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Salty Surprises: Developmental and behavioral responses to environmental salinity reveal higher tolerance of inland rather than coastal *Bufo spinosus* tadpoles

Short title: Salinity impacts on development

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Abstract

Salinization is predicted to intensify due to climate change, impacting biodiversity and ecosystem functioning. Amphibians, particularly embryos and larvae, are highly susceptible to environmental salinity. Yet, local adaptation may cause differing vulnerabilities between coastal and inland populations. In this study, we investigated the physiological, behavioural, and life-history responses to environmental salinity (0, 2 and 4 g.l⁻¹) of embryos and larvae of a widespread amphibian species (spined toad, *Bufo spinosus*) from salt-exposed (coastal) and salt-free (inland) populations. Moderate salinity (4 g.l⁻¹) altered embryonic and larval development in both populations, causing increased malformations, decreased body size and survival, and altered behavior, but did not affect telomere length or oxidative status. Individuals exposed to low salinity (2 g.l⁻¹) performed better across most traits. However, moderate salinity had stronger negative effects on coastal individuals, indicating a lack of local adaptation and overall lower performance compared to their inland counterparts. These findings suggest that increasing salinity will have varied impacts on organisms depending on their population origins and developmental stages.

Key-Words: Behaviour, Eggs, Larvae, Oxidative status, Salinization, Telomere length

1. INTRODUCTION

Salinization - the increase in environmental salinity - is predicted to intensify due to climate change (Reid et al., 2019) and increased water demand (Cañedo-Argüelles et al., 2013). Many inland freshwater bodies are becoming more saline due to human activities (Williams, 2001), which include groundwater pumping (Reid et al., 2019), use of salt to de-ice impervious surfaces (Findlay and Kelly, 2011), reduction in freshwater flow and droughts (Reid et al., 2019). In addition to these causes, coastal ecosystems are expected to experience further salinization because of the forecasted sea level rise (Bakker et al., 2017), and the increase in frequency and intensity of marine storms and associated marine submersions (Dettinger, 2011).

Salinization is expected to have severe consequences for biodiversity and ecosystem functioning (Cañedo-Argüelles et al., 2013; Herbert et al., 2015). Indeed, most organisms have to osmoregulate (Bradley, 2009) and activate physiological mechanisms to regulate water and ion fluxes to maintain internal homeostasis (Evans and Kültz, 2020). Yet, the mechanisms involved in the regulation of the hydro-mineral balance are metabolically expensive (Herbert et al., 2015), negatively influencing critical organismal functions and survival (Cañedo-Argüelles et al., 2013; Herbert et al., 2015). Additionally, elevated salinity can detrimentally affect reproductive performance (Hart et al., 2003). For instance, salinity has been shown to decrease sperm performance (Green et al., 2021) and fecundity (Pinder et al., 2005), and early life stages usually do not tolerate as elevated salinity as adults (Liu et al., 2022; Walker et al., 2023).

Amphibians are particularly susceptible to environmental salinity due to their highly permeable skin used for gas exchange, and for ion and water transport (Shoemaker and Nagy, 1984), and because of their comparatively limited ability to osmoregulate (Katz, 1989). Early-life stages (i.e. embryos and larvae) are more susceptible to salinity than adults (Albecker and McCoy, 2017). Amphibian embryos have very low osmoregulatory abilities (Karraker and Gibbs, 2011), and embryos and larvae are often entirely dependent on - and thus restricted to - the aquatic environment compared to adults (Wells, 2007). Embryos and larvae are thus highly sensitive to moderate salinity levels, which can affect the duration of embryonic development (Haramura, 2016; Tornabene et al., 2021a) and the developmental stage at hatching (Karraker and Gibbs, 2011; Tornabene et al., 2021a). In larvae, salinity may depress thyroid hormones (Gomez-Mestre et al., 2004) and induce changes in hormones involved in osmoregulation such as corticosterone and aldosterone (Tornabene et al., 2022, 2021b), but also in antioxidant enzymes (Burraco and Gomez-Mestre, 2016). These processes can lead to prolonged larval development (Gomez-Mestre and Tejedo, 2003; Lukens and Wilcoxon, 2020) and reduced growth (Bernabò et al., 2013; Gomez-Mestre et al., 2004; Gomez-Mestre and Tejedo, 2003; Haramura, 2016; Lukens and Wilcoxon, 2020; Tornabene et al., 2021a; Wood and Welch, 2015; Wu and Kam, 2009). In addition to these physiological mechanisms, exposure to salinity can also induce behavioral alterations. Indeed, larvae exposed to higher salinity express reduced activity and foraging behavior (Hall et al., 2017; Tornabene et al., 2021a), low responses to stimuli (Sanzo and Hecnar, 2006), low swimming performance (Haramura, 2016), erratic

movements (Tornabene et al., 2021a), and reduced speed (Denoël et al., 2010). Ultimately, increasing salinity has been shown to increase embryonic and larval deformities (Brady, 2013; Hopkins et al., 2013; Sanzo and Hecnar, 2006; Tornabene et al., 2021a) and to decrease survival (Karraker et al., 2008; Sanzo and Hecnar, 2006).

Taken together, these elements point to detrimental impacts of salinization on amphibian populations (Karraker et al., 2008). Yet, it is noteworthy that exposure to moderate levels of salinity can induce an adaptive context and select for increased tolerance to salinity in coastal amphibians (Gomez-Mestre and Tejedo, 2003; Tornabene et al., 2021a). As a consequence, individuals originating from brackish coastal habitats should be more tolerant to increased salinity compared to naïve individuals originating from strictly freshwater habitats. However, it is also plausible that parents living and breeding in salty environments might produce eggs of lower quality due to the costs associated with chronic exposure to salinity (Lorrain-Soligon et al., 2023a). This could result in a compounded negative effect on offspring development in coastal populations, where both parents and eggs are adversely affected by salinity, leading to additive detrimental impacts. Yet, the effects of local adaptation on the developmental and behavioral responses of coastal amphibian larvae to salinity remains poorly investigated.

Additionally, because osmoregulation is energetically demanding and ATP consuming function (Kirschner, 1995), increased ATP consumption can induce an

increased production of reactive oxygen species (ROS). A large quantity of ROS is generally toxic to the organism, leading to detrimental oxidative stress (Halliwell and Gutteridge, 2015; Netto and Antunes, 2016). In turn, increased oxidative stress can damage telomeres (Armstrong and Boonekamp, 2023), which are known to shorten in response to environmental and physiological stress (Angelier et al., 2018; Chatelain et al., 2020). Importantly, telomere length can represent a relevant proxy of longevity and fitness in many wild vertebrate species (Angelier et al., 2019; Wilbourn et al., 2018), including some amphibians (Burraco et al., 2017). Both markers (oxidative status and telomere length) are expected to respond strongly to environmental stress during development (Burraco et al., 2020b, 2017; Cheron et al., 2022), but interestingly the effect of salinity during development, or difference between habitat (i.e. coastal versus inland populations) have never been assessed.

In this study, we investigated impacts of salinity in embryos and larvae of a widespread amphibian species (spined toad, *Bufo spinosus*) originating from salt-exposed (coastal) and salt-free (inland) populations. We experimentally tested the effect of different salinities (0, 2 and 4 g.l⁻¹) mimicking those measured in salt-exposed (coastal) environments. During the embryonic development, we recorded survival (hatching rate) and duration of development (time to hatching). Upon hatching, we assessed oxidative status and telomere length as markers of physiological alterations (Angelier et al., 2018; Armstrong and Boonekamp, 2023; Chatelain et al., 2020; Halliwell and Gutteridge, 2015; Netto and Antunes, 2016). During the larval development, we assessed survival, duration of development, malformation,

morphology and growth. We also investigated behavioral characteristics (activity, swimming speed, space use, behavioral complexity). Finally, once metamorphosis was completed, we investigated the consequences of the salinity experienced during embryonic and larval development on metamorphic individuals. We predicted that the highest salinity treatment should negatively affect individuals, and that these effects should be more pronounced in naïve individuals originating from salt-free environments due to local adaptation in coastal individuals (Gomez-Mestre and Tejedo, 2003). We also predicted that individuals originating from salt-free (inland) and salt-exposed (coastal) populations should perform better in freshwater (Gomez-Mestre and Tejedo, 2003). Finally, we predicted that the effects of early - pre-metamorphosis - exposure to salinity should carry over and influence performance in newly-metamorphosed individuals.

2. METHODS

2.1. Study species and egg collection

The spined toad (*Bufo spinosus*) is a widespread amphibian species found in Western Europe, breeding in late winter, during which adults converge to reproductive ponds where they mate and produce elongated egg strings that contains thousands of eggs. Egg laying, embryonic and larval developments occur in water and are therefore directly impacted by the salinity of breeding ponds.

Already composed pairs (amplexus) were captured in 3 coastal and 4 inland locations (Lorrain-Soligon et al., 2023a). As coastal and inland populations were geographically separated by more than 50 km, amplexus collected within these locations should be sufficiently distant to provide an accurate assessment of population-level differences in their susceptibility to salinity. Captures occurred at night (between 8 pm and 4 am) from 16/02/2022 to 02/03/2022. For inland populations, four ponds were prospected (coordinates: 46.1707, -0.4561; 46.1558, -0.4831; 46.0489, -0.2373; 46.1458, -0.4248), where salinity was 0.0 g.l⁻¹. For coastal populations, three ponds were prospected (coordinates: 46.0469, -1.0516; 46.0359, -1.05307; 46.039504, -1.049723), where salinity ranged from 1.8 to 3.9 g.l⁻¹ (mean=2.67 g.l⁻¹ ± 0.69 SE).

For each population (coastal/inland), 20 amplexus were captured by hand, placed in a transport box (14*16*9 cm), and brought to the laboratory immediately after field sessions.

At the laboratory, each pair was placed in containers (35*55*26 cm) containing freshwater (~20 L, salinity 0.3 g.l⁻¹) and branches for laying support, in a thermally controlled room with temperature set at 17 °C and controlled photoperiod (12 h dark–12 h light). Amplexus were left in these tanks until the egg strings were laid (min time=10 h, max=175 h; mean=66 h ± 45 h, no difference between inland and coastal individuals (Lorrain-Soligon et al., 2023a)). Upon laying, 6 segments of each egg string, containing 30 eggs each (i.e. a total of 180 eggs for each egg string) were selected randomly and kept for our experiment. Adults and the remaining eggs (i.e. 1500–8500) were released at their site of origin.

2.2. Experimental design

2.2.1. Treatment salinity

Our goal was to mimic the range of salinity found *in natura*, and we produced 3 treatments: control (0 g.l⁻¹ salinity: 0.3 ± 0.0 g.l⁻¹), low (2 g.l⁻¹ salinity 2.0 ± 0.05 g.l⁻¹) and moderate (4 g.l⁻¹ salinity 4.0 ± 0.06 g.l⁻¹) which correspond to salinity levels found in coastal environments (see above).

2.2.2. Egg segments (n=240)

Each clutch was represented by 2 segments of 30 eggs in each of the experimental treatments (i.e. for each clutch: 2 segments in the control treatment, 2 segments in the

low treatment, and 2 segments in the moderate treatment). Each segment was placed in a glass tank containing 2 L of water, and water was changed once a week. To prevent osmotic shock, and because natural salinity fluctuations do not exceed $\pm 2 \text{ g.l}^{-1}$ a day, segments exposed to the low or moderate treatments were gradually exposed to salinity with an increase of 1 g.l^{-1} a day. Following this procedure, segments in the low treatment reached 2 g.l^{-1} in 2 days, and segments exposed to moderate treatment reached 4 g.l^{-1} in 4 days. Each segment was monitored twice a day until tadpoles hatched and reached a free feeding stage (Gosner stage 25 (Gosner, 1960), hereafter GS25).

2.2.3. Tadpoles ($n=226$)

Upon hatching, undeformed tadpoles of each segment (e.g. 6 tadpoles per clutch, 2 from the control, 2 from the low treatment, and 2 from the moderate treatment) were selected randomly, placed individually in a glass tank containing 2 L of water with the same salinity than during their embryonic development, and their development was monitored until the end of the metamorphosis. Additional 4 tadpoles per segment were kept at -80°C for physiological measurements (oxidative status and telomeres length, see below). All other tadpoles were released at the site where their parents were captured. In 14 segments (7 control, 2 low and 5 moderate) representing 9 clutches, no individuals survived.

During the whole larval development, water was changed once a week, and tadpoles were fed *ad libitum* with thawed organic spinach. Individuals were checked

twice a day for their developmental stage. We selected Gosner stages 25, 30, 37, 41 and 42 (Gosner 1960, hereafter, GS 25, GS 30, GS 37, GS 41 and GS 42, respectively).

Individuals were kept until metamorphosis, which occurred at Gosner stage 45 (hereafter GS45). At this stage, individuals were transferred in a plastic box (14*16*9 cm) containing a humid paper towel (moistened with water of the same salinity as their treatment) and a shelter. Three coastal individuals in the control treatment never reached metamorphosis (two of them were at GS39 after more than 108 days after GS37, and one of them was at GS41 and did not develop to GS42 for more than 18 days, while it usually take less than 10 days) and were removed from the experiment. At the end of this experimental procedure, individuals were released at the site where their parents were captured.

2.3. Morphological and survival measurements

At GS 25, we assessed hatching success (proportion of eggs that have hatched) and deformation rates (number of larvae that hatched but were malformed divided by the total number of hatched individuals). We quantified visually for different types of malformations: spinal and tail deformities, bent body or tail, edema, underdeveloped individuals, and presence of cysts.

For each larval stage (GS 25, GS 30, GS 37, GS 41 and GS 42), we measured total length, body length, tail length, and head width. Additionally, from GS 37 to GS 42, we also measured tail height, tail muscle height, body height and hindlimb length.

Upon metamorphosis (GS45), we measured the snout-to-vent length (SVL) of toadlets as well as their body width and body height. Five days after metamorphosis (to allow entire tail resorption and skin drying), toadlets were weighted (OHAUS scale, precision $\pm 0.001\text{g}$).

For all measurements, each tadpole and metamorphic individual were put individually into a Petri dish, with the water from its own tank (tadpoles) or without water (metamorphic individuals), and photographed from above and laterally, by placing a millimeter scale next to the individuals. Morphological measurements were performed with the software ImageJ (Schneider et al., 2012).

All individuals were checked daily and abnormalities or mortality were recorded.

2.4. Physiological measurements

120 tadpoles (20 tadpoles per treatment and per site) were used for telomere analysis, and 180 tadpoles (30 tadpoles per treatment and per site) were used for analysis of oxidative status markers.

For telomere analysis, whole individuals were digested with proteinase K, and DNA was extracted using the Nucleospin Tissue Kit (MachereyNagel). The DNA concentration and purity were assessed with a Nanodrop ND2000 spectrophotometer

(Thermo Scientific). Telomere length was determined by quantitative PCR (qPCR) using a BioRad CFX 96 (Bio-Rad, USA) as previously described (Cheron et al., 2022). The control single-copy gene Recombination Activating Gene 1 (RAG-1) was selected and amplified using specific primers designed for the spined toad: RAG1-F 5' - GGGTCCTCTGATAGCCGAAA-3' and RAG1-R 5' - CATCATAACCTGTACCCCGGA-3'. The telomere and single-copy gene primers were used at a concentration of 800 nM. A standard curve (serial dilutions of DNA from a pooled sample of several tadpoles) was run for controlling the amplifying efficiency of the qPCR reactions. For both telomere and RAG-1 amplification, the melt curves displayed single sharp curves, validating amplification specificity of these primers. All samples were run in 4 plates. The relative telomere length (expressed as T/S ratio) was calculated as the telomere copy number (T) relative to single-copy gene (S; RAG1).

