often exceeding natural background levels in disturbed waters (<2 mg L^{-1}; Gomez-Isaza & Rodgers 2022). Nitrate exposure has been shown to induce a physiological stress response in amphibians (Wang et al. 2015; Poulsen et al. 2018; Ruthsatz et al. 2022), increasing CORT levels, an effect we previously demonstrated using WB-CORT collection under laboratory conditions (Ruthsatz et al. 2023). However, it remains unclear whether this response is equally detectable in wild amphibians, where individuals have already been exposed to multiple environmental stressors. If validated in wild populations, WB-CORT sampling could provide a reliable, non-invasive tool for assessing the physiological impact of nitrate pollution in amphibian breeding ponds and serve as an early-warning tool for amphibian conservation (Ricci et al. 2025).

Here, we evaluated whether WB CORT sampling, a method previously validated in laboratory-reared and captive-bred amphibian larvae, can be reliably applied to wild-caught individuals across different life stages. Using the early-breeding European common frog (*Rana temporaria*) as a model species, we tested the utility of WB CORT to assess physiological stress responses to nitrate pollution during the aquatic larval stage. Specifically, we aimed to: (1) determine whether wild-caught larvae exhibit higher baseline CORT levels and lower body mass than their lab-reared counterparts, potentially reflecting exposure to additional environmental stressors such as predation, pollution, and limited food availability; (2) assess whether the developmental trajectories of CORT release and body mass across Gosner stages are consistent between wild and lab-reared groups despite differing environmental conditions; (3) investigate whether prior exposure to multiple stressors in the wild reduces the larvae's

sensitivity to acute nitrate exposure relative to lab-reared tadpoles; and (4) evaluate the reliability of WB CORT sampling for detecting nitrate-induced stress in wild-caught larvae by comparing it with tissue CORT levels.

2. Material & Methods

2.1 Ethics statement

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 from Stadt Braunschweig (Stadt Braunschweig-Fachbereich Umwelt und Naturschutz, Richard-Wagner-Straße 1, 38 106 Braunschweig; Gz. 68.11-11.8-3.3).

2.2 Study species, field sampling, and experimental design

The European common frog (*R. temporaria)* was chosen as the model species because it is the most widespread anuran species in Europe and occurs in natural as well as anthropogenic habitats. While the International Union for Conservation of nature lists *R. temporaria* in the "least concern" category, agricultural pollution is recognized as a specific threat for this species (IUCN 2021).

To address the study hypotheses, we combined two datasets collected during different breeding periods. The first dataset was gathered in 2021 and includes data from various ontogenetic stages of *R. temporaria*, including larvae and metamorphs from both laboratory and field conditions, as well as adults collected exclusively from the field. The second dataset, collected in 2022, consists of laboratory- and field-reared larvae before and after exposure to three different nitrate concentrations (0, 50, 100 mg/L). The integration of both datasets allowed us to examine differences in CORT release rate and body mass across developmental stages, comparing field- and laboratory-reared individuals, while also assessing the potential effects of nitrate exposure on stress responses.

Fieldwork was conducted at Kleiwiesen (52.328°N, 10.582°E), a site in central Germany near Braunschweig, Lower Saxony. Although located close to the city, the area is surrounded by agricultural land. The site features a system of ponds bordered by meadows and mixed deciduous beech forest, providing a suitable habitat for a large population of *R. temporaria*. The nitrate concentration of the pond water at this site was measured using the AQUA-Check 2 photometer (Söll GmbH, Germany) during both experimental years at the time of animal collection (Table S1).

2.2.1 2021 experiment

The 2021 dataset includes data from both laboratory and field environments. During the breeding season, adult *Rana temporaria* (both males, N=3, and females, N=8) were collected at the study site during the night on 13 and 22 March 2021. Later in the season, on 16 and 17 June, larvae at eleven different ontogenetic stages (Gosner stage 33 to the juvenile stage; N = 37) were collected during the day for WB CORT measurements, followed by the determination of dry-blotted body mass (see Section 2.3). Field-collected larvae hatched and developed under natural conditions in a pond at the site.

For lab-reared animals, the experimental design followed those of Ruthsatz et al. (2023) and Ruthsatz et al. (2022) but without nitrate exposure. Briefly, three amplectant pairs were collected on 22 March and transported to the Zoological Institute of the TU Braunschweig, where each pair was placed in a plastic container filled with 8 cm of pond water, some leaves,

and branches until spawning occurred. Egg masses were collected within 12 hours of oviposition, and the adults were then released back into their original pond at Kleiwiesen. The experiment was conducted in a climate chamber (Kälte-Klimatechnik-Frauenstein GmbH, Germany) under a 14:10 h light:dark cycle. Temperature was maintained at 10 ± 0.2°C during hatching and increased to 18 ± 0.1°C after larvae reached Gosner stage 25 (free-swimming larvae; Gosner 1960). After hatching, larvae were allowed to develop to stage 25 before being transferred to three standard 12-L aquaria (15 larvae per aquarium; total N = 45). Larvae from the different clutches were equally mixed during this transfer, and the initial larval density was 1.66 larvae per liter. Larvae were fed *ad libitum* with a 50:50 mixture of high-protein powdered fish food (Sera Micron, Sera, Germany) and *Spirulina* algae. Upon completing metamorphosis at Gosner stage 46, all surviving juveniles were transferred to individual plastic containers with a small amount of water to prevent desiccation. Juveniles were fed *ad libitum* with adult *Drosophila melanogaster* for ten days before undergoing final measurements. At 15 different ontogenetic stages (Gosner stage 22 to the juvenile stage; N=168), WB CORT was measured first, followed by the determination of dry-blotted body mass (see Section 2.3).

Ontogenetic stages were determined for both wild and lab-reared larvae based on the progression of key morphological features following Gosner (1960). Developmental stages were assessed through visual inspection with the naked eye or, when necessary, under a binocular microscope (Keyence X-500).

2.1.1 2022 experiment

The 2022 dataset includes both a laboratory and a field experiment to compare the acute stress response to nitrate exposure in larvae that developed under either controlled laboratory conditions or natural field conditions. For the field experiment, larvae hatched and developed under natural conditions in a pond at the Kleiwiesen study site. On 23 and 31 May 2022, when they reached Gosner stage 37–40 (i.e., pro-metamorphic larvae with fully separated toes; Gosner 1960), 27 individuals were collected and temporarily housed in buckets with pond water in the field until measurements were completed. For lab-reared animals, the experimental design followed Eterovick et al. (2024) and Sinai et al. (2024). Briefly, five clutches were collected at the Kleiwiesen and transported to the Zoological Institute of the Technische Universität Braunschweig. The eggs were kept at 14 ± 0.2°C in five 12-L plastic buckets filled with 5 L of fresh pond water. After hatching, larvae were allowed to develop to Gosner stage 25 before three animals from each clutch were moved to three standard 12-L aquaria, each filled with 9 L of dechlorinated water (3 replicates × 15 larvae = 45 individuals). Larval density was 1.66 larvae/L in the beginning of the experiment. The experiment was conducted in a climate chamber at the Zoological Institute of the TU Braunschweig with a 14:10 h light:dark cycle at 18 ± 0.1°C, corresponding to the average temperatures encountered in the field throughout development. Larvae were fed following the same protocol as the lab-reared larvae in 2021.

At Gosner stage 38 for lab-reared larvae (N=27) and Gosner stage 37–40 for field-collected larvae (N=27), WB CORT was measured first to assess baseline CORT release rates, followed by the determination of dry-blotted mass to the nearest 0.001 g using an electronic balance (Professional Digital Jewellery Gold Scale Balance, GandG, Germany) (see Section 2.3). Following these measurements, all the 54 larvae were individually and randomly assigned to 4.4-L buckets containing 2 L of dechlorinated, ultrafiltered tap water with one of three nitrate concentrations (0, 50, and 100 mg/L). Tap and pond water samples (50 mL; Table S2) were taken for background WB CORT measurements before and after nitrate exposure (see Section 2.3.1). The buckets were covered with cling film with perforations for ventilation. The experiment was conducted at the respective rearing temperature (Lab: 18 ± 0.1°C) or

temperature at collection (Field: 15.31 ± 2.10°C). Lab and field larvae were exposed to acute nitrate treatments for 48 h without water changes and were fed *ad libitum* using the same food as in previous stages. At the end of the exposure period, WB CORT was measured to assess CORT release rates in response to acute nitrate pollution stress, followed by dry-blotted body mass (see Section 2.3). For field larvae, all measurements were conducted on-site in the field. After the measurements, field larvae were grouped into three buckets according to their treatment and transported to the Zoological Institute of the TU Braunschweig for tissue CORT measurements (see Section 2.3).

