Title:

Bringing the Lab to the Field: Exploring Water-Borne Corticosterone as a Conservation Tool in Captive and Wild Common Frog Larvae (Rana temporaria)

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

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Lay summary:

We tested water-borne corticosterone (WB-CORT) as a non-invasive indicator of corticosterone levels in wild and laboratory-reared tadpoles exposed to the common pollutant. Wild tadpoles showed declining body mass during the experiment regardless of pollutant exposure, with no corresponding change in corticosterone. WB-CORT reflected internal hormone levels but requires broader validation.

Abstract

Assessing physiological conditions in wild populations is critical for detecting early environmental deterioration and informing conservation before population declines occur. Non-invasive tools such as water-borne corticosterone (WB-CORT) sampling show promise for monitoring amphibian stress physiology, but their ecological relevance and limitations must be carefully evaluated, particularly when laboratory-developed methods are applied to wild individuals in complex environments. Here, we tested the utility of WB-CORT sampling in larvae of the European common frog (*Rana temporaria)* by comparing wild-caught and laboratory-reared individuals. First, we examined whether environmental origin influenced baseline physiological traits by modelling ontogenetic changes in WB-CORT and body mass, an integrative measure of growth. Wild larvae exhibited higher baseline WB-CORT levels and lower body mass than their laboratory-reared counterparts, suggesting divergent corticosterone profiles and growth trajectories between both environments. Next, we evaluated the sensitivity of WB-CORT and body mass to acute (48h) nitrate exposure, a widespread agricultural pollutant commonly found in amphibian breeding ponds. Nitrate exposure did not alter WB-CORT levels in either origin group. Body mass declined in wild larvae across all treatments, including controls, whereas laboratory-reared larvae maintained stable mass except at the highest nitrate concentration, indicating origin-dependent responses not mirrored by corticosterone release. Finally, we explored WB-CORT as a proxy for internal corticosterone by examining its relationship with tissue CORT concentrations. WB-CORT and tissue levels were positively related in both wild and laboratory-reared larvae. This association supports the use of WB-CORT as a non-invasive measure of corticosterone under the specific conditions tested. Together, our findings show that WB-CORT can reflect variation in corticosterone levels between laboratory-reared and field-collected larvae, but that its responsiveness to environmental challenges is context dependent, highlighting both the potential and limitations of WB-CORT. We conclude by emphasizing the need for further evaluation across species, life stages, and stressors before broader application in amphibian conservation.

1. Introduction

Understanding how organisms respond to biological, physical, or chemical environmental stressors is fundamental for predicting the impacts of global change on biodiversity. Stress can be broadly defined as a state in which environmental demands exceed an organism's capacity to maintain or rapidly restore physiological homeostasis, thereby eliciting coordinated physiological and behavioral responses (Sapolsky et al. 2000; Romero 2004). 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, when stressors are chronic, intense, or unpredictable, stress responses 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, exploring, and validating non-invasive approaches for monitoring stress in wild amphibian populations is crucial for conservation efforts.

Assessing physiological stress, defined here as variation in glucocorticoid-mediated endocrine activity in response to environmental conditions, 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 (WBCORT) sampling has emerged as a promising non-invasive technique for assessing glucocorticoid-mediated physiological responses 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 in laboratory settings for some amphibian species and life-history stages (e.g., Forsburg et al. 2019; McClelland & Woodley 2021; Ruthsatz & Rico-Millan 2023), validation outcomes and approaches vary among taxa, developmental stages, and study designs, and the method has not been consistently validated across species. Its application in wild populations therefore remains limited and context dependent (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). However, many of these conditions are evolutionarily familiar, and wild animals are often adapted to cope with them (Hose & Van den Brink 2004). Nonetheless, 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, unpredictable 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).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 amphibian larvae (Wang et al. 2015; Poulsen et al. 2018; Ruthsatz et al. 2022), including increasedCORT levels We previously observed this effect under laboratory conditions using WB-CORT, following exposure throughout the entire larval period (Ruthsatz et al. 2023). However, it remains unclear whether this response is equally detectable in wild amphibians, where individuals experience more heterogeneous and less controlled environmental conditions. 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 provide meaningful information on corticosterone variation in wild-caught individuals. Using the early-breeding European common frog (*Rana temporaria*) as a model species, we explored the utility of WB-CORT to assess physiological responses to nitrate exposure during the aquatic larval stage. Specifically, we aimed to: (1) examine whether wild-caught larvae differ in baseline CORT levels and body mass, a key integrative trait linked to growth and developmental performance, compared to their lab-reared counterparts, recognizing that any observed differences may reflect multiple environmental and developmental factors; (2) assess whether the developmental trajectories of CORT and body mass across Gosner stages are broadly consistent between origin groups (3) investigate whether prior exposure to a more complex environment in the wild reduces the larvae's sensitivity to acute nitrate exposure relative to lab-reared tadpoles; and (4) compare WB-CORT with tissue CORT to evaluate its context-specific reliability as a non-invasive measure.