For oxidative status analysis, a pool of 3 tadpoles was collected for each segment. The pool of tadpoles was lysed using a TissueLyser II (Qiagen) in Dulbecco's PBS solution supplemented with 1 mM PMFS. We measured the concentration of thiols using the -SHp test (Diacron International); the level of the non-enzymatic antioxidant defenses (OXY) using the OXY-Absorbent assay, the activity of the antioxidant enzymes superoxide dismutase (SOD) using the Ransod assay (RANDOX Laboratories, France), and catalase (CAT) using the OxiSelect CAT Activity Assay (Euromedex, France). We ran all analyses in duplicate or triplicate. We standardized

values of markers by the amount of proteins as quantified using the Bradford protein assay with albumin as reference standard (Sigma-Aldrich, France).

2.5. Activity, locomotion, and behavioral complexity

Upon hatching (GS25) but before tadpoles were transferred to their individual aquaria, we assessed general activity of the whole batch of individuals belonging to each segment. We used a scale of 1 to 5 to assess the proportion of live individuals that were active (moving; 1=0-20%, 2=20-40%, 3=40-60%, 4=60-80%, 5=80-100%) over a 1-min period, following (Lorrain-Soligon et al., 2024).

Just after hatching (GS25), once individuals were transferred to their individual aquaria, we assessed their position in the water column (at the bottom of their aquarium, or within the water column). We also performed a test of escape from predation: we gently stimulated (touched) tadpoles with a pipette, and their response (escape or no escape) was recorded.

At GS37, a pivotal stage characterized by the peak of activity and associated behavior (Cheron et al., 2021), we performed a comprehensive set of behavioral measures. Tadpoles were removed from their tank and placed in an individual arena filled with the water from their tank (Petri dish, diameter 13.5 cm, water level 1.5 cm). Tadpoles were left acclimated for 20 min, and then video recorded for 35 min, using a camera placed above the arena (GoPro HERO; GoPro, San Mateo, CA, USA). Videos

were analyzed with the software ToxTrac (Rodriguez et al., 2018). We used a white background to create a contrast that allowed the software to follow the tadpoles. Using 'Tracking RealSpace' data, we determined the total duration of activity; the total distance travelled (both absolute and relative to body size), mean swimming speed (both absolute and relative to body size), and frequency of positioning at less than 50 mm to the center of the arena. Additionally, we conducted DFA (Detrended Fluctuation Analysis) and DFAC (corrected Detrended Fluctuation Analysis) using the *fractal* package (version 2.0-4, (Constantine and Percival, 2017)). DFA is inversely related to the fractal dimension, a classical index of structural complexity (Mandelbrot, 1982), and thus smaller values reflect greater complexity.

At GS45, toadlets were positioned on a 2 m² plane surface, and were touched with a pipette. Activity was recorded with a scale ranging from 1 to 5 (from amorphous to individual that moved energetically, following (Lorrain-Soligon et al., 2022)). Additionally, we recorded their posture (0: prostrate, 1: stand straight), and their ability to jump (0: no jump, 1: at least one jump). For individuals that did jump, we further assessed jumping performance by measuring the distance travelled for each jump during 6 consecutive jumps across which mean distance was extracted.

2.6. Statistical analyses

We investigated the differences between sites (coastal and inland) and between treatments (0, 2 or 4 g.l⁻¹ salinity), as well as the interaction between treatment and

sites. We used LMMs (Linear Mixed Models) or GLMMs (Generalized Linear Mixed Models), with clutch as a random effect as 6 individuals (or 6 segments) of each clutch were assigned to the treatments. For all models computed, models accuracy was tested using the `check_model` function from the *performance* package (Lüdtke et al., 2020), as well as normality of residuals for LMMs.

To test for the effect of treatment and site on larval mortality, we used a Cox mixed-effects models fitted by maximum likelihood with clutch as a random effect (R package 'coxme' (Therneau, 2024)). We also computed Kaplan-Meier curves of survival using the *survival* package (Therneau, 2022). Numbers of dead and malformed individuals of each stage and for each treatment are presented in Appendix A.

For all Gosner stages, as morphological variables were all highly correlated (see Appendix B), we performed PCA on all the morphological metrics taken at each developmental stage, and extracted the coordinates of the first axis (explaining most part of the variation, see Appendix B), hereafter termed as "morphology score". An increase of the score indicates an increase of all the morphological parameters. To test for the effect of treatment and site on the morphology score at each stage we performed LMMs. Results for total length are also presented for clarity. The same procedure was applied to the behavioral variables at GS37 (see Appendix C), hereafter termed as "behavior score". An increase of the score indicates an increase of total distance, mean speed and total duration of activity, but a decrease in DFA and of the frequency of

positioning at less than 50 mm to the center. For clarity, we also detailed the result found for each behavioral variable.

To test for differences in hatching success, malformation rate and general activity (all during embryonic development), position within the water column and escape from predation (both binomial variables with 0/1 responses, measured at hatching: GS25), in positioning at less than 50 mm to the center (at GS37) and differences in activity (measured at metamorphosis [GS45], using the score as a proportion compared to 5 being the maximum value for activity), between sites and treatment, we performed binomial GLMMs.

To investigate the effect on time to hatching, as well as larval development duration (time in day between hatching and the other specific developmental stages), total distance traveled and duration of activity (measured at GS37), we performed negative binomial GLMMs.

To test for the effect of treatment and site on the behavior score as well as on DFAc and average speed (measured at GS37) as well as effects on the morphology scores at all life stages, body mass and mean jump distance (measured at GS45), we performed LMMs.

We also tested if telomere length and oxidative status (thiols concentration, Oxy, SOD and CAT) at the end of embryonic development were influenced by treatment, site, and their interaction, as well as tadpoles' size (mean tadpoles' size for oxidative status as 4 tadpoles were pooled for these analyses), embryonic development duration and hatching success. As tadpoles' size, embryonic development duration hatching success were correlated to treatment and site (high variance inflation factor [VIF], package *car* (Fox and Weisberg, 2018)) we used two different LMMs for each variable, one testing treatment, site, and their interaction as dependent variables, and the other testing tadpoles' size, embryonic development duration and hatching success as dependent variables, and clutch as a random effect. For these analyses, best variables were retained using a top-down selection, and only the retained variables are presented.

All data analysis were performed using R 3.6.3 (R Core Team, 2020) and Rstudio v1.1.419. Results of statistical analyses are presented in Appendices D (results of the Anova of each test), E (Post-hoc analyses for the differences between sites), F (Post-hoc analysis for the differences within site between the different treatments) and G (Post-hoc analysis for the differences within treatment between the different sites).

3. RESULTS

3.1. Embryonic development

Hatching success was higher in the 2 g.l⁻¹ and 4 g.l⁻¹ salinity treatments compared to the control (0 g.l⁻¹ treatment), with no differences between coastal or inland individuals (Fig 1A, Appendices DEFG). However, malformation rate was higher in the 4 g.l⁻¹ salinity treatments compared to the 0 g.l⁻¹ and 2 g.l⁻¹ salinity treatments, and higher in coastal individuals compared to inland ones in the 4 g.l⁻¹ salinity treatment (Fig 1B, Appendices DEFG). General activity was also higher in the 0 g.l⁻¹ and 2 g.l⁻¹ salinity treatments compared to the 4 g.l⁻¹ treatment, with no differences between coastal or inland individuals (Fig 1C, Appendices DEFG). For inland individuals, time to hatching did not vary according to salinity, but increased in the 4 g.l⁻¹ salinity treatment in coastal individuals (Appendices DEFGH).

Upon hatching, thiols concentration was higher in inland individuals as compared to coastal ones (Estimate=0.020, SE=0.009, t-value=2.220, p-value=0.033, Fig 2A) and was positively correlated to embryonic development duration (Estimate=0.006, SE=0.003, t-value=2.113, p-value=0.040, Fig 2A). OXY was also positively correlated to embryonic development duration (Estimate=0.047, SE=0.010, t-value=4.634, p-value<0.001, Fig 2B). None of them were influenced by the treatment (top-down selection). SOD was influenced neither by treatment, site, tadpoles' size, embryonic development duration nor hatching success (Fig 2C). CAT was negatively related to tadpoles' size (Estimate=-9.496, SE=4.662, t-value=-2.037, p-value=0.047), and marginally negatively related to embryonic development duration (Estimate=-6.900,

SE=3.912, t-value=-1.764, p-value=0.084, Fig 2D). CAT was not influenced by treatment, site, or their interaction. Telomere length was influenced by treatment, site and their interaction (Fig 1D, Appendices DEFG), and was positively correlated to embryonic development duration (Estimate=0.042, SE=0.008, t-value=5.272, p-value<0.001, Fig 2E) and tadpole size (Estimate=0.031, SE=0.008, t-value=3.825, p-value<0.001, Fig 2F).

3.2. Larval development

3.2.1. *Survival*

Survival across the whole larval development was influenced by treatment (Chisq=206.143, p-value<0.001) and site (Chisq=10.202, p-value=0.001). Indeed, overall, survival was lower in coastal individuals as compared to inland ones (Estimate=-0.901, SE=0.282, z-value=-3.194, p-value=0.001), and was lower in the 4 g.l⁻¹ salinity treatment as compared to the 0 g.l⁻¹ (Estimate=3.165, SE=0.274, z-value=11.569, p-value<0.001) and 2 g.l⁻¹ (Estimate=3.722, SE=0.304, z-value=12.253, p-value<0.001) salinity treatments, but similar between the 0 g.l⁻¹ and 2 g.l⁻¹ (Estimate=-0.558, SE=0.323, z-value=-1.728, p-value=0.194) salinity treatments (Fig 3, Appendix A). In the 4 g.l⁻¹ salinity treatment, no coastal individuals survived to GS41 (Fig 3A), while 4 inland individuals survived to GS45 (Fig 3B). Malformation rate was higher in the 4 g.l⁻¹ salinity treatment (see Appendix A).

3.2.2. *Morphology*

Overall, across the larval development, inland individuals had a larger morphology score (Fig 4A, Appendices DEFG) and body length (Figure 4B) compared to coastal ones. At the beginning of the larval development, individuals in the 0 and 2 g.l⁻¹ treatments had a similar morphology score and body length, both being larger compared to individuals in the 4 g.l⁻¹ salinity treatment. At the end of the larval development, individuals in the 2 g.l⁻¹ treatment had a larger morphology score and body length compared to individuals in the 0 g.l⁻¹ treatment (Fig 4AB, Appendices DEFG), and inland individuals in the 4 g.l⁻¹ treatment seemed to have a larger morphology score and body length compared to individuals in the other treatments (Fig 4AB), although this was not significant (Appendices DEFG).

3.2.3. Development duration

During early stages of development (GS30-GS37), inland individuals were slower to develop (Fig 4C, Appendices DEFG). Conversely, at later developmental stages (GS41-GS45), coastal individuals were slower to develop (Fig 4C, Appendices DEFG).

Salinity (4 g.l⁻¹) affected development duration in inland individuals during early developmental stages solely (GS30-GS37), but this effect was transient and at later developmental stages, we found no significant differences between treatments (Fig 4C, Appendices DEFG).

3.2.4. Behavior

At GS25, individuals in the 4 g.l⁻¹ treatment were positioned at the bottom of their aquarium more often while individuals in the 0 g.l⁻¹ and 2 g.l⁻¹ salinity treatments moved within the water column (Appendices DEFGI). Similarly, individuals in the 4 g.l⁻¹ treatment expressed fewer escape response to predation compared to individuals in the 0 g.l⁻¹ and 2 g.l⁻¹ salinity treatments (Appendices DEFGI). Coastal and inland individuals expressed no differences in these behaviors.

At GS37, the behavior score was always higher in the 2 g.l⁻¹ salinity treatment compared to the 0 g.l⁻¹ and 4 g.l⁻¹ salinity treatments, and always higher in inland individuals compared to coastal ones (Appendices DEFGJ). Specifically, DFA and DFAc were lower (higher behavioral complexity) in the 2 g.l⁻¹ treatment and in inland individuals (Fig 5A, Appendices DEFG). Position to the center of the arena was higher in the 4 g.l⁻¹ salinity treatment compared to the 0 and 2 g.l⁻¹ salinity treatments, and higher in coastal individuals compared to inland ones (Fig 5B, Appendices DEFG). Total distance traveled and average speeds (both corrected by individuals' size) were higher in the 2 g.l⁻¹ salinity treatment and for inland individuals (Fig 5CD, Appendices DEFG).

3.3. Metamorphic individuals

3.3.1. *Survival*

No coastal individuals in the 4 g.l⁻¹ salinity treatment survived to metamorphosis, and only 4 inland individuals in the 4 g.l⁻¹ salinity treatment survived to metamorphosis (Fig 3, Appendix A). The survival at GS45 was also lower in coastal individuals in the

0 g.l⁻¹ salinity treatment (Fig 3B). At metamorphosis, no individuals presented evidence of malformation (Appendix A).

3.3.2. *Morphology*

Morphology score was higher in inland individuals, and particularly higher for inland individuals in the 4 g.l⁻¹ salinity treatment (Fig 4A, Appendices DEFG). The morphology score between individuals in the 0 and 2 g.l⁻¹ salinity treatment was similar (Fig 4A, Appendices DEFG). The same pattern was found for body mass (Fig 6A, Appendices DEFG).

3.3.3. *Behavior*

At GS45, activity between individuals in the 0 and 2 g.l⁻¹ salinity treatment was similar (Fig 6B, Appendices DEFG). In inland individuals, 41.4% individuals in the 0 g.l⁻¹ treatment both stood straight and actively jumped; 61.3% and 54.8% individuals in the 2 g.l⁻¹ salinity treatment respectively stood straight and actively jumped and 100% individuals in the 4 g.l⁻¹ salinity treatment stood straight and actively jumped. By contrast, in coastal individuals, 28.6% and 14.3% individuals in the 0 g.l⁻¹ treatment respectively stood straight and actively jumped; and 66.7% and 50.0% individuals in the 2 g.l⁻¹ salinity treatment respectively stood straight and actively jumped. Mean jumping distance was similar between treatments and between origins, but with a non-significant trend to be higher in inland individuals (Fig 6C, Appendices DEFG).

4. DISCUSSION

In this study, we show that exposure to moderate environmental salinity (4 g.l⁻¹) altered both embryonic and larval development in individuals originating from naïve (salt free) and coastal (salt-exposed) populations. Specifically, higher salinity increased malformations, decreased body size and survival and altered behavior. Contrary to our predictions, these effects were more marked in coastal individuals originating from salt-exposed populations compared to inland ones. Interestingly, irrespective of origin, individuals exposed to the low salinity treatment (2 g.l⁻¹) performed better for most traits investigated.