Nitrate concentrations were achieved by adding the respective amount of reagent-grade sodium nitrate ($NaNO_3$, >99% pure; Carl Roth, Germany; Oromí et al. 2009; Wang et al. 2015) to the water before introducing the larvae. Previous research on nitrate toxicity in amphibians has shown that sodium nitrate is less toxic than ammonium nitrate (NH_4NO_3), which has been used in several amphibian studies (e.g., Johansson et al. 2001; Garriga et al. 2017). The selected nitrate concentrations fall within environmental ranges observed in both surface and groundwater in Germany (Sundermann et al. 2020), align with levels measured in amphibian breeding habitats (e.g., De Wijer et al. 2003; Rouse et al. 1999; Johansson et al. 2001; rev. in Sinai et al. 2024), and have been shown to induce an increase in WB-CORT levels in lab-reared larvae (Ruthsatz et al. 2023; Eterovick et al. 2024).

2.3 Corticosterone collection and assay

2.3.1 CORT sample collection

Water-borne CORT was collected immediately after capture to avoid handling stress, following the procedure of Gabor et al. (2013). Briefly, each specimen was placed in a separate 250-mL glass beaker (for larvae, metamorphs, and juveniles) or a 500-mL polypropylene beaker (for adults) containing 50 or 100 mL of ultrafiltered tap water, respectively (20 mL for juveniles to prevent drowning), for 1 hour to collect water-borne hormones. The sampling beakers were placed in a water bath (lab-reared animals; 18°C) or into the pond (field-collected animals; 15.31 ± 2.10°C for the field larvae in 2022) to maintain a constant temperature during hormone collection.

After the collection period, animals were gently removed, weighed using either a portable electronic balance in the field (Professional Digital Jewellery Gold Scale Balance, GandG, Germany) or a stationary precision balance in the lab (Sartorius A200 S, Germany). All samples were collected during the daytime, except for adult samples, which were taken between 22:00 and 02:00 h, corresponding to their peak activity period. Field-collected animals from the 2021 experiments were subsequently released at the site, whereas lab-reared animals were moved back to their aquaria and released at the Kleiwiesen site after completing metamorphosis following the requirements of the permitting authority. All water samples were stored at −20°C until processing.

In the 2022 experiment, all 54 larvae (both lab-reared and field-collected) were individually placed in a 250-mL glass beaker and anesthetized in 50 mL of buffered ultrapure water containing 2 g/L tricaine methanesulfonate (MS-222; Ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich) until they no longer responded to external stimuli, following the measurements. Then, each specimen was then immediately placed in a sterile 1.5-mL tube and snap-frozen in liquid nitrogen for tissue CORT analysis to validate WB-CORT measures. Frozen samples were stored at −80°C until processing.

To measure CORT concentrations in pond water as well as in filtered tap water, three 50-mL samples were collected per water source and experiment in both years. In 2022, pond and tap water samples were collected before and after nitrate exposure.

2.3.2 CORT sample processing

Water-borne and tissue CORT samples were processed following the procedure of Ruthsatz & Rico-Millan et al. (2023) in the respective sampling year.

Thawed water samples were first filtered through Q8 Whatman filter paper to remove suspended particles and feces, then passed through C18 solid-phase extraction columns (Oasis Vac Cartridge HLB 3 cc/60 mg, 30 µm; Waters, Inc., Switzerland) using a vacuum manifold (Visiprep Vacuum Manifold; Sigma-Aldrich, Germany). The manifold was cleaned before each use with 4 mL of HPLC-grade ethanol and 4 mL of nanopure water. Columns were stored at −20°C until hormones were eluted with 4 mL of HPLC-grade methanol. Samples were transferred into 5-mL Eppendorf tubes, and methanol was evaporated using a sample concentrator (Stuart SBHCONC/1; Cole-Parmer, UK) under a fine N_2 stream at 45°C with a block heater (Stuart SBH130D/3; Cole-Parmer, UK). Dried samples were stored at −20°C until enzyme-immunoassay (EIA) analysis. Before analysis, samples were re-suspended in 125 µL of a 5% ethanol (95% lab grade) and 95% EIA buffer solution and frozen at −20°C until hormonal level measurements.

Tissue CORT samples were shipped on dry ice via overnight express to Doñana Biological Station (EBD-CSIC) in Seville, Spain. Samples were randomly thawed, weighed to the nearest 0.0001 g, and homogenized in 16 × 100 mm glass tubes with 500 µL PBS buffer (AppliChem Panreac, Germany) using a homogenizer at ∼17,000 rpm (Miccra D-1, Germany). The tissue blender was rinsed with an additional 500 µL PBS buffer to collect residual material, then cleaned with ddH_2O and 96% EtOH between samples. After homogenization, 4 mL of a 30:70 petroleum ether:diethyl ether mixture (Sigma-Aldrich, Germany) was added, vortexed for 60 s, and centrifuged at 1800 g and 4°C for 15 min. The organic top layer containing CORT was collected and placed in a new 13 × 100 mm glass tube. This extraction was repeated to ensure maximum CORT recovery, and pooled ether fractions were evaporated using a sample concentrator (Techne FSC400D; Barloworld Scientific, UK) under a constant nitrogen flow. Lipids were re-suspended in 315 µL of EIA buffer (DetectX Corticosterone ELISA kit, K014-H5, Arbor Assays, Ann Arbor, MI, USA) via vortexing and incubated overnight at 4°C.

Hormone levels were measured using DetectX Corticosterone ELISA kits (K014-H5, Arbor Assays, USA), previously validated for *R. temporaria* (Burraco et al., 2017; Ruthsatz et al., 2023). WB CORT and tissue CORT were analyzed using 50-µL and 100-µL assay formats, respectively. CORT concentrations were measured in duplicates (WB CORT) and triplicates (tissue CORT) on 96-well plates following the manufacturer's instructions. Plates were read at 450 nm using a Tecan Spark® Microplate Reader (Tecan, Switzerland) in Braunschweig and a multimode microplate reader (MB-580, Heales) in Seville.

Hormonal concentrations were calculated using MyAssays online tools based on calibration standards provided with the DetectX kit (https://www.myassays.com/arbor-assays-corticosterone-enzyme-immunoassay-kit-improved-sensitivity.assay). A new standard curve was generated for each plate. For WB CORT, the mean coefficient of variation of duplicates was 25.65% in 2021 and 10.92% in 2022. A pooled WB CORT sample served as a positive control to calculate intra- and inter-plate variation: intraplate variation was 21.64% (2021) and 15.36% (2022), and interplate variation averaged 29.83% (2021) and 18.34% (2022). For tissue CORT, triplicates with a coefficient of variation ≤30.0% or an absolute mean-median difference ≤2.5 pg were retained; outliers were removed from triplicates that did not meet these criteria (Ruthsatz & Rico-Millan et al. 2023). The mean coefficient of variation for triplicates was 8.53%, with intra-plate and inter-plate variations of 8.1% and 11.35%, respectively. Each ELISA plate included a negative control, and background corticosterone levels were subtracted

from hormone samples. The average R^2 for the 4PLC fitting curve was 0.974 (2021) and 0.992 (2022) for WB CORT, and 0.997 for tissue CORT.

WB CORT was expressed as the release rate (pg/g/h; Gabor et al. 2013), while tissue CORT was expressed in pg.

2.4 Statistical analysis

All statistical analyses were conducted in R (v.4.3.2; R Core Team 2023). Data analysis and visualization were performed in R using ggplot2 (Wickham 2016) and ggpattern (Mike FC & Davis 2022) for graphic creation, readxl (Wickham & Bryan 2025) for importing Excel data; dplyr (Wickham et al. 2023) and tidyr (Wickham & Müller 2024) for data manipulation; patchwork (Pedersen 2024) for combining plots; and car (Fox & Weisberg, 2019), along with performance (Lüdecke et al. 2021) for regression diagnostics and model performance assessment. Linear models were fitted using the *lm*() function. Model assumptions were evaluated visually through residual plots and tested statistically with the Shapiro-Wilk test (*shapiro.test()*) for normality and the Breusch-Pagan test (*bptest()* function from lmtest package; Zeileis & Hothorn 2002) for homoscedasticity. Model performance was assessed using adjusted R^2 values and diagnostic plots.