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 2022).

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 (i.e., Hypotheses 1 and 2; Fig. S1). 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; i.e., Hypothesis 3 and 4; Fig. S1). 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 CORT and body mass 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 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 = 35) were collected from the field 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, and each individual was measured only once. Larvae were temporarily held in a bucket during sampling until all measurements were completed.

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 juveniles (survival rate: 100%) 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. WB-CORT was measured across 14 individual ontogenetic stages (Gosner stage 22 to the juvenile stage), resulting in 168 samples derived from 45 larvae. Individuals were thus sampled repeatedly throughout development, with WB-CORT measured first, followed by 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), assessed by visual inspection 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 short-term changes in CORT levels and body mass following 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 (i.e., no transport to the field). 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; 9 larvae randomly selected from each tank to assign 3 individuals from each tank to each of the nitrate concentrations) 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□ 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 and weighed using a balance with a precision of 0.0001 g, after which the entire tissue sample was homogenized in 136 × 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□O 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. Because corticosterone is a lipophilic steroid hormone, it partitions into the lipid fraction. Therefore, 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. The dried lipid extract was then 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. In total, we used nine and four plates in 2021 and 2022, respectively.

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 supposed to be removed from triplicates that did not meet these criteria (Ruthsatz & Rico-Millan et al. 2023). All triplicates met the criteria in the present study. 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), while tissue CORT was expressed in pg/g.

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 mixed-effect models were fitted using the *lmer*() 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. During the model assumption evaluation, nine samples from the 2021 dataset were determined as extreme outliers and removed from the dataset (N removed: GS 25=4; GS 27= 1; GS35=5; juvenile stage= 1), resulting in 159 measurement points in the final dataset.

To test Hypotheses 1 and 2 (effects of origin and ontogeny on corticosterone levels and body mass), we fitted separate linear mixed-effect 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 account for non-independence among individuals in laboratory aquaria, we included 'Tank identity' as a random intercept for Lab tadpoles only, while Field tadpoles, which were not housed in tanks, were retained in the analysis without a random effect. Gosner stage was treated as a continuous covariate and mean-centered (i.e., GS_c = GS − mean[GS]) to facilitate interpretation of model intercepts and interaction terms. This centering ensured that model intercepts reflected average responses at the mean

developmental stage, thereby avoiding extrapolation to biologically implausible GS values. The Origin × GS_c interaction was included to test whether developmental trajectories of corticosterone levels and body mass differed between field and lab tadpoles.

To test the effects of acute nitrate exposure on WB-CORT release and body mass and whether responses differ by origin (Hypothesis 3), we calculated the change in CORT levels and body mass for each individual as the difference between measurements before and after 48-h of nitrate exposure within each origin. Owing to non-normality (Shapiro test: $p = 0.012$), CORT differences were log-transformed (log[CORT difference + offset], where offset was added to ensure all values were positive) before modeling. Linear mixed-effect models were fitted with Origin, Nitrate treatment (0, 50, and 100 mg/L), and their interaction as fixed effects, as well as tank as a random intercept for lab-reared animals, whereas field larvae were assigned a single level. This random-effects structure accounts for potential non-independence among lab-reared larvae while including field individuals in the analysis.Mass differences met normality assumptions (Shapiro test: $p = 0.673$), 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 (comparing WB CORT with tissue CORT to evaluate its context-specific reliability as a non-invasive measure), we fitted linear mixed-effects models to account for non-independence among lab-reared larvae from the same tank. WB CORT was modeled as the response variable, with tissue CORT, Origin (Field or Lab), and their interaction as fixed effects. Tank identity was included as a random intercept for lab-reared individuals, whereas field-collected larvae were treated as independent observations (i.e., not sharing a random-effect level). This approach allowed us to test whether the relationship between WB CORT and tissue CORT differed by origin while accounting for potential tank effects. Slopes of WB CORT versus tissue CORT were extracted separately for each origin using the *emtrends()* function from the emmeans package to facilitate interpretation of origin-specific relationships. For models including tank as a random effect, singular fit warnings were occasionally observed, indicating that variance attributed to tank identity was near zero. This reflects limited among-tank variability and does not affect inference on fixed effects.