4.1. *Differences in sensitivity between coastal and inland populations*

Higher salinities are known to be harmful to both embryos and larvae (Albecker and McCoy, 2017; Nakkrasae et al., 2016), in both coastal and inland populations (Albecker and McCoy, 2017). Yet, individuals native to brackish environments are expected to express a stronger tolerance to salinity because of local adaptation (Gomez-Mestre and Tejedo, 2003). However, in our study, survival and all metrics of development were severely reduced at 4 g.l⁻¹ in coastal individuals compared to inland ones. By contrast, we found that inland individuals performed better under all treatments. Different hypotheses can explain this counterintuitive lower tolerance to salinity in coastal individuals. First, the reduced development and survival we highlighted in coastal individuals may be the result of their smaller telomere's length, and/or to the smaller size of the eggs from which they hatched (Lorrain-Soligon et al., 2023a). Indeed, coastal

amphibians are often found to be smaller, as a result of osmotic costs (Lorrain-Soligon et al., 2023a; Marangoni et al., 2008), which affect their reproductive output (decreased clutch size and egg size, Lorrain-Soligon et al., 2023a), and could ultimately reduce larval tolerance to salinity. Indeed, larger eggs will produce larger – presumably more resistant – hatchlings. In addition, larger eggs are more likely to successfully hatch, and larger egg size is associated with higher offspring and larval fitness (Xu et al., 2019). Whether this reduction in size is persistent and linked to evolutionary response to exposure to salinity, or genetic differences between inland and coastal populations (Albecker et al., 2021) needs to be studied. In addition, it is also plausible that reduced performance of coastal individuals is linked to the fact that adults produced their eggs in freshwater, a salinity that does not correspond to the salinity of their environment (environmental mismatch paradigm, Telesh et al., 2013). However, this hypothesis is unlikely to explain our results as another study has demonstrated that coastal individuals of the same species did not perform better if they were spawned in brackish water (Lorrain-Soligon et al., 2024). Third, it is possible that, in coastal populations, salinity gradients occur within ponds (e.g., linked to differential density of salt versus fresh water, (Millero and Poisson, 1981)), and salinity also vary spatially and temporally in coastal ponds (Lorrain-Soligon et al., 2023b). As a consequence, such salinity gradients may allow reproductive females to select areas characterized by lower salinities to lay their eggs (Albecker and McCoy, 2017), and/or larvae to select such areas to develop. Future studies should investigate whether such processes occur *in natura*. It is also noteworthy that we considered only one type of salt (NaCl), while the effects of salinity can differ according to the different types of salt (e.g., Na⁺, K⁺,

Cl⁻, CO₃²⁻, SO₄²⁻), which can be combined in coastal habitats (Walker et al., 2023). It is plausible that our findings may differ in response to these other types of salt and/or their combination. Further studies are required to decipher the underlying mechanisms, including investigations of the physiological mechanisms used by individuals from coastal and inland populations to cope with elevated salinity.

4.2. *Embryonic development*

Embryonic development duration did not vary according to salinity in inland individuals. Conversely, in coastal individuals, embryonic development duration increased in individuals exposed to moderate salinity solely. As a consequence, embryonic development duration was longer in inland individuals at the lower salinities (0 and 2 g.l⁻¹), but shorter at 4 g.l⁻¹ compared to coastal individuals. Differences in development time according to salinity have already been reported in amphibians (see (Lukens and Wilcoxon, 2020; Sanzo and Hecnar, 2006; Wu and Kam, 2009)). It is plausible that osmoregulation mechanisms activated to cope with high salinity altered developmental rates, possibly owing to energy diverted from growth towards osmoregulatory mechanisms (Gomez-Mestre et al., 2004). This further suggests a possible energetic trade-off between development and osmoregulation more pronounced in coastal individuals as compared to inland ones.

Importantly, this difference might be related to site-specific differences in antioxidants. Indeed, irrespective of treatment, thiols concentration was higher in

inland individuals as compared to coastal ones. As thiols groups can undergo redox reactions, they can participate in important cellular processes such as energy production and antioxidant defense mechanisms (Tu and Weissman, 2004). The higher concentration of thiols in inland individuals might explain their higher tolerance to saline, stressful environments. Importantly, thiols concentration and OXY were both positively correlated to embryonic development duration. Thiols and OXY markers are indicative of the non-enzymatic antioxidant status (Pamplona and Costantini, 2011), and might be costly to produce, thus inducing higher energy expenditures and longer development duration. On the contrary, catalase activity was negatively correlated to embryonic duration. Catalase is an enzyme that specifically helps to break down hydrogen peroxide, a reactive oxygen species, into water and oxygen (Gebicka and Krych-Madej, 2019). It suggests an interplay between enzymatic and non-enzymatic antioxidant systems in tadpole physiology. Possible explanations include differential regulatory mechanisms and resource allocation trade-offs. Standard metabolic rate needs to be investigated in coastal and inland individuals exposed to different levels of salinity in order to further understand these processes (Burraco and Gomez-Mestre, 2016).

Interestingly, lower hatching success in both coastal and inland individuals were found for freshwater (0 g.l⁻¹ salinity), possibly owing to hormetic mechanisms (Costantini et al., 2010). Indeed, some evidence suggests that low salinity could be beneficial while freshwater may be detrimental (Martinez-Palacios et al., 2004; Robles Mendoza et al., 2009). This could be due to lower parasite and pathogen prevalence at

low salinity levels as compared to freshwater (Clulow et al., 2018). In any case, hatching success may be poorer *in natura* as 4 g.l⁻¹ salinity has been shown to reduce the ability of sperm to survive and swim (Green et al., 2021), but this hypothesis is not supported by a complementary experiment on the same species (Lorrain-Soligon et al., 2024).

In addition, we show that telomere length was smaller in coastal individuals as compared to inland ones, this difference being particularly relevant in the 0 and 2 g.l⁻¹ salinity treatment, but not in the 4 g.l⁻¹ salinity treatment. Telomere length is known to be a reliable measure of cellular aging (Marasco et al., 2022) and exposure to stress (Angelier et al., 2018; Chatelain et al., 2020). These results suggest that coastal individuals undergo more stress than inland ones, which can also explain the lower performance of coastal individuals, in addition to smaller size of eggs and larvae produced by coastal individuals (Lorrain-Soligon et al., 2023a). Indeed, importantly, we found increasing telomere length with increasing tadpoles' size and embryonic development duration (Burraco et al., 2020b, 2017). This is in line with another study suggesting that life-history traits can be major drivers of telomere dynamics, while environmental conditions may have weaker effects (Burraco et al., 2020a). However, it is plausible that environmental salinity influenced telomere length through its effect on hatchling size and embryonic development duration.

4.3. Larval development

During larval development, we found that survival and growth were reduced, and malformation rate increased in brackish water (Hopkins et al., 2013; Lukens and Wilcoxon, 2020; Wu and Kam, 2009). In the 4 g.l⁻¹ salinity treatment, individuals were the smallest. These individuals had a higher frequency of positioning at less than 50 mm of the center of the arena, where tadpoles can be more visible to predators (see (Denoël et al., 2012) and references therein), which can be considered as a “risky” strategy. Complementarily, these individuals also displayed less complex behavior (Higher DFA, Mandelbrot, 1982), leading, in natural environments, to a reduced probability of resource acquisition (Viswanathan et al., 2008), and a tendency to be less active (Sanzo and Hecnar, 2006).

Interestingly, in the control treatment, individuals expressed a higher DFA and thus less complex behavior (Mandelbrot, 1982). Control individuals also displayed a smaller speed and lower distance travelled compared to the 2 g.l⁻¹ salinity treatment. As in other studies, we found that survival and body length increased at low salinities compared to freshwater (Lambret et al., 2021; Nakkrasae et al., 2016). Similarly to what we suggest for embryos (see above), the mechanisms underlying why low salinities may be beneficial during larval development remain to be identified, but might be due to lower osmolality differential between tadpoles and water.

4.4. *Metamorphic individuals*

An intriguing pattern is the lack of difference between treatments in the time to reach metamorphosis while such result has been widely reported (Lukens and Wilcoxon, 2020; Sanzo and Hecnar, 2006; Wu and Kam, 2009). We found a difference in the time to metamorphosis between coastal and inland individuals, with inland individuals reaching metamorphosis sooner than coastal individuals. The optimal timing to metamorphosis involves trade-offs between growth, development, and survival of both the larvae and the future adult (Semlitsch et al., 1988). We found that inland individuals metamorphosed sooner at a larger body size compared to coastal individuals. Interestingly, complete development was not achieved in 3 coastal individuals exposed to the control treatment, which may be attributed to maladaptation of these individuals to freshwater environments ((Brady, 2013), but see (Gomez-Mestre and Tejedo, 2003)). Individuals in coastal and inland populations also differed in size and mass at metamorphosis, which might be linked to parental size (Lorrain-Soligon et al., 2023a) and may, in turn, positively influence juvenile survival, adult size, size at first reproduction, mating success, and reproductive output (Relyea, 2007; Semlitsch et al., 1988).

Importantly, at metamorphosis, individuals in the 4 g.l⁻¹ salinity treatment (all originating from inland populations) had a tendency to perform better, with a seemingly higher activity, size and mass (see also (Dahrouge and Rittenhouse, 2022)). This could be linked to the selection of the few individuals that were able to survive in this harsh environment (Lorrain-Soligon et al., 2024), and once again highlight higher quality of inland individuals. Inland, only the better performing, and thus largest

individuals, survived. However, low sample size (only 4 inland individuals survived in this treatment) precluded robust statistical results. Yet, the effects of high-salinity during larval development can carry-over later during the post-metamorphic juvenile life stage (Dahrouge and Rittenhouse, 2022), so that these surviving inland individuals can experiment detrimental effects later in life, and longer-term monitoring of these individuals during their juvenile and adult life-stages would be valuable.

5. CONCLUSION

In this study, we showed very high sensitivity of larval anurans to salinity (albeit at lower levels than what have been reported in literature, see (Albecker and McCoy, 2017)), but beneficial effects of salinity at low concentrations (2 g.l^{-1}), highlighting a potential threshold effect, which had not yet been documented. In particular, we found that alteration of embryonic development, telomere attrition, and modified activity might be relevant proximate mechanisms underlying the responses of amphibians to increasing salinity. Our results also suggest that (i) conspecific populations may differ in their responses to a same salinity stress, and (ii) there are specific stages of development during which toads are more sensitive to salinity. Interestingly, we also highlighted that coastal individuals performed poorly both in fresh- and brackish water. Our results suggest that the impact of increasing salinity on organisms is likely to differ between conspecific populations and development stages.

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CRedit

LLS & FB have conceptualized the study. LLS, LB & ML participated to data collection. LLS & AK analyzed the data. LLS performed DNA extraction, FA & CR performed telomere's analyses. MS & DC performed oxidative stress analyses. LLS & FB wrote the initial draft. All authors have reviewed and edited the manuscript and approved the final version.

Ethics statement

This work was approved by the French authorities under permits DREAL/2020D/8041 (animals capture) and APAFIS #33592-2021102610033982 (animals husbandry).

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Data archiving statement

Data supporting the finding of the study will be made available by the corresponding author upon request.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Caption to figures

Figure 1 - (A) Hatching success (proportion of eggs that have hatched), (B) Malformation rate (proportion of malformed larvae on the total number of hatched individuals), (C) General activity (on a scale of 1 to 5, describing the proportion of individuals that were active; 1=0-20%, 5=80-100%), and (D) telomeres length for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE.

Figure 2 - (A) Thiols, (B) OXY, (C) SOD activity, (D) Catalase activity, and (E) telomeres length according to embryonic development duration, as well as (F) telomeres length according to tadpoles' size. Points are the observed values. Linear trend line \pm SE. The dashed line represent a non-significant relationship.

Figure 3 -Curves of Kaplan-Meier for estimated larval survival of (A) coastal and (B) inland individuals in the three treatments (0, 2 and 4 g.l⁻¹).

Figure 4 - (A) Morphology score, extracted for the first axis of the PCA between all the different metrics variables for all developmental stages (see text and Appendix B for details), (B) body length, and (C) time between Gosner stage 25 (hatching) and each studied stage, for each stage and the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE.

Figure 5 - Behavioral metrics studied at Gosner stage 37. (A) Corrected DFA (DFAc: Detrended fluctuation analysis, indicating the complexity of the movements, the higher it is, the less the movement is complex), (B) Frequency of individuals positioning at less than 50 mm to the center of the arena, (C) total travelled distance corrected by individuals' total length (number of time an individual travelled its total length), and

(D) Average speed, corrected by individuals' total length (number of time an individual travelled its total length/second), for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE.

Figure 6 - Metrics studied at J+5 after metamorphosis (Gosner stage 45). (A) Individuals body mass, (B) Activity (measured from 1 to 5 according to (Lorrain-Soligon et al., 2022): 1 individuals did not react to stimuli, 5: individuals are very active and realized multiple jumps), and (C) mean jump distance, for individuals that have realized jumps, for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE.