To test Hypotheses 1 and 2 (effects of origin and ontogeny on corticosterone levels and body mass), we fitted separate linear models for WB CORT and body mass (g) as dependent variables. Each model included Origin (Field or Lab), Gosner stage (GS), and their interaction as fixed effects. To aid interpretation of model intercepts and interaction terms, Gosner stage was treated as a continuous covariate and mean-centered (i.e., GS_c = GS − mean[GS]). This centering ensured that model intercepts reflected average responses at the mean developmental stage, thereby avoiding extrapolation to biologically implausible GS values. The interaction term between Origin and mean-centered Gosner stage (GS_c) was included to assess whether the developmental trajectories of CORT levels and body mass differed between Field and Lab tadpoles, allowing us to test if changes across developmental stages varied by origin.

To test the effects of acute nitrate exposure on stress physiology and whether responses differ by origin (Hypothesis 3), we calculated the change in corticosterone levels and body mass for each individual as the difference between measurements before and after 48 hours of nitrate exposure within each origin. Owing to non-normality, CORT differences were log-transformed (log[CORT difference + 1]) before modeling. Linear models were fitted with Origin, Nitrate treatment (0, 50, and 100 mg/L), and their interaction as fixed effects. Mass differences met normality assumptions and were modeled without transformation. Post hoc pairwise comparisons were conducted using Tukey's HSD via the *emmeans()* function from the emmeans package (Lenth 2023), with adjustment for multiple testing.

To address Hypothesis 4 (validity of WB as a proxy for tissue CORT), we first assessed linear correlations between WB CORT and tissue CORT overall and by origin using Pearson's correlation (*cor.test()* function). Then, a linear model was fitted with WB CORT as the response, tissue CORT, Origin, and their interaction as predictors, to test if the relationship differed by origin. Predictor significance was evaluated using Type III ANOVA via the *Anova()* function from the car package.

3. Results

3.1 The effects of environmental origin and ontogeny on baseline CORT levels and body mass

CORT levels – There was a significant main effect of origin on CORT concentrations (Fig. 1A; Table 1). Lab-reared larvae exhibited lower baseline CORT levels than wild-caught larvae (Estimate = –411.4 ± 161.1 pg/g/h, $t = -2.55$, $p = 0.0115$). The main effect of ontogenetic stage was not significant (Estimate = 1.41 ± 28.1, $p = 0.96$), indicating no clear change in CORT across GS in the field group (Fig. 1A; Fig. S2A; Table 1). However, there was a significant origin × GS_c interaction (Estimate = –67.6 ± 28.9, $t = -2.34$, $p = 0.0206$), suggesting divergent ontogenetic trajectories of CORT between groups (Table 1). Post hoc comparisons at the mean GS_c revealed that wild-caught larvae had significantly higher CORT levels than lab-reared counterparts (difference = 425 pg/g/h, $p = 0.0076$).

Body mass – Lab-reared larvae were significantly heavier than wild-caught larvae at the mean developmental stage (Estimate = 0.139 ± 0.053 g, $t = 2.63$, $p = 0.0093$; Fig. 1B; Table 1). The main effect of ontogenetic stage on mass in the field group was not significant (Estimate = –0.0023 ± 0.0092, $p = 0.81$), but the interaction between origin and GS_c was significant (Estimate = 0.033 ± 0.0095, $t = 3.47$, $p = 0.00065$; Table 1). Lab-reared larvae showed an increase in mass with advancing GS (Fig. S2B), whereas wild-caught larvae did not.

Adult common frogs were excluded from statistical analyses, but descriptive data show that mean (±SD) water-borne CORT release rates were 0.521 ± 0.672 pg/g/h in females and 0.220 ± 0.102 pg/g/h in males. Mean body mass was 132.70 ± 68.23 g for females and 154.33 ± 38.31 g for males.

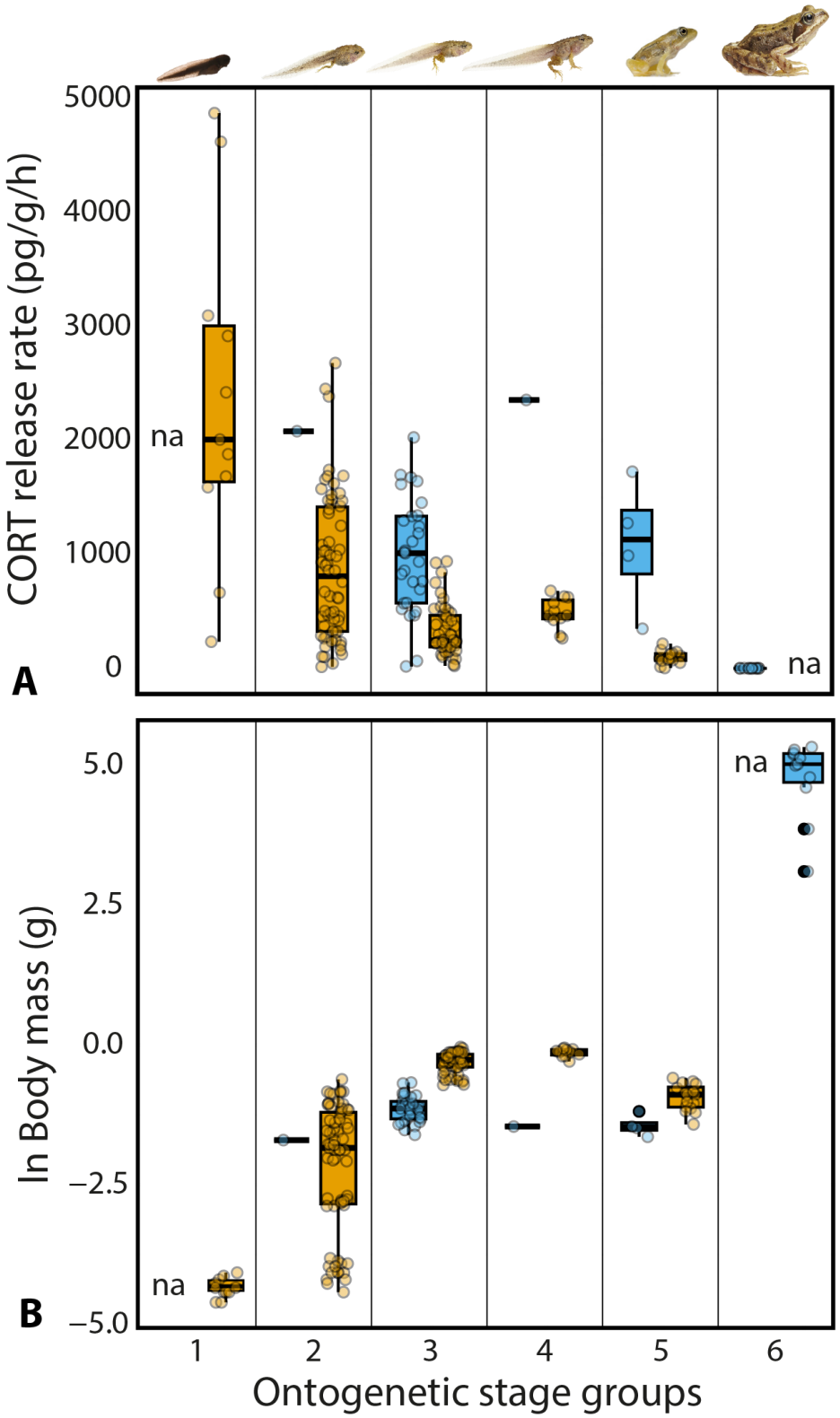

Figure 1. Effects of environmental origin (lab-reared vs field-collected) and ontogeny on **A** water-borne baseline corticosterone (CORT) release rates (pg/g/h) and **B** ln body mass (g) of *Rana temporaria*. Boxes and whiskers show 25th to 75th and 10th to 90th percentiles, respectively; black lines indicate the median. Blue for field-collected animals. Orange for lab-reared animals. Ontogenetic stage groups sensu Gosner (1960): 1 = newly hatched (GS 20–22); 2 = pre-metamorphic (GS 23–34); 3 = pro-metamorphic (GS 35–41); 4= metamorphic (GS 42–46); **5** = post-metamorphic/juvenile (>GS 46); **6** = adults (indicated through nuptial pads in males and

eggs in females; adults were not included in the statistical analysis). Further details on the methods in the main text.

3.2 Prior exposure to multiple stressors in the wild influences mass loss, but not CORT response, to acute nitrate stress

For CORT difference, we performed a log transformation because the data were not normally distributed (Shapiro test: $p = 0.012$). Mass difference, on the other hand, was normally distributed (Shapiro test: $p = 0.673$), and no further adjustments were necessary. We then fitted linear models testing for the effects of origin (Field or Lab), nitrate concentration (Nitrate A–C), and their interaction.