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 = –424.9 ± 155.6 pg/g/h, t = –2.73, p = 0.0069).The main effect of ontogenetic stage was not significant (Estimate = 1.41 ± 27.8.1, p = 0.96), indicating no clear change in CORT across Gosner stages (Fig. 1A; Fig. S2A; Table 1). However, there was a significant origin × GS_c interaction (Estimate = –67.6 ± 28.6, t = –2.36, p = 0.019), 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 = 0.020).

Body mass – Lab-reared larvae were significantly heavier than wild-caught larvae at the mean developmental stage (Estimate = 0.150 ± 0.051 g, t = 2.94, p = 0.0037; Fig. 1B; Table 1). The main effect of ontogenetic stage on mass in the field group was not significant (Estimate = –0.0023 ± 0.0091, p = 0.80), but the interaction between origin and GS_c was significant (Estimate = 0.033 ± 0.0094, t = 3.54, p = 0.00051; Table 1). Lab-reared larvae showed an increase in mass with advancing GS (Fig. S2B), whereas wild-caught larvae did not.

The models returned a singular fit warning for the random effect of Tank, indicating that Tank variance was near zero. This does not affect the interpretation of the fixed effects, which capture the main biological patterns of interest.

Adult common frogs (N= 11; 8 females, 3 males collected from the field 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.