Figure 1

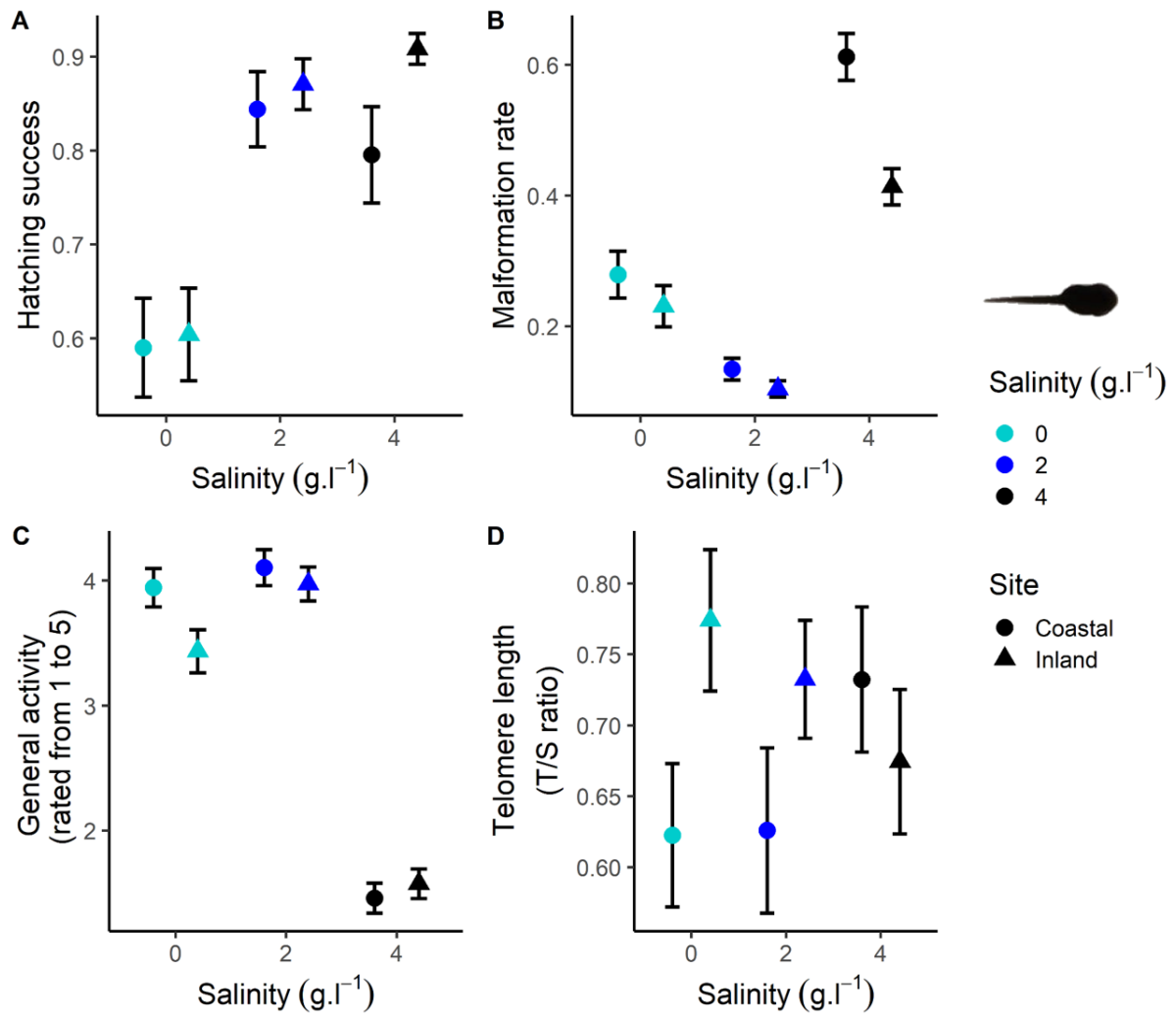


Figure 2

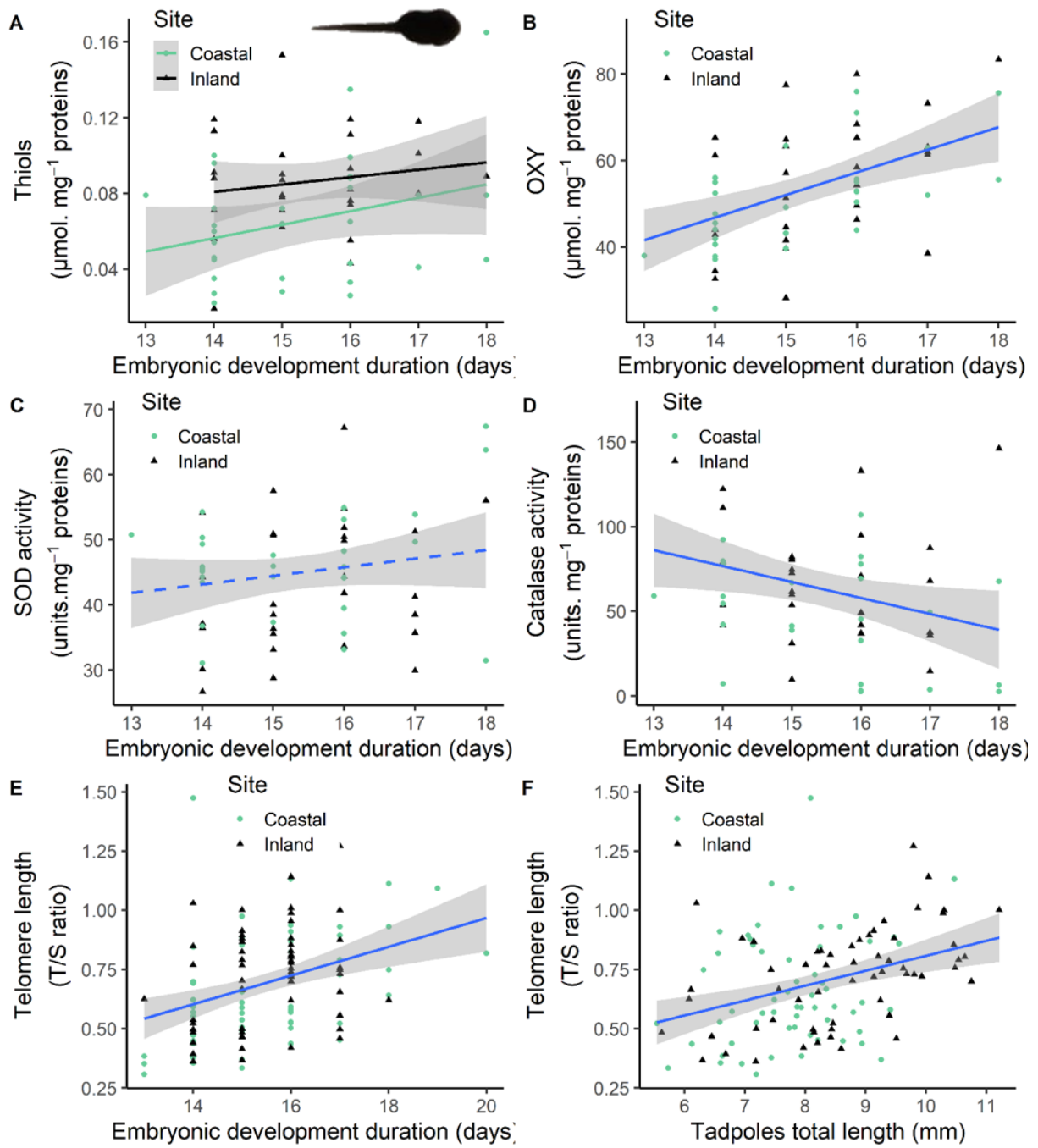


Figure 3

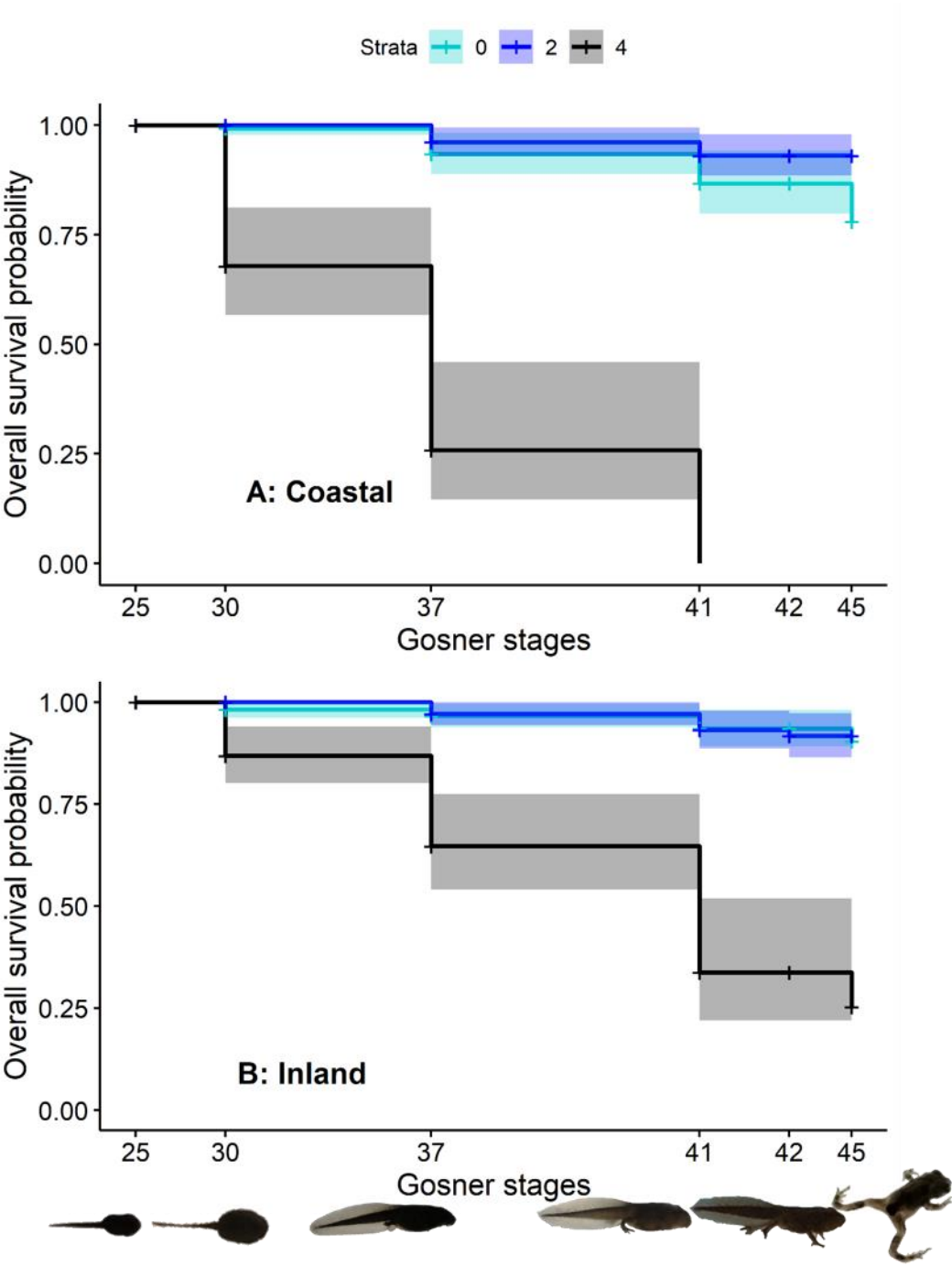


Figure 4

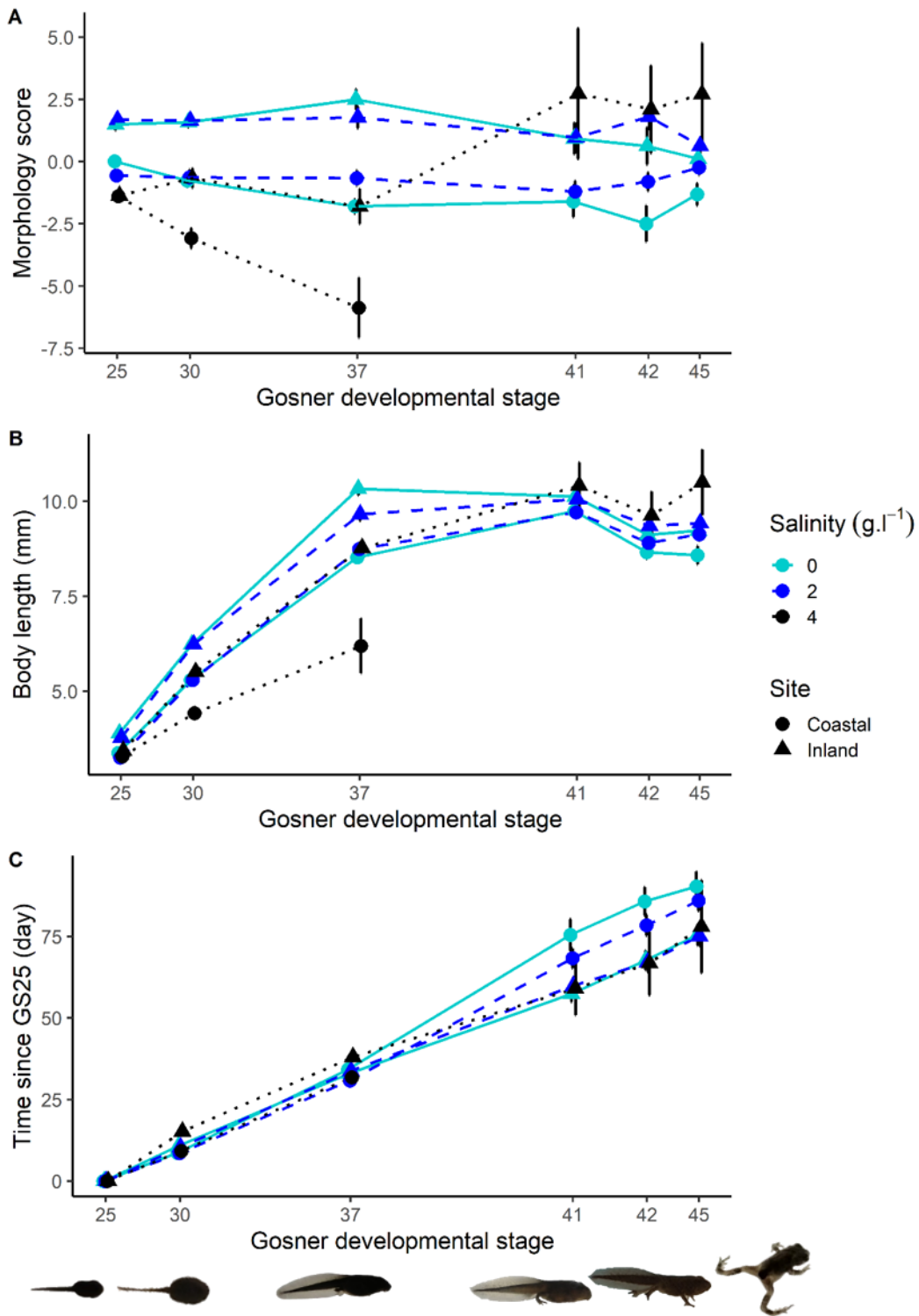


Figure 5

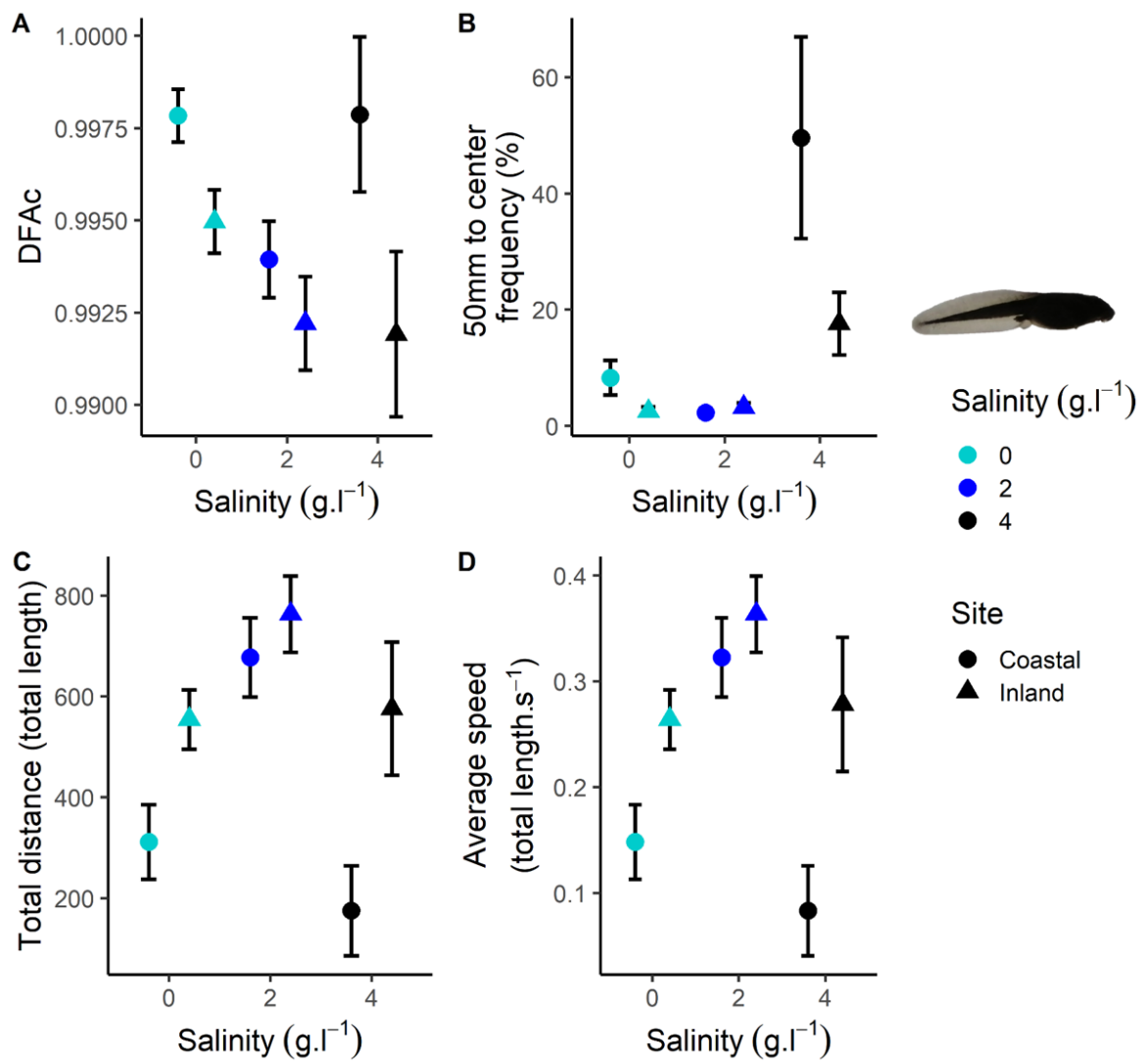
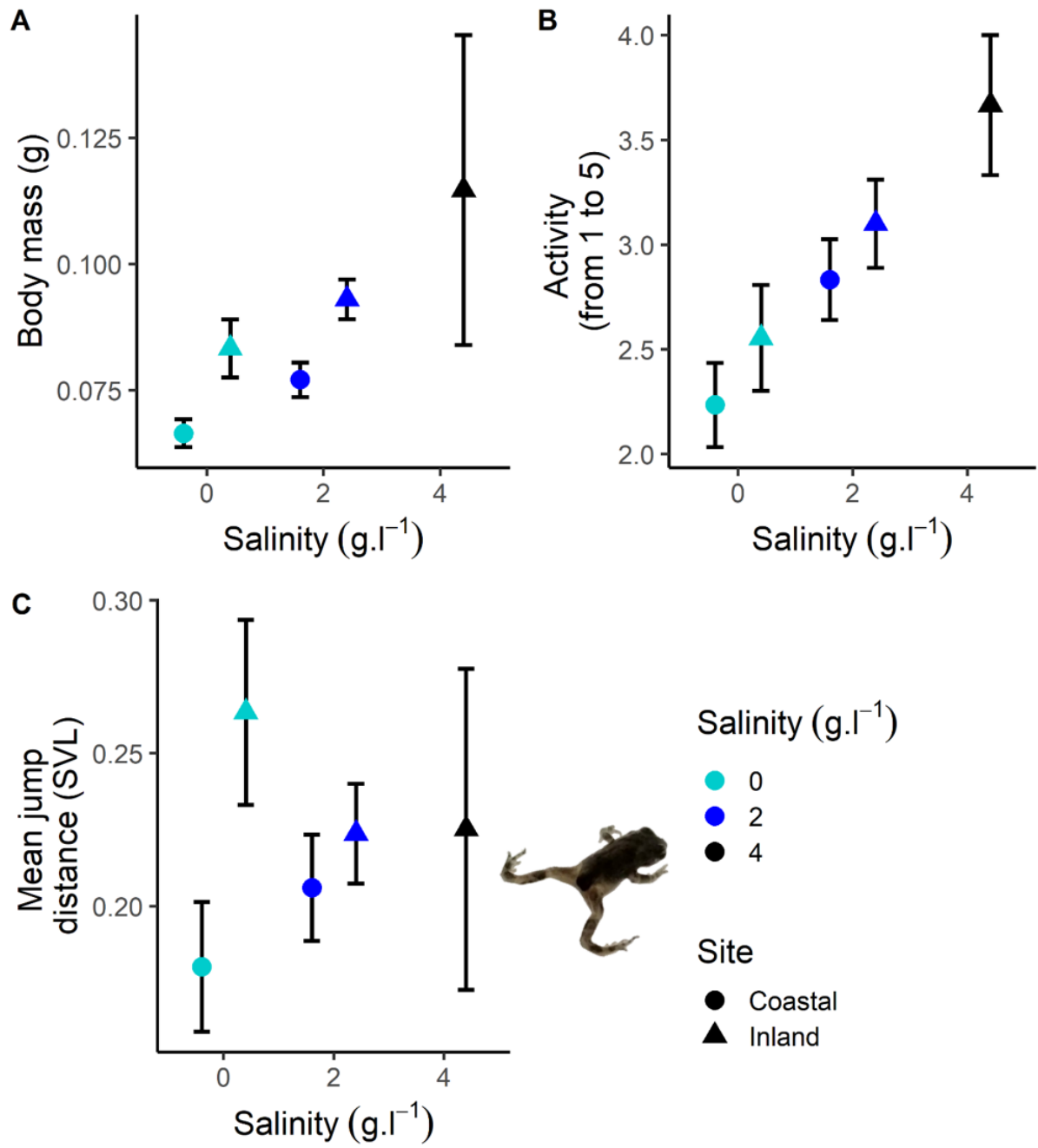


Figure 6



Caption to appendices

Appendix A - Summary corresponding to number of dead individuals, number of malformed individuals, total length, tail length, body length, muscle tail height, tail height, body height, time since hatching, and time since Gosner stage 25, for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE. Note that all groups are not constituted of 40 individuals at hatching, since, for some segments, no individuals have hatched. The N at hatching is presented in the table as well.

Appendix B - PCA for the calculation of the morphology score between all the different morphological variables for all developmental stages: (A) Gosner stage 25 and (B) Gosner stage 30 (for both, PCA between total length, tail length, body length and body width), (C) Gosner stage 37, (D) Gosner stage 41, (E) Gosner stage 42 (for all three, PCA between total length, tail length, body length, body width, tail muscle height, tail height, body height and hindlimbs length), and (F) Gosner stage 45 (PCA between body length, body width and body height).

Appendix C - PCA for the calculation of the behaviour score, between all the different behavioural variables recorded at Gosner stage 37 (DFA, DFAc, total distance, total distance corrected by individual body size, mean speed, mean speed corrected by individuals body size, total duration of activity, frequency of positioning at less than 50 mm to the center).

Appendix D - Analyses on the different variables studied, at the different Gosner developmental stages (GS25, GS30, GS37, GS41, GS42 and GS45), the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Post-hoc analyses are presented in Appendices F and G. Significance levels are given at $\alpha < 0.05$ (*) and $\alpha < 0.1$ (.).

Appendix E - Post-hoc analyses for the differences between the different treatments (0, 2 and 4 g.l⁻¹), on the different variables studied, at the different Gosner developmental stages (GS25, GS30, GS37, GS41, GS42 and GS45), the two sites (Coastal or Inland). Significance levels are given at $\alpha < 0.05$ (*) and $\alpha < 0.1$ (.).

Appendix F - Post-hoc analyses for the differences within sites between the different treatments, on the different variables studied, at the different Gosner developmental stages (GS25, GS30, GS37, GS41, GS42 and GS45), the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Significance levels are given at $\alpha < 0.05$ (*) and $\alpha < 0.1$ (.).

Appendix G - Post-hoc analyses for the differences within treatments between the different sites, on the different variables studied, at the different Gosner developmental stages (GS25, GS30, GS37, GS41, GS42 and GS45), the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Significance levels are given at $\alpha < 0.05$ (*) and $\alpha < 0.1$ (.).

Appendix H - (A) Time to hatching and (B) morphology score at GS25, for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE.