CORT levels – The model testing log-transformed CORT differences revealed no statistically significant main or interaction effects ($F_{(5,42)} = 1.202$, $p = 0.325$; Table 1; Fig. 2A; Fig. S3A). Neither origin nor nitrate concentration significantly affected post-exposure CORT release rates. A weak trend was observed for the Origin × 100 mg/L nitrate interaction, suggesting a possible difference in CORT response between field and lab tadpoles at the highest nitrate concentration, but this result was not statistically significant.

Body mass – In contrast, the model assessing mass differences showed a significant overall effect ($F_{(5,44)} = 7.838$, $p < 0.001$; Table 1; Fig. 2B; Fig. S3B). Lab-reared tadpoles lost less mass overall than field-collected individuals ($p = 0.001$), and a significant Origin × 100 mg nitrate/L interaction ($p < 0.001$) indicated that lab tadpoles exhibited a markedly different mass response at the highest nitrate concentration. Post hoc comparisons confirmed that field tadpoles lost more mass than lab tadpoles at both low and intermediate nitrate levels [Field (0 mg nitrate/L) vs Lab (0 mg nitrate/L): $p = 0.014$; Field (0 mg nitrate/L) vs Lab (50 mg nitrate/L): $p = 0.004$], while lab tadpoles exposed to 100 mg nitrate/L lost significantly more mass than those exposed to lower nitrate concentrations [Lab (0 mg nitrate/L) vs Lab (100 mg nitrate/L): $p < 0.001$; Lab B vs Lab C: $p < 0.001$].

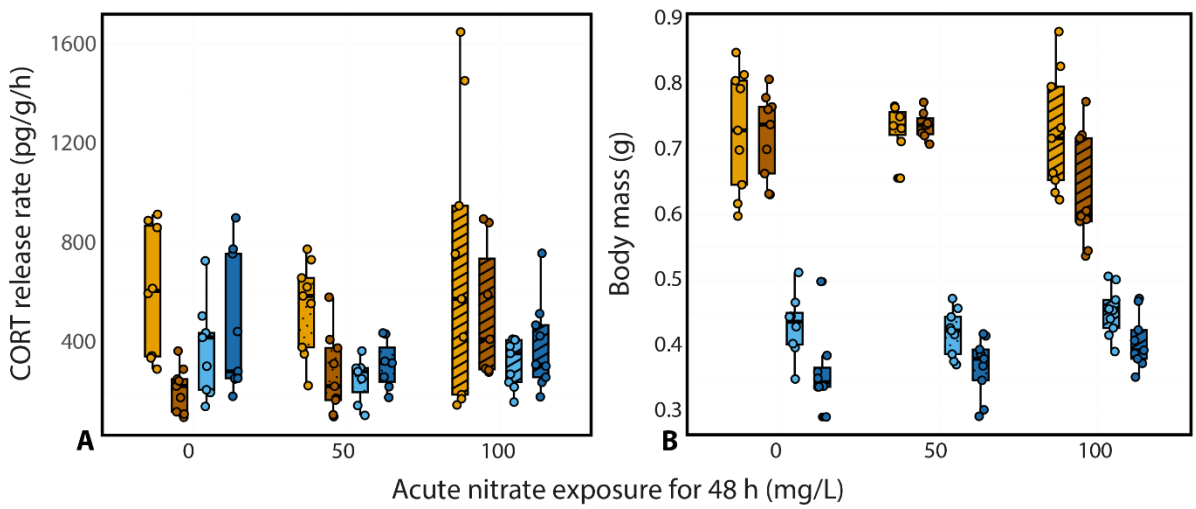

Figure 2. Differences in **A** water-borne corticosterone (CORT) release rate (pg/g/h) and **B** body mass of *Rana temporaria* (g) before and after acute nitrate exposure. Boxes and whiskers show 25th to 75th and 10th to 90th percentiles, respectively; black lines indicate the median. Blue for field-collected animals: bright blue for animals before nitrate exposure, dark blue for animals after nitrate exposure. Orange for lab-reared animals: bright orange for animals before nitrate exposure, dark orange for animals after nitrate exposure. Dotted pattern for exposure to

50 mg/L nitrate. Striped pattern for exposure to 100 mg/L nitrate. Further details on the methods in the main text.

3.3 Water-borne corticosterone reliably reflects tissue corticosterone but shows origin-dependent differences

To evaluate the reliability of water-borne CORT as a non-invasive indicator of nitrate-induced stress, we assessed its relationship with tissue CORT across all samples and by origin (Field vs Lab). Overall, water-borne CORT was strongly and positively correlated with tissue CORT (Pearson's $r = 0.841$, $p < 0.001$), indicating a reliable relationship between the two measures. When analyzed separately by origin, the correlations remained very strong for both field-collected ($r = 0.980$, $p < 0.001$) and lab-reared tadpoles ($r = 0.982$, $p < 0.001$), confirming consistent concordance within groups.

A linear model including tissue CORT, Origin, and their interaction revealed significant main effects of tissue CORT ($\beta = 3.18 \pm 0.11$, $t = 28.09$, $p < 0.001$) and Origin (Lab) ($\beta = 224.02 \pm 64.50$, $t = 3.47$, $p = 0.001$), as well as a significant tissue CORT × Origin interaction ($\beta = -1.81 \pm 0.13$, $t = -13.79$, $p < 0.001$). These effects were confirmed by Type III ANOVA (all $p < 0.01$). The model explained 96.6% of the variance in water-borne CORT (adjusted $R^2 = 0.966$).

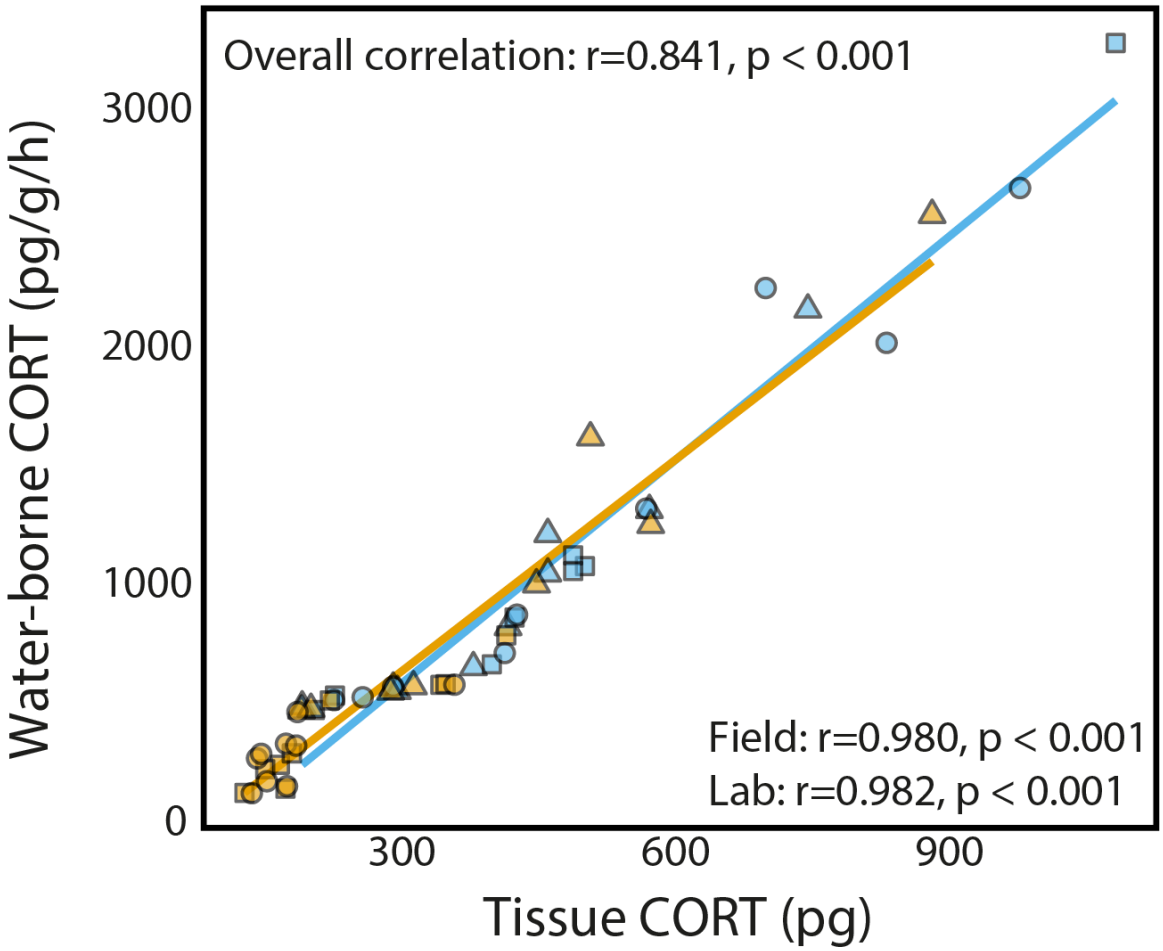

Figure 3. Relationship between tissue corticosterone (pg) and water-borne corticosterone (pg/g/h) in *Rana temporaria* tadpoles. Each point represents an individual tadpole. Blue for field-collected animals. Orange for lab-reared animals. Solid lines show fitted linear

regressions for each origin group. Circle: 0 mg/L nitrate. Square: 50 mg/L nitrate. Triangle: 100 mg/L nitrate. Correlation coefficient and p values for Pearson's correlation. Further details on the methods in the main text.