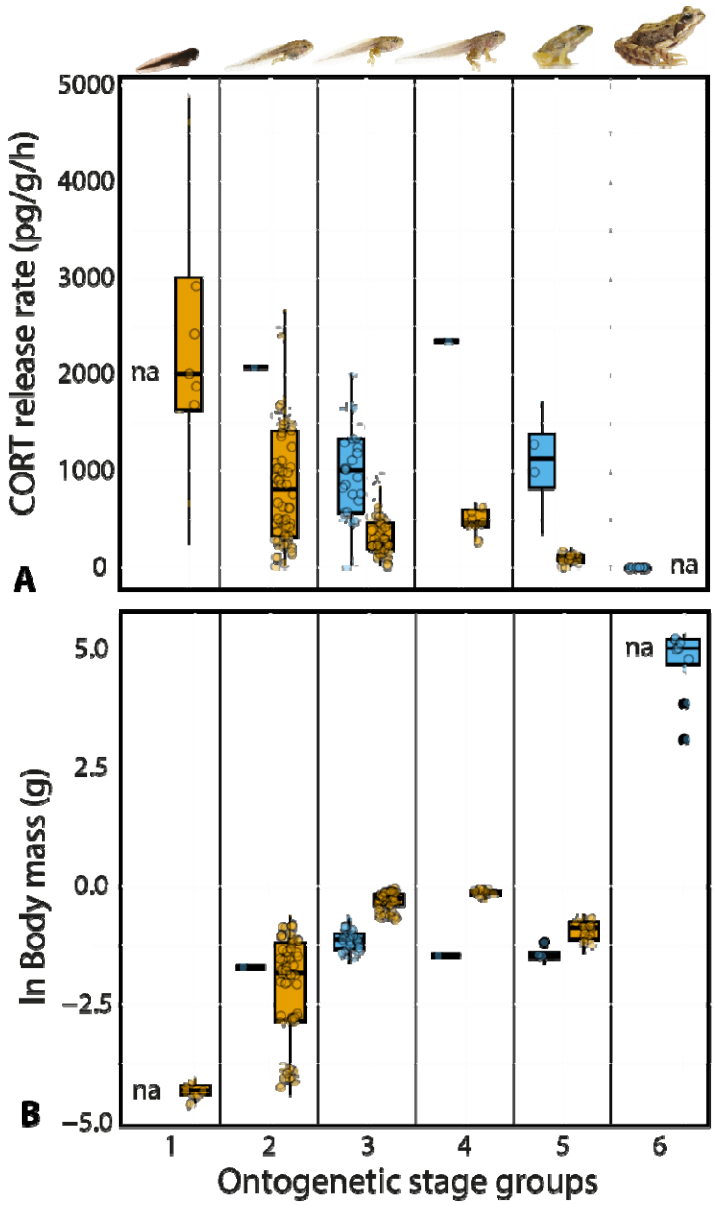


Figure 1. Effects of environmental origin (lab-reared vs field-collected) and ontogeny on **A** water-borne baseline corticosterone (CORT) release rates (pg/g/h) and **B** ln body mass (g). Boxes and whiskers show 25th to 75th and 10th to 90th percentiles, respectively; black lines indicate the median. 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). Numbers indicate sample sizes. Further details on the methods in the main text. Sample sizes (lab/field) for CORT (A) were: stage 1 = 11/0; stage 2 = 62/1; stage 3 = 49/29; stage 4 = 18/3; stage 5 = 8/2; stage 6 = 0/11. Sample sizes (lab/field) for body mass (B) were: stage 1 = 11/0; stage 2 = 67/1; stage 3 = 54/29; stage 4 = 18/3; stage 5 = 9/2; stage 6 = 0/11. Further methodological details are provided in the main text.

3.2 Environmental origin influences mass loss, but not CORT responses, to acute nitrate exposure

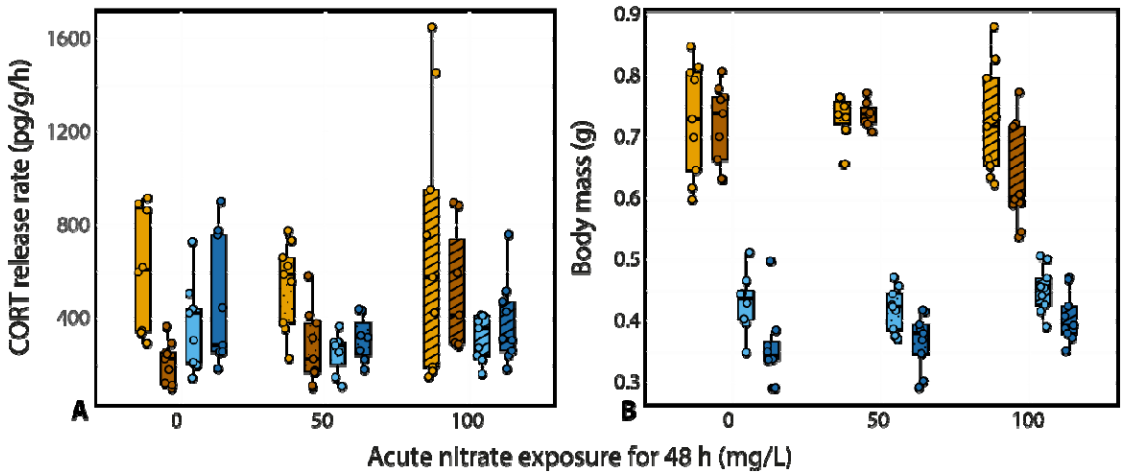
CORT levels – The model testing log-transformed CORT differences revealed no statistically significant main or interaction effects (Table 1; Fig. 2A; Fig. S3A). Neither origin nor nitrate concentration significantly influences post-exposure CORT release rates. A weak, non-significant trend was observed for the Origin × 100 mg/L nitrate interaction, suggesting a possible origin-dependent difference at the highest nitrate concentration, but this effect did not reach statistical significance.

Body mass – Nitrate exposure affected body mass differently in field-collected and lab-reared tadpoles. During the 48-h exposure period, field-collected tadpoles consistently lost body mass regardless of nitrate treatment, including in the control group with no nitrate. In contrast, lab-reared tadpoles maintained relatively stable body mass at low and intermediate nitrate concentrations, and only showed marked mass loss at the highest nitrate concentration (100 mg nitrate/L). Consequently, lab-reared tadpoles lost less mass overall than field-collected individuals. These patterns resulted in significant main effects of Nitrate treatment and origin, as well as a significant Origin × Nitrate interaction (Table 1; Fig. 2B; Fig. S3B). Post hoc tests confirmed that field tadpoles had lower body mass than lab-reared tadpoles at low and intermediate nitrate concentrations, whereas within the lab-reared group, exposure to 100 mg nitrate/L resulted in significantly greater mass loss compared with lower nitrate concentrations (all $p < 0.05$).