Appendix I - Behavioural metrics that have been recorded on individuals at Gosner stage 25 (hatching). (A) Position in the water column (0: if individuals stay at the bottom of their aquarium, 1: if individuals have moved within the water column) and (B) escape from predation, evaluated by stimulating individuals (0: if individuals did not escape, 1 if they try to escape), for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE.

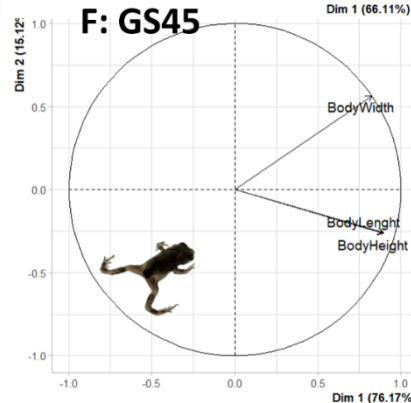
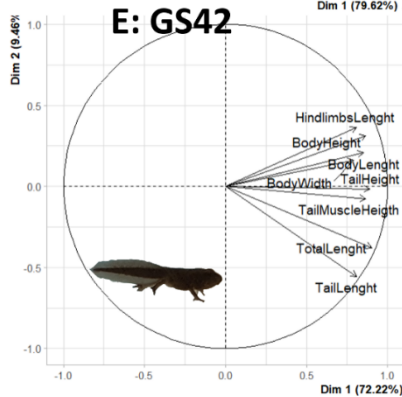
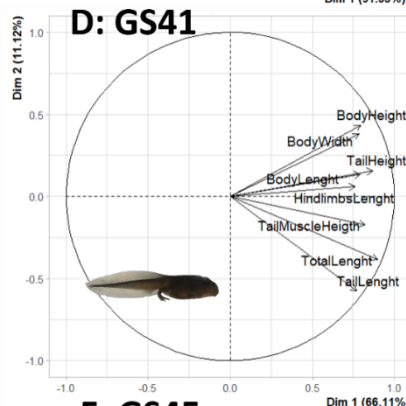
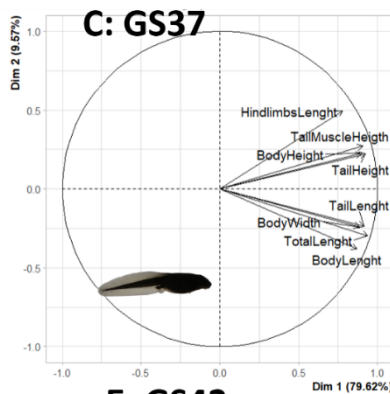
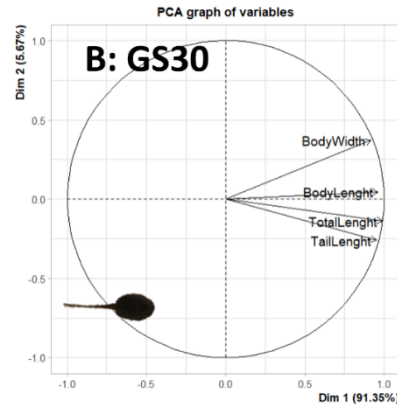
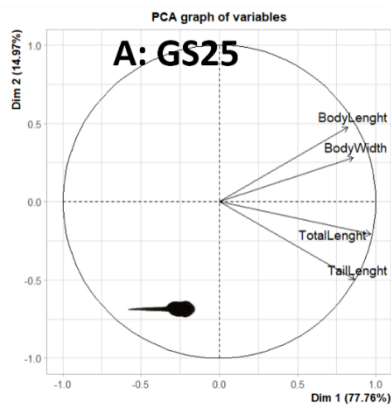
Appendix J - Behaviour score, extracted for the first axis of the PCA between all the different metrics variables for Gosner stage 37 (DFA, DFAC, total distance, total distance corrected by individual body size, mean speed, mean speed corrected by individuals body size, total duration of activity), for the two sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE.

		0	38	8	0	26.84	0.37	1.99	0.04	4.54	0.12	4.57	0.10	57.37	2.40	73.03	2.40
	Inland	2	40	8	1	26.53	0.35	2.01	0.04	4.74	0.11	4.55	0.08	59.84	2.72	75.41	2.68
		4	40	36	0	26.74	1.40	2.05	0.15	5.17	0.32	4.93	0.44	59.00	8.07	74.00	8.34
42	Coastal	0	35	12	1	21.31	0.73	1.52	0.05	3.55	0.15	3.45	0.08	85.75	4.36	100.40	4.35
		2	38	8	0	23.33	0.28	1.70	0.03	3.90	0.07	3.51	0.05	78.43	3.23	93.07	3.23
		4	35	35	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Inland	0	38	8	1	24.07	0.62	1.70	0.06	4.02	0.14	3.66	0.09	67.69	3.00	83.38	2.99
		2	40	9	1	24.77	0.32	1.87	0.04	4.41	0.11	3.80	0.06	67.03	2.93	82.58	2.88
		4	40	36	0	24.71	1.06	1.89	0.11	4.65	0.15	3.70	0.23	66.75	9.66	81.75	9.92
45	Coastal	0	35	14	0	NA	NA	NA	NA	NA	NA	3.24	0.08	90.33	4.59	105.06	4.57
		2	38	8	0	NA	NA	NA	NA	NA	NA	3.48	0.06	86.00	3.14	100.63	3.14
		4	35	35	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Inland	0	38	9	0	NA	NA	NA	NA	NA	NA	3.48	0.10	75.47	3.24	91.13	3.21
		2	40	9	0	NA	NA	NA	NA	NA	NA	3.63	0.06	75.13	3.04	90.68	2.99
		4	40	37	0	NA	NA	NA	NA	NA	NA	3.98	0.28	78.00	14.29	93.00	14.73

Gosner stage	Site	Treatment (g.l ⁻¹)	DFAc	SE DFac	Total distance (mm)	SE total distance	Mean speed (mm/s)	SE Mean speed	Total duration of activity (s)	SE total duration of activity	SE Frequency of positioning	
											Frequency of positioning at less than 50 mm from the center	Frequency of positioning at less than 50 mm from the center
25	Coastal	0	0.98	0.00	6457.68	1477.42	48.99	9.26	157.33	55.86	8.19	2.88
		2	0.99	0.00	14976.43	1822.89	54.17	9.43	304.43	42.28	2.30	0.70
		4	0.98	0.01	1900.26	1690.51	29.12	14.56	46.47	38.76	74.79	17.61
	Inland	0	1.00	0.00	14817.72	1610.60	61.31	10.67	247.16	30.12	2.45	0.77
		2	0.99	0.00	19196.19	1904.53	53.59	8.93	383.91	44.44	3.26	0.78
		4	0.99	0.00	13161.60	3099.28	42.49	10.97	317.97	71.43	17.64	5.36

Gosner stage	Site	Treatment (g.l ⁻¹)	Telomere length	SE telomere length	OXY (μmol/mg proteins)	SE OXY	Thiols (μmol/mg proteins)	SE thiols	SOD Units/mg proteins	SE SOD	Catalase Units/mg proteins	SE catalase
25	Coastal	0	0.59	0.06	48.46	4.37	0.06	0.01	46.21	2.24	48.36	14.14
		2	0.56	0.05	47.59	3.07	0.05	0.01	45.36	1.67	70.78	14.96
		4	0.73	0.06	57.16	2.88	0.08	0.01	46.70	3.84	55.12	11.16
	Inland	0	0.76	0.10	58.17	4.60	0.09	0.01	48.20	3.76	73.39	11.95
		2	0.70	0.04	54.38	3.99	0.08	0.01	39.94	2.61	49.87	6.99
		4	0.70	0.09	57.32	6.48	0.09	0.01	43.45	3.80	81.92	12.22