Table 1. Summary of linear model results examining the effects of larval origin (Field vs Lab), ontogenetic stage (centered Gosner stage; GS), and nitrate exposure (0, 50, 100 mg/L) on physiological traits in *Rana temporaria* tadpoles. Separate models were fitted for (1) water-borne corticosterone (CORT) release rates, (2) body mass, log-transformed changes in (3) CORT and (4) body mass following acute nitrate exposure, and (5) the relationship between water-borne (WB) and tissue-based CORT levels. Estimates are presented with standard errors, *t*-values, *p*-values, and significance levels (p <0.05 = *, <0.01 = **, <0.001 = ***). "ns" denotes non-significant effects (p >0.05).

Dependent variable	**Fixed effects**	**Estimate**	**Std. Error**	**t-value**	**p-value**	**Significance**
CORT release rates (pg/g/h)	Intercept (Field)	1064.4	152.7	6.97	<0.001	***
	Lab	−411.4	161.1	−2.55	0.0115	*
	GS	1.41	28.1	0.05	0.96	ns
	Lab*GS	−67.6	28.9	−2.34	0.0206	*
Body mass (g)	Intercept (Field)	0.318	0.05	6.34	<0.001	***
	Lab	0.139	0.053	2.63	0.0093	**
	GS	−0.0023	0.0092	−0.25	0.81	ns
	Lab*GS	0.033	0.0095	3.47	0.00065	**
Log-transformed difference in CORT release rate before and after Nitrate exposure (pg/g/h)	Intercept (Field, 0 mg/L)	7.477	0.595	12.559	<0.001	***
	Lab	0.49	0.868	0.565	0.575	ns
	50 mg/L	0.176	0.941	0.187	0.853	ns
	100 mg/L	0.139	0.842	0.165	0.869	ns
	Lab*50 mg/L	−0.242	1.28	−0.189	0.851	ns
	Lab*100 mg/L	−2.106	1.25	−1.685	0.1	ns
Log-transformed difference in Body mass before and after Nitrate exposure (g)	Intercept (Field, 0 mg/L)	0.0764	0.0147	5.193	<0.001	***
	Lab	−0.0683	0.0196	−3.481	0.001	**
	50 mg/L	−0.0242	0.0196	−1.234	0.224	ns
	100 mg/L	−0.0315	0.0196	−1.607	0.115	ns
	Lab*50 mg/L	0.0101	0.0278	0.364	0.718	ns
	Lab*100 mg/L	0.1174	0.0269	4.37	<0.001	***
WB CORT (pg/g/h)	Intercept (WB CORT)	−183.74	53.29	−3.45	0.0012	**
	Tissue CORT	3.18	0.11	28.09	< 0.001	***
	Lab	224.02	64.5	3.47	0.0011	**
	Tissue CORT*Lab	−1.81	0.13	−13.79	< 0.001	***

4. Discussion

Amphibians, particularly during their aquatic larval stage, are highly susceptible to environmental stressors because of their limited dispersal capacity and their permeable skin (Hayes et al. 2006; Yu et al. 2015). These stressors often elicit sublethal physiological effects that can impair growth, development, and stress resilience, potentially weakening annual recruitment and contributing to long-term, cryptic or silent population declines (Ruthsatz et al. 2022). Non-invasive methods that enable repeated physiological assessments across developmental stages are therefore essential for monitoring the sublethal impacts of environmental change, and may serve as early-warning tools for amphibian conservation (Walls & Gabor 2019). However, a key concern in ecological and conservation physiology is whether tools validated in controlled laboratory settings or on lab-reared animals yield ecologically meaningful estimates when applied to wild populations (Pintanel et al. 2020), which are exposed to complex, fluctuating environments and multiple stressors. In this study, we first compared WB CORT levels and body mass between wild-caught/field-collected and lab-reared amphibian larvae across development, to assess how environmental origin influences baseline physiological traits. We then tested the sensitivity of these traits to acute nitrate pollution, a widespread agricultural stressor in amphibian breeding ponds, and finally evaluated the validity of WB CORT as a non-invasive proxy for endogenous (tissue) CORT levels by testing how reliably WB CORT reflects tissue CORT concentrations in both wild and lab-reared larvae.

4.1 Environmental origin shapes baseline CORT levels and growth patterns in amphibian larvae

Understanding how baseline physiological traits vary between lab-reared and wild individuals is essential for evaluating the ecological relevance of stress metrics, especially when applying tools developed or validated under controlled laboratory conditions to populations exposed to natural environmental complexity (Tornabene et al. 2021). Our study revealed that environmental origin significantly influenced both body mass and baseline WB CORT levels in larvae of *R. temporaria*, with clear divergence between field-collected and lab-reared individuals across developmental stages. Wild larvae exhibited consistently higher WB CORT concentrations and lower body mass than their lab-reared counterparts, suggesting that field conditions impose a greater allostatic load and energetic constraints. Exposure to multiple, chronic, or unpredictable stressors can activate the HPI axis (rev. in Awkerman et al. 2024), resulting in elevated baseline glucocorticoid levels, which are commonly associated with increased metabolic demand and resource reallocation away from growth and toward maintenance (Romero 2004; Rich & Romero 2005; Boonstra 2013). Unlike lab-reared larvae raised under stable conditions with *ad libitum* food access, wild larvae must forage actively under environmental uncertainty, which probably increases both their energy expenditure and physiological stress load (Moyers & Hagger 2023). Together, these constraints help explain the lower body mass observed in field-collected larvae (Bryant et al. 2022). Another potential source of variation in WB CORT measurements is environmental/ambient CORT exposure. Environmental glucocorticoids can originate from both anthropogenic sources (e.g., pharmaceuticals in runoff; Stavreva et al. 2012) and organismal release within high-density groups (Gabor et al. 2018; Tornabene et al. 2021). Such ambient CORT can influence the observed WB CORT levels through passive uptake or feedback mechanisms. For instance, elevated environmental CORT may lead to increased baseline readings, as potentially occurred in our wild-caught larvae. However, other studies have shown that chronic exposure to ambient CORT can suppress endogenous hormone production, leading to lower or even negative net

release rates (Gabor et al. 2018; Tornabene et al. 2021). This suppression could occur via feedback inhibition or equilibrium effects, where organisms absorb CORT from their surroundings and reduce internal synthesis and diffusion. Indeed, amphibians (both larvae and adults) absorb glucocorticoids through their skin and gills (Wack et al. 2010; Middlemis Maher et al. 2013; Gabor et al. 2019), making them especially susceptible to ambient hormonal influences. In our study, the lower WB CORT levels in lab-reared larvae may reflect such dynamics: larvae were housed in groups, potentially leading to gradual accumulation of CORT in the holding water, which in turn could have suppressed endogenous production or release via negative feedback. Unfortunately, we did not quantify CORT concentrations in the rearing water, limiting our ability to directly compare ambient CORT exposure across origin groups. Consequently, our findings emphasize that early environmental conditions have lasting effects on endocrine and somatic development, reinforcing the need to account for environmental origin in physiological assessments, particularly when comparing stress responses across rearing contexts.