3.3 Water-borne corticosterone reliably reflects tissue corticosterone, with origin-dependent scaling

Because body mass decreased in field-collected tadpoles across all treatments, including controls, any origin-specific differences in WB CORT were interpreted independently of nitrate exposure.

The model revealed a significant positive relationship between tissue CORT and WB CORT overall ($\beta = 0.922 \pm 0.046$, $t = 20.23$, $p < 0.001$). There was also a significant tissue CORT ×



Origin interaction ($\beta = 0.847 \pm 0.131$, $t = 6.49$, $p < 0.001$), indicating that the relationship between WB CORT and tissue CORT differed between lab-reared and field-collected tadpoles. Specifically, model-estimated slopes were 0.922 (95% CI: 0.823–1.021) for field-collected tadpoles and 1.770 (95% CI: 1.467–2.072) for lab-reared tadpoles. The main effect of Origin (Lab vs. Field) was not statistically significant ($\beta = -107.62 \pm 184.52$, $t = -0.58$, $p = 0.566$).

Figure 2. Differences in **A** water-borne corticosterone (CORT) release rate (pg/g/h) and **B** body mass (g) before and after acute nitrate exposure in *Rana temporaria* tadpoles at GS 37-40 (field) and 38 (lab) (repeated measurements on the same individuals). 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.

Table 1. Summary of linear mixed-effects model results examining the effects of larval origin (Field vs. Lab), ontogenetic stage (centered Gosner stage; GS), acute nitrate exposure (0, 50, and 100 mg/L), and the relationship between tissue-based and water-borne corticosterone (CORT) in *Rana temporaria* tadpoles. Separate models were fitted for (1) water-borne CORT release rates and (2) body mass as functions of origin and GS (Hypotheses 1–2), (3) log-transformed changes in CORT release rates and (4) changes in body mass following 48-h of nitrate exposure (Hypothesis 3), and (5) the relationship between tissue CORT and water-borne CORT, including origin-specific slopes (Hypothesis 4). Tank identity was included as a random intercept for laboratory-reared tadpoles, while field-collected individuals were assigned a separate level with no associated random effect. Estimates represent fixed-effect coefficients with associated standard errors, t-values, and p-values.

Hypothesis	Dependent variable	Fixed effects	Estimate	Std. Error	t-value	p-value
1 & 2	CORT release rates (pg/g/h)	Intercept (Field)	1064.6	147.1	7.24	<0.001
		Lab	−424.9	155.6	−2.73	0.0069
		GS	1.41	27.78	0.05	0.96
		Lab*GS	−67.6	28.61	−2.36	0.019
1 & 2	Body mass (g)	Intercept (Field)	0.318	0.0483	6.58	<0.001
		Lab	0.150	0.0511	2.94	0.0037
		GS	−0.0023	0.00913	−0.25	0.80
		Lab*GS	0.0333	0.0094	3.54	0.00051
3	Log-transformed difference in CORT release rate before and after Nitrate exposure (pg/g/h)	Intercept (Field, 0 mg/L)	7.477	0.557	13.426	<0.001
		Lab	0.49	0.812	0.604	0.549
		50 mg/L	0.176	0.881	0.199	0.843
		100 mg/L	0.139	0.788	0.177	0.860
		Lab*50 mg/L	−0.242	1.198	−0.202	0.841
		Lab*100 mg/L	−2.106	1.170	−1.801	0.078
3	Difference in Body mass before and after Nitrate exposure (g)	Intercept (Field, 0 mg/L)	0.0764	0.0138	5.536	<0.001
		Lab	−0.0683	0.0184	−3.711	0.0005
		50 mg/L	−0.0242	0.0184	−1.315	0.195
		100 mg/L	−0.0315	0.0184	−1.713	0.093
		Lab*50 mg/L	0.0101	0.0260	0.388	0.700
		Lab*100 mg/L	0.1174	0.0252	4.659	<0.001
4	WB-CORT (pg/g/h)	Intercept (WB CORT)	−87.21	69,65	−1.25	0.221
		Tissue CORT	0.922	0.046	20.23	< 0.001
		Lab	−152.44	184.52	−0.58	0.566
		Tissue CORT*Lab	0.847	0.131	6.49	< 0.001