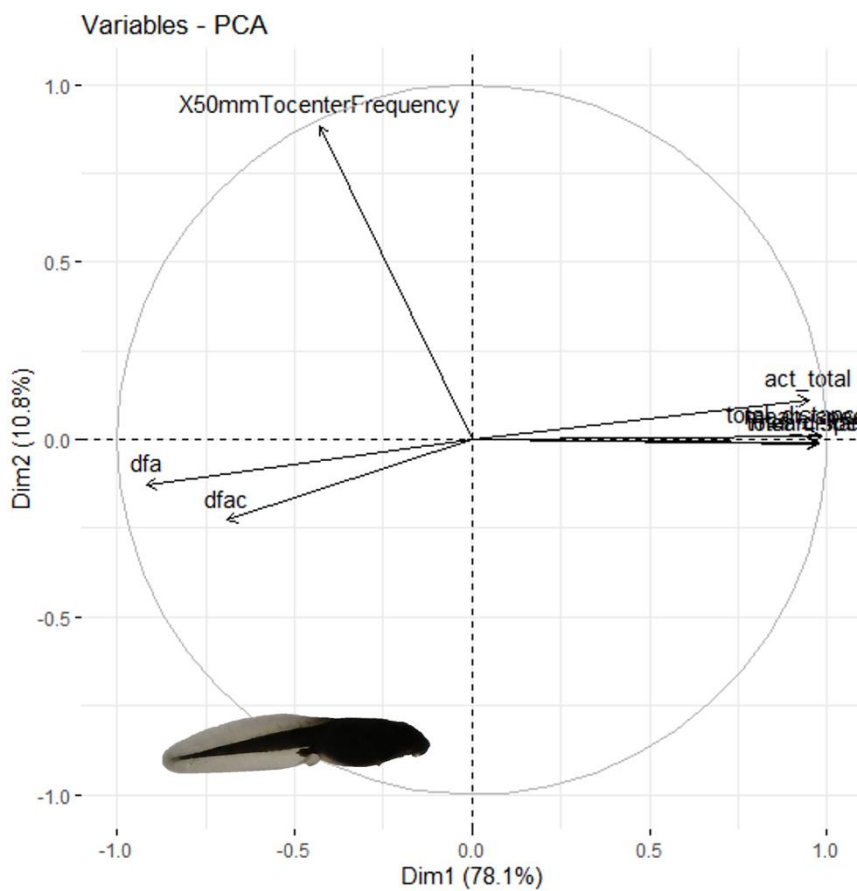
1 Appendix B - PCA for the calculation of the morphology score between all the
 2 different morphological variables for all developmental stages: (A) Gosner stage 25
 3 and (B) Gosner stage 30 (for both, PCA between total length, tail length, body length
 4 and body length, and body width), (C) Gosner stage 37, (D) Gosner stage 41, (E) Gosner
 5 stage 42 (for all three, PCA between total length, tail length, body length, body width,
 6 tail muscle height, tail height, body height and hindlimbs length), and (F) Gosner stage
 7 45 (PCA between body length, body width and body height).



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10 Appendix C - PCA for the calculation of the behaviour score, between all the different
11 behavioural variables recorded at Gosner stage 37 (DFA, DFAC, total distance, total
12 distance corrected by individual body size, mean speed, mean speed corrected by
13 individuals body size, total duration of activity, frequency of positioning at less than
14 50 mm to the center).



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17 Appendix D - Analyses on the different variables studied, at the different Gosner
 18 developmental stages (GS25, GS30, GS37, GS41, GS42 and GS45), the two sites (Coastal
 19 or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Post-hoc analyses are presented in
 20 Appendices F and G. Significance levels are given at $\alpha < 0.05$ (*) and $\alpha < 0.1$ (.)

Developmental stage	Metrics	Covariates	Df	SumSq	F-value	Chisq	p-value	Significance
GS25	Hatching success	Treatment	2	-	-	37.19	<0.001	*
		Site	1	-	-	0.053	0.819	
		Treatment:Site	2	-	-	1.831	0.4	
	Malformation rate	Treatment	2	-	-	244.237	<0.001	*
		Site	1	-	-	1.898	0.168	
		Treatment:Site	2	-	-	2.702	0.259	
	General activity	Treatment	2	-	-	34.425	<0.001	*
		Site	1	-	-	0.756	0.384	
		Treatment:Site	2	-	-	0.677	0.713	
	Time to hatching	Treatment	2	1.901	-	11.056	0.004	*
		Site	1	0.065	-	3.424	0.064	.
		Treatment:Site	2	6.521	-	9.274	0.01	*
	Morphology score	Treatment	2	204.369	116.912	-	<0.001	*
		Site	1	12.183	13.939	-	<0.001	*
		Treatment:Site	2	48.054	27.49	-	<0.001	*
	Position within the water column	Treatment	2	-	-	19.148	<0.001	*
		Site	1	-	-	1.005	0.316	
		Treatment:Site	2	-	-	0.852	0.653	
Predation test	Treatment	2	-	-	18.602	<0.001	*	
	Site	1	-	-	2.306	0.129		
	Treatment:Site	2	-	-	0.824	0.662		
Telomeres' length	Treatment	2	-	-	6.833	0.033	*	
	Site	1	-	-	5.211	0.022	*	
	Treatment:Site	2	-	-	10.475	0.005	*	
GS30	Time between GS25 and GS30	Treatment	2	36.874	-	0.503	0.778	
		Site	1	22.537	-	8.609	0.003	*
		Treatment:Site	2	7.778	-	8.641	0.013	*
Morphology score	Treatment	2	201.998	87.705	-	<0.001	*	
	Site	1	39.077	33.933	-	<0.001	*	
	Treatment:Site	2	0.976	0.424	-	0.655		
GS37	Time between GS25 and GS37	Treatment	2	8.684	-	5.982	0.05	.
		Site	1	2.155	-	0.858	0.024	*
		Treatment:Site	2	6.864	-	7.426	<0.001	*
Morphology score	Treatment	2	104.549	13.869	-	<0.001	*	
	Site	1	83.346	22.113	-	<0.001	*	

	Treatment:Site	2	31.972	4.241	-	0.017	*	
Behavior score	Treatment	2	147.87	8.895	-	<0.001	*	
	Site	1	83.379	10.031	-	0.002	*	
	Treatment:Site	2	17.59	1.058	-	0.349		
DFA	Treatment	2	0.008	6.992	-	0.001	*	
	Site	1	0.005	7.133	-	0.008	*	
	Treatment:Site	2	0.001	0.707	-	0.495		
DFAc	Treatment	2	<0.001	4.607	-	0.012	*	
	Site	1	<0.001	6.613	-	0.011	*	
	Treatment:Site	2	<0.001	0.65	-	0.523		
50 mm to center frequency	Treatment	2	-	-	248.21	<0.001	*	
	Site	1	-	-	6.357	0.012	*	
	Treatment:Site	2	-	-	75.35	<0.001	*	
Total distance (size)	Treatment	2	14.709	-	19.292	<0.001	*	
	Site	1	8.776	-	7.371	0.007	*	
	Treatment:Site	2	5.732	-	5.731	0.057	.	
Mean speed (size/mm)	Treatment	2	0.707	8.838	-	<0.001	*	
	Site	1	0.279	6.979	-	0.009	*	
	Treatment:Site	2	0.086	1.069	-	0.346		
Total duration of activity	Treatment	2	0.707	-	13.301	0.001	*	
	Site	1	0.279	-	4.63	0.031	*	
	Treatment:Site	2	0.086	-	3.741	0.154		
GS41	Time between GS25 and GS41	Treatment	2	0.08	-	2.301	0.316	
		Site	1	12.3	-	13.205	<0.001	*
		Treatment:Site	2	2.288	-	2.266	0.132	
GS41	Morphology score	Treatment	2	7.334	0.453	-	0.637	
		Site	1	95.32	11.792	-	0.002	*
		Treatment:Site	2	1.748	0.216	-	0.643	
GS42	Time between GS25 and GS42	Treatment	2	0.72	-	2.44	0.295	
		Site	1	13.139	-	11.835	<0.001	*
		Treatment:Site	2	1.115	-	1.109	0.292	
GS42	Morphology score	Treatment	2	37.987	3.361	-	0.04	*
		Site	1	83.45	14.766	-	<0.001	*
		Treatment:Site	2	4.621	0.818	-	0.369	
GS45	Time between GS25 and GS45	Treatment	2	0.425	-	1.78	0.411	
		Site	1	9.925	-	8.869	0.003	*
		Treatment:Site	2	0.798	-	0.794	0.373	
GS45	Morphology score	Treatment	2	19.715	3.087	-	0.051	.
		Site	1	21.305	6.672	-	0.013	*
		Treatment:Site	2	5.204	1.63	-	0.205	
GS45	Mass at 5 days	Treatment	2	0.003	3.893	-	0.024	*
		Site	1	0.003	7.735	-	0.008	*
		Treatment:Site	2	<0.001	0.236	-	0.628	
GS45	Activity	Treatment	2	-	-	1.721	0.423	
		Site	1	-	-	0.299	0.585	

	Treatment:Site	2	-	-	0.02	0.887
Mean jump distance	Treatment	2	0.948	1.268	-	0.294
	Site	1	1.343	3.592	-	0.067
	Treatment:Site	2	0.391	1.047	-	0.312

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22 Appendix E - Post-hoc analyses for the differences between the different treatments (0,
 23 2 and 4 g.l⁻¹), on the different variables studied, at the different Gosner developmental
 24 stages (GS25, GS30, GS37, GS41, GS42 and GS45), the two sites (Coastal or Inland).
 25 Significance levels are given at $\alpha < 0.05$ (*) and $\alpha < 0.1$ (.)

Developmental stage	Metrics	Comparison	Estimate	SE	z/t ratio	p-value	Significance
GS25	Hatching success	2 g.l ⁻¹ - 0 g.l ⁻¹	0.37241	0.0648	5.747	<0.001	*
		4 g.l ⁻¹ - 0 g.l ⁻¹	0.31639	0.06512	4.859	<0.001	*
		4 g.l ⁻¹ - 2 g.l ⁻¹	-0.05601	0.06171	-0.908	0.635	
	Malformation rate	2 g.l ⁻¹ - 0 g.l ⁻¹	-0.77924	0.12583	-6.193	<0.001	*
		4 g.l ⁻¹ - 0 g.l ⁻¹	0.8358	0.09799	8.529	<0.001	*
		4 g.l ⁻¹ - 2 g.l ⁻¹	1.61504	0.10861	14.87	<0.001	*
	General activity	2 g.l ⁻¹ - 0 g.l ⁻¹	0.03996	0.15579	0.257	0.964	
		4 g.l ⁻¹ - 0 g.l ⁻¹	-0.99424	0.19111	-5.203	<0.001	*
		4 g.l ⁻¹ - 2 g.l ⁻¹	-1.0342	0.18867	-5.481	<0.001	*
	Time to hatching	2 g.l ⁻¹ - 0 g.l ⁻¹	-0.001809	0.044838	-0.04	0.9991	
		4 g.l ⁻¹ - 0 g.l ⁻¹	0.134189	0.042658	3.146	0.00451	*
		4 g.l ⁻¹ - 2 g.l ⁻¹	0.135998	0.051331	2.649	0.02175	*
	Morphology score	2 g.l ⁻¹ - 0 g.l ⁻¹	-0.5496	0.2197	-2.501	0.0331	*
		4 g.l ⁻¹ - 0 g.l ⁻¹	-1.3761	0.2251	-6.113	<0.001	*
		4 g.l ⁻¹ - 2 g.l ⁻¹	-0.8266	0.2197	-3.762	<0.001	*
	Position within the water column	2 g.l ⁻¹ - 0 g.l ⁻¹	-0.5978	0.498	-1.201	0.45157	
		4 g.l ⁻¹ - 0 g.l ⁻¹	-2.4918	0.5841	-4.266	<0.001	*
		4 g.l ⁻¹ - 2 g.l ⁻¹	-1.894	0.556	-3.407	0.002	*
	Predation test	2 g.l ⁻¹ - 0 g.l ⁻¹	-1.3595	0.8531	-1.594	0.243287	
		4 g.l ⁻¹ - 0 g.l ⁻¹	-3.1754	0.8374	-3.792	<0.001	*
		4 g.l ⁻¹ - 2 g.l ⁻¹	-1.8159	0.5765	-3.15	0.004475	*
Telomeres' length	2 g.l ⁻¹ - 0 g.l ⁻¹	-0.003	0.033	-0.092	0.995		
	4 g.l ⁻¹ - 0 g.l ⁻¹	0.073	0.033	2.23	0.066	.	
	4 g.l ⁻¹ - 2 g.l ⁻¹	0.076	0.033	2.284	0.058	.	
GS30	Time between GS25 and GS30	2 g.l ⁻¹ - 0 g.l ⁻¹	-0.001343	0.072657	-0.018	1	
		4 g.l ⁻¹ - 0 g.l ⁻¹	0.060383	0.092024	0.656	0.787	
		4 g.l ⁻¹ - 2 g.l ⁻¹	0.061725	0.094832	0.651	0.79	
Morphology score	2 g.l ⁻¹ - 0 g.l ⁻¹	0.2166	0.2547	0.851	0.669		
	4 g.l ⁻¹ - 0 g.l ⁻¹	-2.6339	0.3365	-7.828	<0.001	*	
	4 g.l ⁻¹ - 2 g.l ⁻¹	-2.8505	0.3311	-8.61	<0.001	*	
GS37	Time between GS25 and GS37	2 g.l ⁻¹ - 0 g.l ⁻¹	-0.10755	0.04427	-2.429	0.0368	*
		4 g.l ⁻¹ - 0 g.l ⁻¹	-0.07579	0.09059	-0.837	0.6692	
		4 g.l ⁻¹ - 2 g.l ⁻¹	0.03177	0.0912	0.348	0.9325	
Morphology score	2 g.l ⁻¹ - 0 g.l ⁻¹	1.0548	0.5048	2.089	0.0848	.	
	4 g.l ⁻¹ - 0 g.l ⁻¹	-1.8174	1.1226	-1.619	0.2242		