4.2 Acute nitrate stress reduces body mass in field-collected but not in lab-reared amphibian larvae without altering CORT responses

We found that acute exposure to nitrate significantly reduced body mass in field-collected *R. temporaria* tadpoles, while lab-reared individuals showed no such response. Interestingly, this reduction in growth occurred without a corresponding change in WB CORT release rates, suggesting that body mass may be a more sensitive indicator of short-term environmental stress than hormonal output (Ruthsatz et al. 2023), particularly under complex field conditions. A possible explanation is that field-collected larvae, which already exhibited elevated baseline CORT levels, may have had limited capacity to mount an additional hormonal response to acute nitrate exposure. Previous work has shown that animals chronically exposed to environmental stressors can exhibit reduced responsiveness of the HPI axis to novel stressors (Romero 2004; Rich & Romero 2005; Gabor et al. 2018). In our study, this may have suppressed the CORT response to nitrate in field-collected individuals, even though the stressor was sufficient to alter growth trajectories. Nitrate was not present as a stressor at the time of field collection, suggesting that the observed patterns are not a result of prior adaptation to nitrate exposure. In contrast, lab-reared tadpoles, raised under controlled, low-stress environments, may have been better able to buffer or compensate for the short-term challenge without detectable physiological costs as growth was not impaired. It is also possible that nitrate exposure led to subtle increases in endogenous CORT that were not detectable in water-borne samples (Ruthsatz et al. 2022, 2023). Unfortunately, we measured endogenous (tissue) CORT levels only at the end of the 48-hour nitrate exposure period and did not collect paired pre- and post-exposure samples. As a result, we were unable to directly assess individual-level changes or confirm whether CORT levels increased over the course of the exposure. This limits our ability to conclusively determine whether the observed growth reduction was mediated by hormonal shifts or by other stress-related physiological processes. WB CORT integrates free, circulating hormone excreted over time and may not capture transient or low-magnitude hormonal responses, especially in organisms with already elevated baseline levels. Alternatively, growth suppression under nitrate exposure could reflect increased metabolic costs unrelated to CORT release *per se*. Nitrate pollution can impair osmoregulatory function (Ortiz-Santaliestra et al. 2006), disrupt mitochondrial respiration (Xie et al. 2024), impair blood oxygen transport causing tissue hypoxia (Yang et al. 2019; Gomez Isaza et al. 2020, 2021), and increase oxidative stress in aquatic animals such as amphibian larvae (Wang et al. 2023), all of which may disrupt energy homeostasis (rev. in McLean & Rodgers 2023) and thus, compromise energy allocation to growth. Our previous research on *R. temporaria* also found

growth suppression under chronic nitrate exposure (Ruthsatz et al. 2023), suggesting that both acute and long-term exposure may compromise developmental trajectories. Consequently, our results highlight that nitrate pollution can reduce performance-related traits even in the absence of clear endocrine signals, and underscore the importance of integrating multiple physiological and morphological indicators when assessing the impacts of environmental stressors on wild populations.

4.3 Non-invasive WB corticosterone assay is a valid tool to assess the physiological state in wild and captive-bred amphibian larvae

Reliable, non-invasive methods for assessing physiological stress are essential for monitoring amphibian health *in situ*, especially in sensitive larval stages. WB CORT sampling is increasingly recognized as a promising tool for such assessments, with several studies validating the relationship between WB CORT and endogenous (tissue) hormone concentrations in amphibians under laboratory conditions (e.g., Forsburg et al. 2019; McClelland & Woodley 2021; Ruthsatz & Rico-Millan et al. 2023). In the present study, we observed strong positive correlations between WB and tissue CORT concentrations in *R. temporaria* larvae, providing further support for the validity of this approach. Importantly, this relationship held for both lab-reared and field-collected individuals, indicating that WB CORT reliably reflects internal hormone levels even in the face of natural environmental complexity and stressor exposure.

Here, we focused our sampling on pro-metamorphic larvae, a developmental stage previously shown to exhibit particularly strong concordance between WB and tissue CORT levels (Ruthsatz & Rico-Millan et al. 2023). At this stage, endogenous CORT concentrations are naturally elevated (Denver 1998; Reguera et al. 2022), and the larval skin remains permeable because of limited keratinization and the presence of functional gills (Shi 2000), facilitating transdermal hormone release. This physiological configuration probably contributed to the robust WB–tissue correlation observed in the present study. However, this balance between internal hormone production and transdermal release may not hold at other ontogenetic stages, where lower CORT levels (i.e., earlier developmental stages; Denver 1998; Reguera et al. 2022), reduced skin permeability (i.e., Nishikawa et al. 1989), and the degeneration of the gills (i.e., Burggren & West 1982) could diminish WB CORT signal strength. In wild larvae especially, additional stressors such as pollutants or skin pathogens may disrupt this balance (Gabor et al. 2018; Forsburg et al. 2021), potentially leading to mismatches between WB and tissue hormone measures. Such stage- and condition-specific limitations influencing the strength and reliability of WB CORT collection must be carefully considered when applying this method in field-based monitoring frameworks, to avoid misinterpreting physiological baselines or stress responses (rev. in Ruthsatz & Rico-Millan et al. 2023).

4.4 Implications for amphibian conservation and concluding remarks

In the face of ongoing environmental change, early detection of physiological stress is critical for identifying at-risk populations before demographic declines become evident. Our effort highlights the strong potential of non-invasive WB CORT sampling as a practical and ethical tool for monitoring the physiological state of amphibian larvae in both captive and wild populations. By enabling repeated, minimally disruptive sampling across developmental stages, this approach offers a particular promise for amphibian conservation, as elevated CORT levels can serve as early indicators of exposure to anthropogenic stressors, such as agrochemical pollution, habitat degradation, or climate-induced shifts in hydroperiod, long before these stressors manifest in population declines. Our results demonstrate that WB CORT levels differ

systematically between wild and lab-reared individuals, and that somatic traits like body mass also reflect environmental sensitivity. In some cases, body mass proved more responsive to acute environmental stress, consistent with previous findings (e.g., Ruthsatz et al. 2023). Integrating WB CORT into existing monitoring frameworks can therefore enhance our ability to interpret changes in somatic traits by providing physiological context, helping to determine whether observed shifts in body mass reflect underlying stress responses. We thus recommend the combined use of endocrine (e.g., CORT) and morphological (e.g., body mass) metrics to achieve a more holistic and reliable assessment of amphibian health, physiological resilience, and environmental quality. To fully realize the potential of WB CORT sampling as a tool in amphibian conservation, we encourage future research to deepen our mechanistic understanding of how CORT is released into water across species and developmental stages, and to establish calibration standards under a range of environmental conditions. These efforts will enhance the reliability and scalability of WB CORT as a physiological biomarker for conservation applications. A critical next step could be to conduct cross-species and cross-population, stage-specific validation studies under controlled and (semi-)natural conditions, to establish how environmental variables, and biological traits, influence the relationship between tissue and WB CORT, laying the groundwork for standardized application in amphibian conservation contexts.

5. Author contributions

FB: Conceptualization (lead); Methodology (equal); Data curation (equal); Formal analysis (equal); Investigation (lead); Writing – original draft (equal); Writing – review and editing (equal). **KR:** Conceptualization (supporting); Methodology (equal); Data curation (equal); Formal analysis (equal); Investigation (supporting); Writing – original draft (equal); Writing – review and editing (equal). Supervision (lead); Funding raised (lead). All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

6. 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.

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8. Data availability

Data, including all original measurements, and codes will be deposited in Figshare under DOI:XXX after acceptance.

9. Statement of ethics

The authors have no ethical conflicts to disclose.

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12. Figure legends

Figure 1. Effects of environmental origin (lab-reared vs field-collected) and ontogeny on **A** water-borne baseline CORT release rates (pg/g/h) and **B** ln body mass (g). Boxes and whiskers show 25th to 75th and 10th to 90th percentiles, respectively; black lines indicate the median. Blue for field-collected animals. Orange for lab-reared animals. Ontogenetic stage groups sensu Gosner (1960): 1 = newly hatched (GS 20-22); 2 = pre-metamorphic (GS 23-34); 3 = pro-metamorphic (GS 35-41); 4= metamorphic (GS 42-46); **5** = post-metamorphic/juvenile (>GS 46); **6** = adults (indicated through nuptial pads in males and eggs in females; adults were not included in the statistical analysis). Further details on the methods in the main text.

Figure 2. Differences in **A** water-borne CORT release rate (pg/g/h) and **B** body mass (g) before and after acute nitrate exposure. Boxes and whiskers show 25th to 75th and 10th to 90th percentiles, respectively; black lines indicate the median. Blue for field-collected animals: bright blue for animals before nitrate exposure, dark blue for animals after nitrate exposure. Orange for lab-reared animals: bright orange for animals before nitrate exposure, dark orange for animals after nitrate exposure. Dotted pattern for exposure to 50 mg/L nitrate. Striped pattern for exposure to 100 mg/L nitrate. Further details on the methods in the main text.