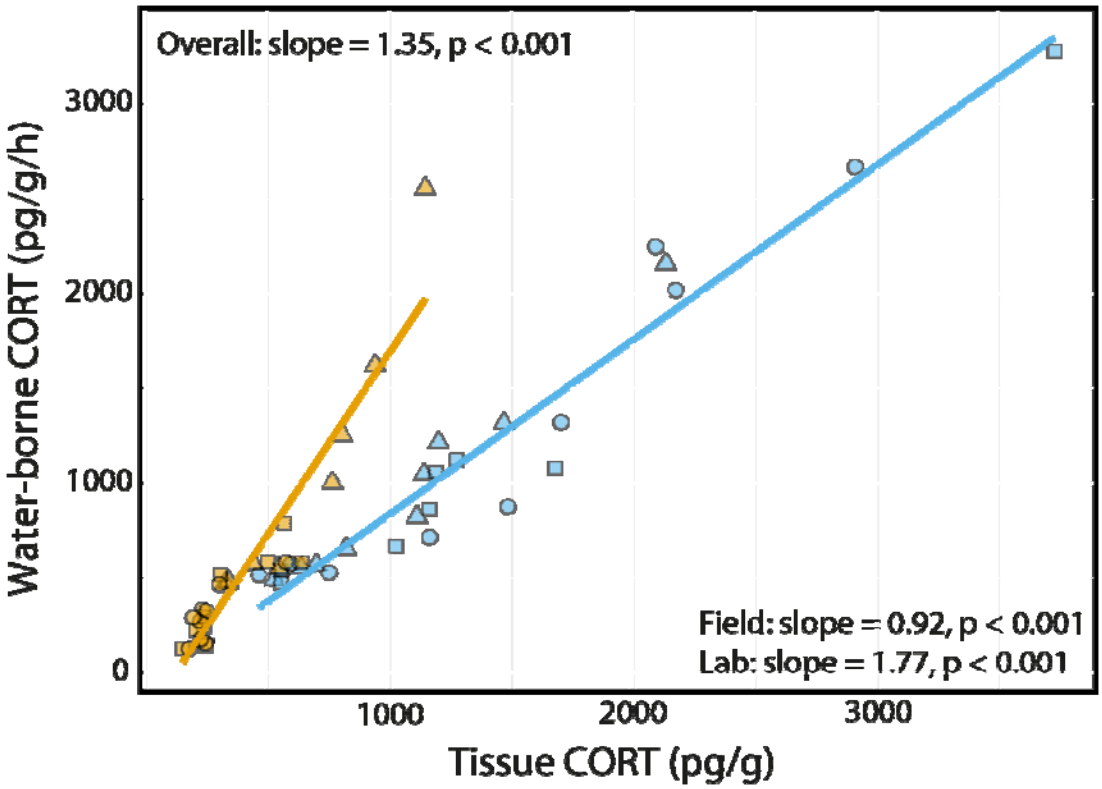


Figure 3. Relationship between tissue corticosterone (pg/g) and water-borne corticosterone (pg/g/h) in *Rana temporaria* tadpoles at Gosner stages 37–40 (field) and 38 (lab). Each point represents an individual tadpole. Blue symbols indicate field-collected animals, and orange symbols indicate lab-reared animals. Solid lines show model-estimated relationships between tissue and water-borne corticosterone for each origin, derived from a linear mixed-effects model. Circles indicate 0 mg/L nitrate, squares 50 mg/L nitrate, and triangles 100 mg/L nitrate. The relationship between tissue and water-borne corticosterone differed significantly between origin groups (tissue CORT × origin interaction, $p < 0.001$). Further details on statistical analyses are provided in the main text.

4. Discussion

4.1 Environmental origin shapes baseline CORT levels and growth patterns in amphibian larvae (Hypotheses 1 &2)

Understanding how baseline physiological traits vary between lab-reared and field-collected 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. Field-collected larvae exhibited consistently higher WB-CORT concentrations and lower body mass than their lab-reared

counterparts, suggesting consistent differences in physiological state between field-collected and lab-reared larvae across the developmental stages sampled.