		4 g.l-1 - 2 g.l-1	-2.8721	1.108	-2.592	0.0225	*
Behavior score		2 g.l-1 - 0 g.l-1	2.4675	0.7482	3.298	0.00271	*
		4 g.l-1 - 0 g.l-1	-1.1189	1.5446	-0.724	0.73966	
		4 g.l-1 - 2 g.l-1	-3.5864	1.5264	-2.35	0.0456	*
DFA		2 g.l-1 - 0 g.l-1	-0.0209175	0.0065716	-3.183	0.00389	*
		4 g.l-1 - 0 g.l-1	0.0006141	0.0135677	0.045	0.99882	
		4 g.l-1 - 2 g.l-1	0.0215316	0.0134076	1.606	0.23112	
DFAc		2 g.l-1 - 0 g.l-1	-3.90E-03	1.60E-03	-2.432	0.0364	*
		4 g.l-1 - 0 g.l-1	3.14E-05	3.31E-03	0.01	0.9999	
		4 g.l-1 - 2 g.l-1	3.93E-03	3.27E-03	1.202	0.438	
50 mm to center frequency		2 g.l-1 - 0 g.l-1	-1.3182	0.1411	-9.344	<0.001	*
		4 g.l-1 - 0 g.l-1	2.0536	0.1943	10.572	<0.001	*
		4 g.l-1 - 2 g.l-1	3.3718	0.2162	15.598	<0.001	*
Total distance (size)		2 g.l-1 - 0 g.l-1	0.7772	0.2109	3.686	<0.001	*
		4 g.l-1 - 0 g.l-1	-0.5766	0.4363	-1.322	0.36905	
		4 g.l-1 - 2 g.l-1	-1.3538	0.4311	-3.14	0.00408	*
Mean speed (size/mm)		2 g.l-1 - 0 g.l-1	0.17434	0.05189	3.36	0.00199	*
		4 g.l-1 - 0 g.l-1	-0.065	0.10714	-0.607	0.80917	
		4 g.l-1 - 2 g.l-1	-0.23934	0.10588	-2.261	0.05696	.
Total duration of activity		2 g.l-1 - 0 g.l-1	0.17434	0.05189	3.36	0.00174	*
		4 g.l-1 - 0 g.l-1	-0.065	0.10714	-0.607	0.80917	
		4 g.l-1 - 2 g.l-1	-0.23934	0.10588	-2.261	0.0564	.
GS41	Time between GS25 and GS41	2 g.l-1 - 0 g.l-1	-0.08777	0.06209	-1.414	0.315	
		4 g.l-1 - 0 g.l-1	0.06652	0.12305	0.541	0.842	
		4 g.l-1 - 2 g.l-1	0.15429	0.13747	1.122	0.48	
	Morphology score	2 g.l-1 - 0 g.l-1	0.3995	0.8228	0.486	0.871	
		4 g.l-1 - 0 g.l-1	1.3448	1.5738	0.854	0.652	
		4 g.l-1 - 2 g.l-1	0.9453	1.7759	0.532	0.847	
GS42	Time between GS25 and GS42	2 g.l-1 - 0 g.l-1	-0.08653	0.05792	-1.494	0.276	
		4 g.l-1 - 0 g.l-1	0.05241	0.11626	0.451	0.887	
		4 g.l-1 - 2 g.l-1	0.13894	0.12969	1.071	0.512	
	Morphology score	2 g.l-1 - 0 g.l-1	1.6548	0.7073	2.339	0.0458	*
		4 g.l-1 - 0 g.l-1	-0.0547	1.3654	-0.04	0.9991	
		4 g.l-1 - 2 g.l-1	-1.7095	1.5378	-1.112	0.4871	
GS45	Time between GS25 and GS45	2 g.l-1 - 0 g.l-1	-0.04957	0.05487	-0.903	0.619	
		4 g.l-1 - 0 g.l-1	0.11778	0.12014	0.98	0.569	
		4 g.l-1 - 2 g.l-1	0.16735	0.13199	1.268	0.392	
	Morphology score	2 g.l-1 - 0 g.l-1	1.1174	0.5434	2.056	0.0897	.
		4 g.l-1 - 0 g.l-1	2.0083	1.151	1.745	0.1739	
		4 g.l-1 - 2 g.l-1	0.8909	1.2728	0.7	0.7496	
	Mass at 5 days	2 g.l-1 - 0 g.l-1	0.009477	0.003986	2.377	0.0398	*
		4 g.l-1 - 0 g.l-1	0.022336	0.012629	1.769	0.1633	
		4 g.l-1 - 2 g.l-1	0.012858	0.012617	1.019	0.5426	
Activity	2 g.l-1 - 0 g.l-1	0.2371	0.2377	0.997	0.561		
	4 g.l-1 - 0 g.l-1	0.361	0.4236	0.852	0.655		

	4 g.l-1 - 2 g.l-1	0.1239	0.4858	0.255	0.962
	2 g.l-1 - 0 g.l-1	0.02867	0.04679	0.613	0.804
Mean jump distance	4 g.l-1 - 0 g.l-1	-0.07655	0.04067	-1.882	0.135
	4 g.l-1 - 2 g.l-1	-0.10522	0.06199	-1.697	0.195

27 Appendix F - Post-hoc analyses for the differences within sites between the different
 28 treatments, on the different variables studied, at the different Gosner developmental
 29 stages (GS25, GS30, GS37, GS41, GS42 and GS45), the two sites (Coastal or Inland) and
 30 the three treatments (0, 2 and 4 g.l⁻¹). Significance levels are given at $\alpha < 0.05$ (*) and
 31 $\alpha < 0.1$ (.).

Developmental stage	Metrics	Site	Comparison	Estimate	SE	z/t ratio	p-value	Significance
GS25	Hatching success	Coastal	0 g.l-1 - 2 g.l-1	-0.372	0.065	-5.747	<0.001	*
			0 g.l-1 - 4 g.l-1	-0.316	0.065	-4.859	<0.001	*
			2 g.l-1 - 4 g.l-1	0.056	0.062	0.908	0.635	
		Inland	0 g.l-1 - 2 g.l-1	-0.376	0.064	-5.928	<0.001	*
			0 g.l-1 - 4 g.l-1	-0.421	0.063	-6.66	<0.001	*
			2 g.l-1 - 4 g.l-1	-0.044	0.06	-0.746	0.736	
	Malformation rate	Coastal	0 g.l-1 - 2 g.l-1	0.779	0.126	6.193	<0.001	*
			0 g.l-1 - 4 g.l-1	-0.836	0.098	-8.529	<0.001	*
			2 g.l-1 - 4 g.l-1	-1.615	0.109	-14.87	<0.001	*
		Inland	0 g.l-1 - 2 g.l-1	0.779	0.135	5.766	<0.001	*
			0 g.l-1 - 4 g.l-1	-0.639	0.106	-6.044	<0.001	*
			2 g.l-1 - 4 g.l-1	-1.418	0.116	-12.183	<0.001	*
	General activity	Coastal	0 g.l-1 - 2 g.l-1	-0.04	0.156	-0.257	0.964	
			0 g.l-1 - 4 g.l-1	0.994	0.191	5.203	<0.001	*
			2 g.l-1 - 4 g.l-1	1.034	0.189	5.481	<0.001	*
		Inland	0 g.l-1 - 2 g.l-1	-0.146	0.155	-0.943	0.613	
			0 g.l-1 - 4 g.l-1	0.78	0.183	4.264	<0.001	*
			2 g.l-1 - 4 g.l-1	0.926	0.179	5.162	<0.001	*
	Time to hatching	Coastal	0 g.l-1 - 2 g.l-1	0.002	0.045	0.04	0.999	
			0 g.l-1 - 4 g.l-1	-0.134	0.043	-3.146	0.005	*
			2 g.l-1 - 4 g.l-1	-0.136	0.0513	-2.649	0.022	*
Inland		0 g.l-1 - 2 g.l-1	0.03186	0.0526	0.605	0.8172		
		0 g.l-1 - 4 g.l-1	0.05809	0.0531	1.093	0.5182		
		2 g.l-1 - 4 g.l-1	0.02623	0.055	0.477	0.8821		
Morphology score	Coastal	0 g.l-1 - 2 g.l-1	0.55	0.22	2.501	0.035	*	
		0 g.l-1 - 4 g.l-1	1.376	0.225	6.112	<0.001	*	
		2 g.l-1 - 4 g.l-1	0.827	0.22	3.762	<0.001	*	
	Inland	0 g.l-1 - 2 g.l-1	-0.173	0.212	-0.815	0.694		
		0 g.l-1 - 4 g.l-1	2.866	0.212	13.505	<0.001	*	
		2 g.l-1 - 4 g.l-1	3.039	0.209	14.537	<0.001	*	
Coastal	0 g.l-1 - 2 g.l-1	0.598	0.498	1.201	0.453			
	0 g.l-1 - 4 g.l-1	2.492	0.584	4.266	<0.001	*		

			2 g.l-1 - 4 g.l-1	1.894	0.556	3.407	0.002	*
			0 g.l-1 - 2 g.l-1	0.405	0.628	0.646	0.795	
		Inland	0 g.l-1 - 4 g.l-1	3.114	0.71	4.387	<0.001	*
			2 g.l-1 - 4 g.l-1	2.708	0.7	3.867	<0.001	*
			0 g.l-1 - 2 g.l-1	1.359	0.853	1.594	0.248	
		Coastal	0 g.l-1 - 4 g.l-1	3.175	0.837	3.792	<0.001	*
			2 g.l-1 - 4 g.l-1	1.816	0.576	3.15	0.005	*
			0 g.l-1 - 2 g.l-1	0.428	0.576	0.743	0.738	
		Inland	0 g.l-1 - 4 g.l-1	2.573	0.602	4.277	<0.001	*
			2 g.l-1 - 4 g.l-1	2.145	0.553	3.876	<0.001	*
			0 g.l-1 - 2 g.l-1	0.003	0.034	0.092	0.995	
		Coastal	0 g.l-1 - 4 g.l-1	-0.073	0.033	-2.23	0.073	.
			2 g.l-1 - 4 g.l-1	-0.076	0.034	-2.283	0.064	.
			0 g.l-1 - 2 g.l-1	0.021	0.033	0.64	0.798	
		Inland	0 g.l-1 - 4 g.l-1	0.065	0.033	1.986	0.123	
			2 g.l-1 - 4 g.l-1	0.044	0.033	1.346	0.375	
			0 g.l-1 - 2 g.l-1	0.00134	0.0727	0.018	0.9998	
			-	-	-	-	-	
		Coastal	0 g.l-1 - 4 g.l-1	0.06038	0.092	-0.656	0.7889	
			-	-	-	-	-	
			2 g.l-1 - 4 g.l-1	0.06173	0.0948	-0.651	0.7919	
			0 g.l-1 - 2 g.l-1	0.04325	0.0695	0.623	0.8077	
			-	-	-	-	-	
		Inland	0 g.l-1 - 4 g.l-1	0.34317	0.0699	-4.913	<0.001	*
			-	-	-	-	-	
			2 g.l-1 - 4 g.l-1	0.38642	0.0689	-5.611	<0.001	*
			0 g.l-1 - 2 g.l-1	-0.217	0.255	-0.85	0.672	
		Coastal	0 g.l-1 - 4 g.l-1	2.634	0.337	7.817	<0.001	*
			2 g.l-1 - 4 g.l-1	2.851	0.332	8.598	<0.001	*
			0 g.l-1 - 2 g.l-1	-0.062	0.25	-0.249	0.966	
		Inland	0 g.l-1 - 4 g.l-1	2.398	0.276	8.69	<0.001	*
			2 g.l-1 - 4 g.l-1	2.46	0.268	9.171	<0.001	*
			0 g.l-1 - 2 g.l-1	0.1076	0.0443	2.429	0.0401	*
		Coastal	0 g.l-1 - 4 g.l-1	0.0758	0.0906	0.837	0.6803	
			2 g.l-1 - 4 g.l-1	-0.0318	0.0912	-0.348	0.9353	
			0 g.l-1 - 2 g.l-1	-0.0261	0.0414	-0.63	0.8037	
		Inland	0 g.l-1 - 4 g.l-1	-0.1438	0.0512	-2.808	0.0138	*
			2 g.l-1 - 4 g.l-1	-0.1178	0.0506	-2.329	0.0519	.
			0 g.l-1 - 2 g.l-1	-1.055	0.505	-2.087	0.097	.
		Coastal	0 g.l-1 - 4 g.l-1	1.817	1.132	1.605	0.247	
			2 g.l-1 - 4 g.l-1	2.872	1.117	2.571	0.03	*
			0 g.l-1 - 2 g.l-1	0.732	0.474	1.546	0.273	
		Inland	0 g.l-1 - 4 g.l-1	4.439	0.622	7.135	<0.001	*
			2 g.l-1 - 4 g.l-1	3.707	0.614	6.036	<0.001	*
			0 g.l-1 - 2 g.l-1	-2.468	0.75	-3.292	0.004	*
		Coastal	0 g.l-1 - 4 g.l-1	1.119	1.576	0.71	0.758	

			2 g.l-1 - 4 g.l-1	3.586	1.558	2.303	0.059	.
			0 g.l-1 - 2 g.l-1	-1.416	0.702	-2.018	0.113	
		Inland	0 g.l-1 - 4 g.l-1	-0.068	0.909	-0.075	0.997	
			2 g.l-1 - 4 g.l-1	1.349	0.891	1.513	0.288	
			0 g.l-1 - 2 g.l-1	0.021	0.007	3.177	0.005	*
		Coastal	0 g.l-1 - 4 g.l-1	<-0.001	0.014	-0.044	0.999	
			2 g.l-1 - 4 g.l-1	0.022	0.014	-1.574	0.26	
			0 g.l-1 - 2 g.l-1	0.011	0.006	1.846	0.159	
		Inland	0 g.l-1 - 4 g.l-1	0.002	0.008	0.289	0.955	
			2 g.l-1 - 4 g.l-1	-0.009	0.008	-1.159	0.48	
			0 g.l-1 - 2 g.l-1	0.004	0.002	2.428	0.044	*
		Coastal	0 g.l-1 - 4 g.l-1	<-0.001	0.003	-0.009	1	
			2 g.l-1 - 4 g.l-1	-0.003	0.003	-1.178	0.469	
			0 g.l-1 - 2 g.l-1	0.003	0.002	1.834	0.163	
		Inland	0 g.l-1 - 4 g.l-1	0.003	0.002	1.567	0.264	
			2 g.l-1 - 4 g.l-1	<0.001	0.002	0.154	0.987	
			0 g.l-1 - 2 g.l-1	1.32	0.141	9.344	<0.001	*
		Coastal	0 g.l-1 - 4 g.l-1	-2.05	0.194	-10.572	<0.001	*
			2 g.l-1 - 4 g.l-1	-3.37	0.216	-15.598	<0.001	*
			0 g.l-1 - 2 g.l-1	-0.32	0.148	-2.16	0.078	.
		Inland	0 g.l-1 - 4 g.l-1	-2.02	0.14	-14.468	<0.001	*
			2 g.l-1 - 4 g.l-1	-1.7	0.127	-13.444	<0.001	*
			0 g.l-1 - 2 g.l-1	-0.7772	0.211	-3.686	0.0007	*
		Coastal	0 g.l-1 - 4 g.l-1	0.5766	0.436	1.322	0.3831	
			2 g.l-1 - 4 g.l-1	1.3538	0.431	3.14	0.0048	*
			0 g.l-1 - 2 g.l-1	-0.3199	0.197	-1.622	0.2364	
		Inland	0 g.l-1 - 4 g.l-1	-0.0381	0.254	-0.15	0.9877	
			2 g.l-1 - 4 g.l-1	0.2818	0.25	1.129	0.4959	
			0 g.l-1 - 2 g.l-1	-0.174	0.052	-3.354	0.003	*
		Coastal	0 g.l-1 - 4 g.l-1	0.065	0.109	0.595	0.823	
			2 g.l-1 - 4 g.l-1	0.239	0.108	2.215	0.072	.
			0 g.l-1 - 2 g.l-1	-0.099	0.049	-2.044	0.106	
		Inland	0 g.l-1 - 4 g.l-1	-0.014	0.063	-0.228	0.972	
			2 g.l-1 - 4 g.l-1	0.085	0.062	1.377	0.356	
			0 g.l-1 - 2 g.l-1	-0.1743	0.052	-3.354	0.0031	*
		Coastal	0 g.l-1 - 4 g.l-1	0.065	0.1093	0.595	0.8232	
			2 g.l-1 - 4 g.l-1	0.2393	0.108	2.215	0.0721	.
			0 g.l-1 - 2 g.l-1	-0.0995	0.0487	-2.044	0.1064	
		Inland	0 g.l-1 - 4 g.l-1	-0.0144	0.0631	-0.228	0.9716	
			2 g.l-1 - 4 g.l-1	0.0851	0.0618	1.377	0.356	
			0 g.l-1 - 2 g.l-1	0.088	0.062	1.414	0.158	
		Coastal	0 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
			2 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
			0 g.l-1 - 2 g.l-1	-0.0396	0.0573	-0.69	0.7693	
		Inland	0 g.l-1 - 4 g.l-1	-0.0665	0.123	-0.541	0.8513	
GS41	Time between GS25 and GS41							