Figure 3. Relationship between tissue corticosterone (pg) and water-borne corticosterone (pg/g/h) in *Rana temporaria* tadpoles. Each point represents an individual tadpole. Blue for field-collected animals. Orange for lab-reared animals. Solid lines show fitted linear regressions for each origin group. Circle: 0 mg/L nitrate. Square: 50 mg/L nitrate. Triangle: 100 mg/L nitrate. Correlation coefficient and p values for Pearson's correlation. Further details on the methods in the main text.

13. Table legends

Table 1. Summary of linear model results examining the effects of larval origin (Field vs Lab), ontogenetic stage (centered Gosner stage; GS), and nitrate exposure (0, 50, 100 mg/L) on physiological traits in *Rana temporaria* tadpoles. Separate models were fitted for (1) water-borne corticosterone (CORT) release rates, (2) body mass, log-transformed changes in (3) CORT and (4) body mass following acute nitrate exposure, and (5) the relationship between water-borne (WB) and tissue-based CORT levels. Estimates are presented with standard errors, *t*-values, *p*-values, and significance levels ($p < 0.05$ = *, < 0.01 = **, < 0.001 = ***). "ns" denotes non-significant effects ($p > 0.05$).

14. Supplementary material

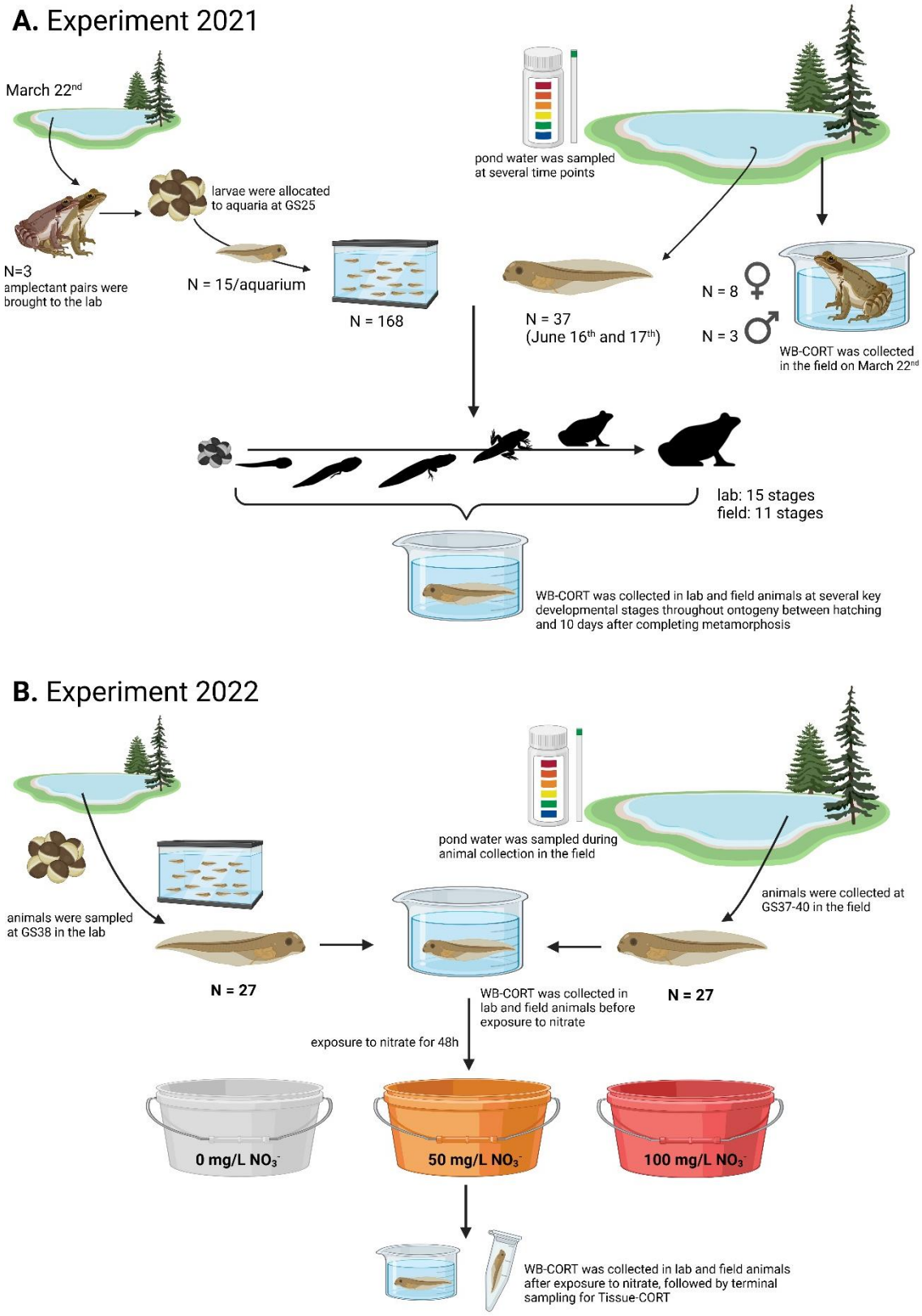

Figure S1. Experimental design and sampling. See main text for details.

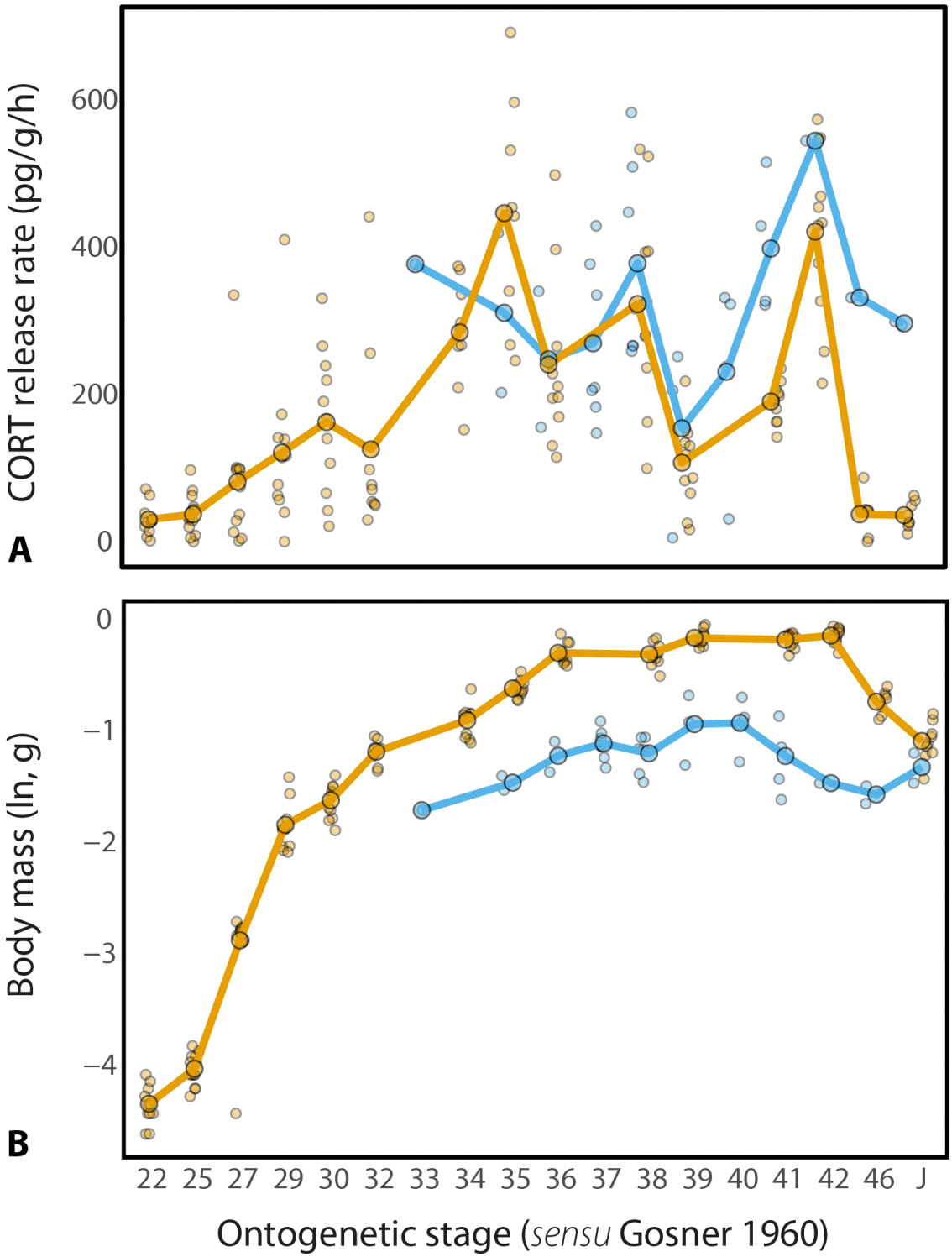

Figure S2. Ontogenetic patterns of water-borne corticosterone (CORT) release rates and body mass in *Rana temporaria* tadpoles. **A**: Water-borne CORT release rates (pg/g/h) across Gosner (1960) developmental stages. **B**: Body mass (ln-transformed, g) across the same stages. Blue lines and dots represent field-collected tadpoles; orange lines and dots represent laboratory-reared tadpoles. Lines show mean values per developmental stage; small dots represent individual data points.