Unpredictable or chronic stressors can activate the HPI axis, elevating baseline glucocorticoids and diverting resources from growth to maintenance (Awkerman et al. 2024; Romero 2004; Rich & Romero 2005; Boonstra 2013). Unlike lab-reared larvae raised under stable conditions with *ad libitum* food, wild larvae must forage actively under environmental uncertainty, likely increasing energy expenditure and physiological demand (Moyers & Hagger 2023), which helps explain their lower body mass (Bryant et al. 2022). However, interpretation of these origin effects should be tempered by uneven developmental stage representation in the present study, particularly among field-collected larvae, where early (GS 20–34) and late (GS 42) stages were scarce. This reflects well-known sampling challenges in natural ponds, as early stages occupy cryptic microhabitats and late-stage larvae are less detectable. Although Gosner stage was included as a covariate and was not a significant predictor of WB-CORT, limited sample sizes at stage extremes may have reduced power to detect subtle ontogenetic trends. Nonetheless, the consistent elevation of WB-CORT in field-collected larvae across sampled stages suggests that origin effects are not solely driven by stage composition.

Another potential, but untested, source of variation is exposure to ambient glucocorticoid-like compounds in the surrounding water. Environmental glucocorticoids can originate from anthropogenic sources (e.g., pharmaceuticals; Stavreva et al. 2012) or from organisms within high-density groups (Gabor et al. 2018; Tornabene et al. 2021). ELISA-based methods cannot distinguish biologically active corticosterone from cross-reactive compounds, so ambient signals may reflect false positives. Chronic exposure to exogenous CORT can suppress endogenous production, potentially reducing net release (Gabor et al. 2018; Tornabene et al. 2021). Amphibians absorb glucocorticoids via skin and gills (Wack et al. 2010; Middlemis Maher et al. 2013; Gabor et al. 2019), increasing susceptibility to ambient hormones. In our study, we did not quantify environmental CORT from the aquaria or verify chemical identity, so interpretation remains speculative. This highlights a methodological challenge and underscores the need for combining immunoassays with chemical analyses (e.g., LC-MS) to characterize background glucocorticoid signals in aquatic environments and their physiological relevance.

4.2 Acute nitrate exposure and context-dependent mass loss in field- and lab-reared larvae (Hypothesis 3)

Acute exposure to nitrate significantly reduced body mass in field-collected *R. temporaria* tadpoles, while lab-reared individuals showed no such response. Interestingly, growth reduction occurred without detectable changes in WB-CORT release rates, suggesting body mass may be a more sensitive short-term stress indicator than hormonal output (Ruthsatz et al. 2023).

Primarily, field-collected tadpoles lost body mass across all treatments, including controls, whereas lab-reared tadpoles remained stable except at the highest nitrate concentration. This

pattern likely reflects general stress from capture rather than a nitrate-specific effect (e.g., handling and environmental transition stress can disrupt feeding and water balance). Although not extensively quantified in *R. temporaria*, handling, confinement, and novel conditions can activate stress pathways in amphibian larvae, affecting growth, behavior, and energy balance independently of experimental stressors (e.g., Narayan et al. 2012).

Additionally, field-collected larvae had elevated baseline CORT levels, which may have limited their capacity to mount further hormonal responses to acute nitrate exposure. Previous work has shown that animals chronically exposed to environmental stressors can exhibit reduced HPI/A axis responsiveness to novel stressors (Romero 2004; Rich & Romero 2005; Gabor et al. 2018). In our study, this may have contributed to the lack of detectable WB-CORT response in field-collected individuals, even though growth trajectories were affected. Importantly, nitrate was not present at the time of collection, indicating that this pattern does not reflect prior adaptation. In contrast, lab-reared tadpoles, raised under controlled, relatively simplified conditions, were better able to buffer or compensate for the short-term challenge, as growth was not impaired.

It is also possible that nitrate exposure led to subtle increases in endogenous CORT undetectable in water-borne samples (Ruthsatz et al. 2022, 2023). Because tissue CORT was measured only at the end of the 48-h exposure, we could not assess individual-level changes or confirm transient hormonal responses. Consequently, we cannot determine whether the observed growth reduction was mediated by hormonal shifts. WB-CORT integrates free, circulating hormone excreted over time and may not capture low-magnitude or transient responses, especially in organisms with already elevated baseline levels.

Alternatively, growth suppression under nitrate exposure could reflect increased metabolic costs unrelated to CORT. 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 disrupt energy homeostasis (rev. in McLean & Rodgers 2023) and reduce allocation to growth. Our previous research on *R. temporaria* also found growth suppression under chronic nitrate exposure (Ruthsatz et al. 2023), suggesting both acute and long-term exposure may compromise development.

Together, 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, such as body mass, when assessing the impacts of environmental stressors on wild populations.

4.3 Non-invasive WB-CORT assay is a valid tool to assess the endogenous CORT level in wild and captive-bred larvae of Rana temporaria (Hypothesis 4)

Reliable, non-invasive methods are essential for monitoring amphibian health *in situ*, especially during sensitive larval stages. WB-CORT sampling is increasingly recognized as a

promising tool , with 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 a strong positive relationship 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 heterogeneity..

Here, we focused on pro-metamorphic larvae, a developmental stage with naturally elevated CORT (Denver 1998; Regueira et al. 2022) and high skin permeability due to limited keratinization and functional gills (Shi 2000), facilitating transdermal release. This physiological configuration probably contributed to the robust WB–tissue relationshipobserved in the present study. At other stages, however, this balance between internal hormone production and transdermal release may not hold, where lower CORT levels (i.e., earlier developmental stages; Denver 1998; Regueira 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. Field-collected larvae may also experience additional stressors (pollutants, pathogens) that disrupt hormone balance (Gabor et al. 2018; Forsburg et al. 2021). These stage- and condition-specific limitations influencing the strength and reliability of WB-CORT collection must be carefully considered when using this method in field monitoring, 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 occur. Our study highlights both the potential and the current limitations of non-invasive WB-CORT sampling as a tool for monitoring physiological variation in amphibian larvae. While WB-CORT allows for repeated, minimally disruptive sampling across developmental stages, our results indicate that CORT levels may not reliably detect short-term environmental challenges, such as the 48-hour nitrate exposure tested here, as no increase was observed. This context-dependence underscores that WB-CORT should not be interpreted in isolation.

In the present study, for instance, body mass as an integrative measure of growth and environmental sensitivity responded more consistently than WB-CORT under some experimental conditions, reinforcing the value of combining physiological and morphological metrics. This pattern was consistent with previous findings (Ruthsatz et al. 2023). Integrating both measures can provide a more holistic and reliable assessment of amphibian health, physiological resilience, and environmental quality.

We emphasize that the utility of WB-CORT is likely species-, stage-, and context-specific. To fully realize the potential of WB-CORT as a conservation tool, future research should focus on stage-specific and cross-species calibration, mechanistic studies of hormone release,

and validation under controlled and semi-natural conditions. Establishing calibration standards under varying environmental conditions will improve WB-CORT reliability and scalability as a biomarker.

More broadly, our findings are consistent with previous work showing that organismal responses to stressors can differ markedly between laboratory, mesocosm, and field experiments, including effects on survival, development, body mass, morphology, and behaviour (Hose & Van den Brink 2004; Mikó et al. 2015). Several studies suggest that individuals reared under laboratory conditions may exhibit heightened sensitivity to stressors compared to those in more complex environments, which may render laboratory experiments precautionary for conservation assessments (Hose & Van den Brink 2004). At the same time, these venue-dependent differences emphasize the need to validate laboratory-based findings under semi-natural and field conditions before applying them to conservation or management strategies (Mikó et al. 2015).

Ultimately, our findings advance understanding of CORT dynamics in *R. temporaria* larvae and contribute to broader evaluation of non-invasive endocrine tools for amphibian conservation. While promising, WB-CORT's limitations and context-dependence must be acknowledged, and further research is required to define when it can reliably inform conservation strategies.

5. Author contributions

FB: Conceptualization (lead); Methodology (equal); Data curation (equal);; Investigation (lead); Writing – original draft (equal); Writing – review and editing (equal). **KR:** Conceptualization (supporting); Methodology (equal); Data curation (equal); Formal analysis (lead); 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.

7. Funding

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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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