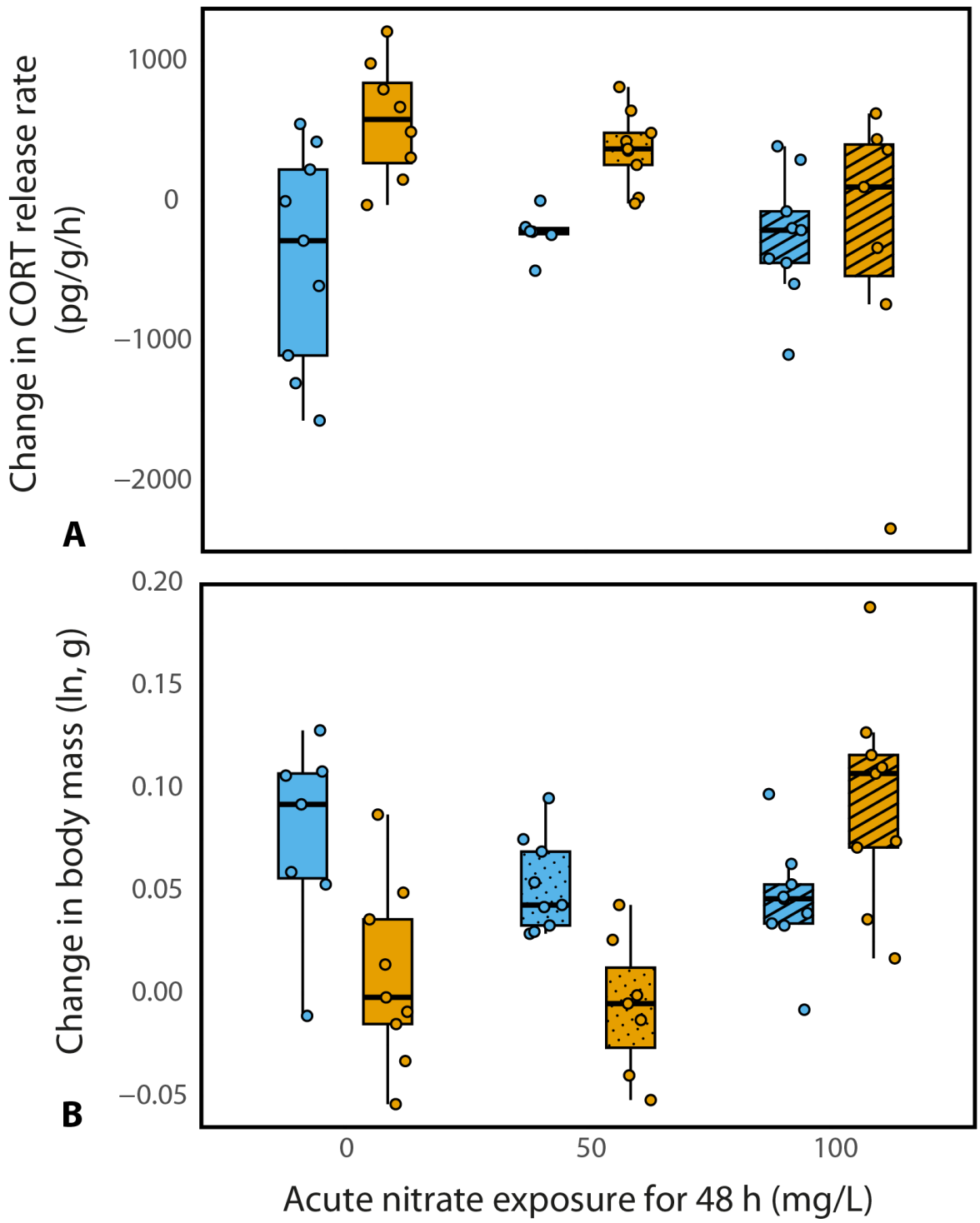

Figure S3. Changes in water-borne corticosterone (CORT) release rates and body mass in *Rana temporaria* tadpoles following acute nitrate exposure (48 h). **A**: Change in CORT release rate (pg/g/h) before and after 48 h nitrate exposure at 0, 50, and 100 mg/L nitrate. **B**: Change in ln-transformed body mass (g) before and after the same exposure. Blue boxes and dots represent field-collected tadpoles; orange boxes represent laboratory-reared tadpoles. Points show individual data. Dotted pattern for exposure to 50 mg/L nitrate. Striped pattern for exposure to 100 mg/L nitrate. Boxes and whiskers show 25th to 75th and 10th to 90th percentiles, respectively; black lines indicate the median.

Table S1. *Rana temporaria* 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}.

Experimental year	Type	Water parameters								
		NO_3^-	NO_2^-	NH_4^+	PO_4^{3+}	Cu^{2+}	Fe	Pb	pH	N
2021	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
2022	Pond water	< 6	0.07 ± 0.02	0	0.29 ± 0.05	<0.02	0.13 ± 0.01	0	6.8 ± 0.3	3
	Tap water used in experiments	< 6	0	0	0.08 ± 0.01	<0.02	<0.1	0	7.2 ± 0.1	20

Table S2. Background water-borne corticosterone (WB CORT) concentrations measured in tap and pond water samples in 2021 and in 2022 before and after nitrate exposure. Samples (50 mL) were collected as procedural blanks to verify that water sources did not contribute detectable CORT levels during the experiment. These values indicate that pond water contains high levels of CORT, suggesting that field-collected *Rana temporaria* larvae are chronically exposed to elevated exogenous CORT, in contrast to the lab-reared larvae.

Year	ID	Nitrate experiment	Origin	CORT (pg/ml)	%CV	CORT correct (pg)
2021	Control Pond 1	NA	Field	NA	NA	NA
2021	Control Pond 2	NA	Field	150.8	2.32	18.85
2021	Control Pond 3	NA	Field	NA	NA	NA
2021	Tap water 1	NA	Lab	284.2	7.81	35.525
2021	Tap water 2	NA	Lab	NA	NA	NA
2021	Tap water 3	NA	Lab	NA	NA	NA
2022	Pond 1	Before	Field	3102	21.1	387.75
2022	Pond 2	Before	Field	2007	20.3	250.875
2022	Pond 3	Before	Field	3224	2.45	403
2022	Pond 4	Before	Field	2681	4.72	335.125
2022	Pond 5	Before	Field	3554	12.8	444.25
2022	Pond 6	Before	Field	3960	0.873	495
2022	Pond 7	After	Field	3018	12.2	377.25
2022	Pond 8	After	Field	2418	5.37	302.25
2022	Pond 9	After	Field	2640	14.2	330
2022	Tap water 1	Before	Field	120.5	19.7	15.0625
2022	Tap water 2	Before	Field	508.7	0.103	63.5875
2022	Tap water 3	Before	Field	95.31	37.5	11.91375
2022	Tap water 4	Before	Field	520.1	43.4	65.0125
2022	Tap water 5	Before	Field	611.9	0.0408	76.4875
2022	Tap water 6	Before	Field	NA	NA	NA
2022	Tap water 7	After	Field	NA	NA	NA
2022	Tap water 8	After	Field	569.3	8.1	71.1625
2022	Tap water 9	After	Field	653.1	8.13	81.6375
2022	Tap water 10	After	Field	688.9	6.45	86.1125
2022	Tap water 11	After	Field	428.7	11.7	53.5875
2022	Tap water 12	After	Field	521	34.1	65.125
2022	Tap water 1	Before	Lab	392.8	25.1	49.1
2022	Tap water 2	Before	Lab	377	10.1	47.125
2022	Tap water 3	Before	Lab	NA	NA	NA
2022	Tap water 4	After	Lab	198.5	7.66	24.8125
2022	Tap water 5	After	Lab	185.9	9.22	23.2375
2022	Tap water 6	After	Lab	430.9	27.3	53.8625