			2 g.l-1 - 4 g.l-1	-0.027	0.1224	-0.22	0.9736		
	Morphology score	Coastal	0 g.l-1 - 2 g.l-1	-0.4	0.826	-0.484	0.879		
			0 g.l-1 - 4 g.l-1	NA	NA	NA	NA		
			2 g.l-1 - 4 g.l-1	NA	NA	NA	NA		
		Inland	0 g.l-1 - 2 g.l-1	0.114	0.739	0.154	0.987		
			0 g.l-1 - 4 g.l-1	-1.345	1.589	-0.846	0.675		
			2 g.l-1 - 4 g.l-1	-1.459	1.579	-0.924	0.629		
GS42	Time between GS25 and GS42	Coastal	0 g.l-1 - 2 g.l-1	0.08653	0.0579	1.494	0.1352		
				0 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
				2 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
			Inland	0 g.l-1 - 2 g.l-1	0.00301	0.0542	0.055	0.9983	
				0 g.l-1 - 4 g.l-1	-	0.05241	0.1163	-0.451	0.894
				2 g.l-1 - 4 g.l-1	-	0.05542	0.1155	-0.48	0.8808
		Morphology score	Coastal	0 g.l-1 - 2 g.l-1	-1.655	0.709	-2.33	0.057	
				0 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
				2 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
			Inland	0 g.l-1 - 2 g.l-1	-0.792	0.642	-1.234	0.437	
				0 g.l-1 - 4 g.l-1	0.055	1.373	0.04	0.999	
				2 g.l-1 - 4 g.l-1	0.847	1.362	0.622	0.809	
		Time between GS25 and GS45	Coastal	0 g.l-1 - 2 g.l-1	0.0496	0.0549	0.903	0.3664	
				0 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
				2 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
			Inland	0 g.l-1 - 2 g.l-1	-0.0164	0.0497	-0.33	0.9417	
				0 g.l-1 - 4 g.l-1	-0.1178	0.1201	-0.98	0.5893	
				2 g.l-1 - 4 g.l-1	-0.1014	0.1199	-0.845	0.6748	
GS45	Morphology score	Coastal	0 g.l-1 - 2 g.l-1	-1.117	0.545	-2.05	0.107		
				0 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
				2 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
			Inland	0 g.l-1 - 2 g.l-1	-0.192	0.482	-0.398	0.917	
				0 g.l-1 - 4 g.l-1	-2.008	1.162	-1.728	0.201	
				2 g.l-1 - 4 g.l-1	-1.816	1.168	-1.556	0.27	
		Mass at 5 days	Coastal	0 g.l-1 - 2 g.l-1	-0.012	0.006	-1.912	0.142	
				0 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
				2 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
			Inland	0 g.l-1 - 2 g.l-1	-0.008	0.005	-1.47	0.311	
				0 g.l-1 - 4 g.l-1	-0.022	0.013	-1.663	0.225	
				2 g.l-1 - 4 g.l-1	-0.014	0.013	-1.06	0.541	
		Activity	Coastal	0 g.l-1 - 2 g.l-1	-0.237	0.238	-0.997	0.579	
				0 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
				2 g.l-1 - 4 g.l-1	NA	NA	NA	NA	
			Inland	0 g.l-1 - 2 g.l-1	-0.193	0.198	-0.974	0.593	
				0 g.l-1 - 4 g.l-1	-0.361	0.424	-0.852	0.671	
				2 g.l-1 - 4 g.l-1	-0.168	0.418	-0.401	0.915	

		0 g.l-1 - 2 g.l-1	-0.186	0.513	-0.362	0.931
Mean jump distance	Coastal	0 g.l-1 - 4 g.l-1	NA	NA	NA	NA
		2 g.l-1 - 4 g.l-1	NA	NA	NA	NA
		0 g.l-1 - 2 g.l-1	0.389	0.263	1.479	0.317
	Inland	0 g.l-1 - 4 g.l-1	0.835	0.437	1.912	0.156
		2 g.l-1 - 4 g.l-1	0.447	0.454	0.984	0.593

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34 Appendix G - Post-hoc analyses for the differences within treatments between the
 35 different sites, on the different variables studied, at the different Gosner
 36 developmental stages (GS25, GS30, GS37, GS41, GS42 and GS45), the two sites (Coastal
 37 or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Significance levels are given at
 38 $\alpha < 0.05$ (*) and $\alpha < 0.1$ (.).

Developmental stage	Metrics	Treatment	Comparison	Estimate	SE	z/t ratio	p-value	Significance
GS25	Hatching success	0 g.l-1	Coastal-Inland	0.019	0.084	0.229	0.819	
		2 g.l-1	Coastal-Inland	0.015	0.079	0.194	0.846	
		4 g.l-1	Coastal-Inland	-0.085	0.079	-1.079	0.28	
	Malformation rate	0 g.l-1	Coastal-Inland	0.201	0.146	1.378	0.168	
		2 g.l-1	Coastal-Inland	0.201	0.161	1.251	0.211	
		4 g.l-1	Coastal-Inland	0.398	0.113	3.541	<0.001	*
	General activity	0 g.l-1	Coastal-Inland	0.138	0.159	0.87	0.385	
		2 g.l-1	Coastal-Inland	0.032	0.152	0.213	0.832	
		4 g.l-1	Coastal-Inland	-0.076	0.212	-0.36	0.719	
	Time to hatching	0 g.l-1	Coastal-Inland	-0.085	0.046	-1.85	0.064	.
		2 g.l-1	Coastal-Inland	-0.054	0.053	-1.031	0.303	
		4 g.l-1	Coastal-Inland	0.108	0.052	2.071	0.038	*
	Morphology score	0 g.l-1	Coastal-Inland	-1.513	0.382	-3.956	<0.001	*
		2 g.l-1	Coastal-Inland	-2.235	0.378	-5.919	<0.001	*
		4 g.l-1	Coastal-Inland	-0.023	0.381	-0.06	0.953	
	Position within the water column	0 g.l-1	Coastal-Inland	-0.588	0.586	-1.003	0.316	
		2 g.l-1	Coastal-Inland	-0.78	0.546	-1.428	0.153	
		4 g.l-1	Coastal-Inland	0.034	0.708	0.048	0.962	
	Predation test	0 g.l-1	Coastal-Inland	1.339	0.882	1.518	0.129	
		2 g.l-1	Coastal-Inland	0.407	0.613	0.664	0.507	
		4 g.l-1	Coastal-Inland	0.737	0.553	1.332	0.183	
Telomeres' length	0 g.l-1	Coastal-Inland	-0.104	0.046	-2.283	0.025	*	
	2 g.l-1	Coastal-Inland	-0.086	0.046	-1.871	0.065	.	
	4 g.l-1	Coastal-Inland	0.035	0.046	0.755	0.453		
GS30	Time between GS25 and GS30	0 g.l-1	Coastal-Inland	-0.236	0.081	-2.934	0.033	*
		2 g.l-1	Coastal-Inland	-0.194	0.081	-2.402	0.016	*
		4 g.l-1	Coastal-Inland	-0.519	0.1	-5.208	<0.001	*
	Morphology score	0 g.l-1	Coastal-Inland	-2.43	0.467	-5.201	<0.001	*
		2 g.l-1	Coastal-Inland	-2.27	0.457	-4.975	<0.001	*
		4 g.l-1	Coastal-Inland	-2.67	0.522	-5.104	<0.001	*
GS37	Time between GS25 and GS37	0 g.l-1	Coastal-Inland	0.039	0.043	0.927	0.354	
		2 g.l-1	Coastal-Inland	-0.094	0.042	-2.215	0.027	*

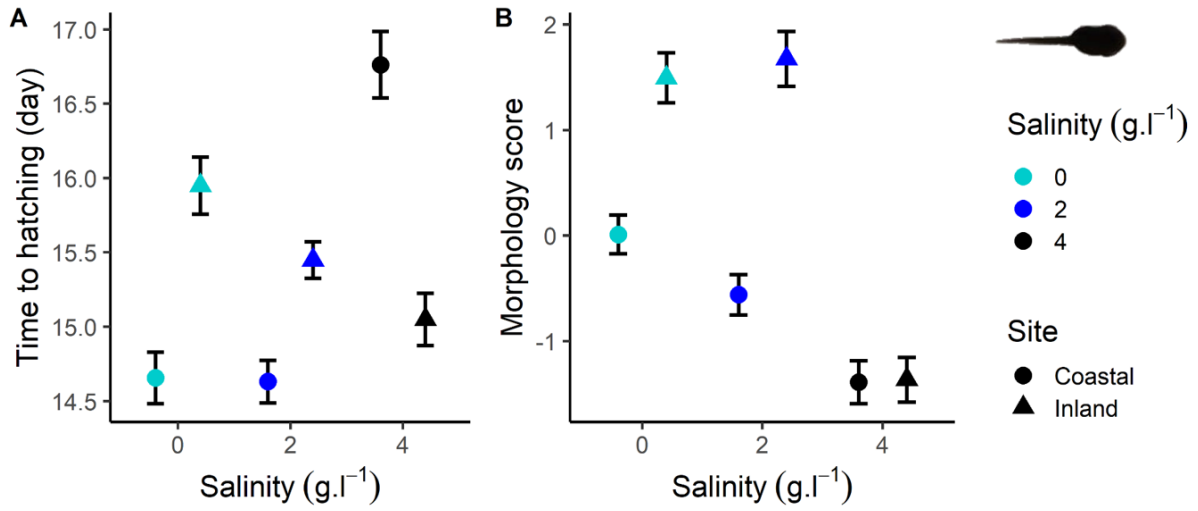
	4 g.l-1	Coastal-Inland	-0.179	0.095	-1.898	0.058	.	
Morphology score	0 g.l-1	Coastal-Inland	-4.2	0.621	-6.764	<0.001	*	
	2 g.l-1	Coastal-Inland	-2.41	0.592	-4.072	<0.001	*	
	4 g.l-1	Coastal-Inland	-1.58	1.242	-1.271	0.206		
Behavior score	0 g.l-1	Coastal-Inland	-1.98	0.756	-2.621	0.01	*	
	2 g.l-1	Coastal-Inland	-0.93	0.696	-1.336	0.184		
	4 g.l-1	Coastal-Inland	-3.17	1.655	-1.914	0.058	.	
DFA	0 g.l-1	Coastal-Inland	0.017	0.007	2.593	0.011	*	
	2 g.l-1	Coastal-Inland	0.008	0.006	1.256	0.212		
	4 g.l-1	Coastal-Inland	0.02	0.015	1.385	0.169		
DFAc	0 g.l-1	Coastal-Inland	0.003	0.002	1.777	0.078	.	
	2 g.l-1	Coastal-Inland	0.002	0.001	1.165	0.247		
	4 g.l-1	Coastal-Inland	0.006	0.004	1.681	0.095	.	
50 mm to center frequency	0 g.l-1	Coastal-Inland	0.925	0.367	2.521	0.012	*	
	2 g.l-1	Coastal-Inland	-0.713	0.372	-1.914	0.056	.	
	4 g.l-1	Coastal-Inland	0.958	0.391	2.452	0.014	*	
Total distance (size)	0 g.l-1	Coastal-Inland	-0.577	0.212	-2.715	0.007	*	
	2 g.l-1	Coastal-Inland	-0.119	0.196	-0.61	0.542		
	4 g.l-1	Coastal-Inland	-1.191	0.458	-2.601	0.009	*	
Mean speed (size/mm)	0 g.l-1	Coastal-Inland	-0.116	0.052	-2.207	0.029	*	
	2 g.l-1	Coastal-Inland	-0.041	0.048	-0.847	0.399		
	4 g.l-1	Coastal-Inland	-0.195	0.115	-1.699	0.092	.	
Total duration of activity	0 g.l-1	Coastal-Inland	-0.116	0.052	-2.207	0.029	*	
	2 g.l-1	Coastal-Inland	-0.041	0.048	-0.847	0.399		
	4 g.l-1	Coastal-Inland	-0.195	0.115	-1.699	0.092	.	
GS41	Time between GS25 and GS41	0 g.l-1	Coastal-Inland	0.262	0.072	3.634	<0.001	*
		2 g.l-1	Coastal-Inland	0.135	0.066	2.045	0.041	*
		4 g.l-1	Coastal-Inland	NA	NA	NA	NA	
	Morphology score	0 g.l-1	Coastal-Inland	-2.64	0.93	-2.833	0.006	*
		2 g.l-1	Coastal-Inland	-2.12	0.845	-2.511	0.014	*
		4 g.l-1	Coastal-Inland	NA	NA	NA	NA	
GS42	Time between GS25 and GS42	0 g.l-1	Coastal-Inland	0.236	0.069	3.44	<0.001	*
		2 g.l-1	Coastal-Inland	0.153	0.063	2.439	0.015	*
		4 g.l-1	Coastal-Inland	NA	NA	NA	NA	
	Morphology score	0 g.l-1	Coastal-Inland	-3.53	0.973	-3.629	<0.001	*
		2 g.l-1	Coastal-Inland	-2.67	0.903	-2.954	<0.001	*
		4 g.l-1	Coastal-Inland	NA	NA	NA	NA	
GS45	Time between GS25 and GS45	0 g.l-1	Coastal-Inland	0.197	0.066	2.978	0.003	*
		2 g.l-1	Coastal-Inland	0.131	0.059	2.209	0.027	*
		4 g.l-1	Coastal-Inland	NA	NA	NA	NA	
	Morphology score	0 g.l-1	Coastal-Inland	-1.744	0.649	-2.688	0.009	*
		2 g.l-1	Coastal-Inland	-0.818	0.581	-1.407	0.164	
		4 g.l-1	Coastal-Inland	NA	NA	NA	NA	
Mass at 5 days	0 g.l-1	Coastal-Inland	-0.018	0.008	-2.41	0.018	*	
	2 g.l-1	Coastal-Inland	-0.014	0.007	-2.41	0.037	*	

	4 g.l-1	Coastal-Inland	NA	NA	NA	NA
Activity	0 g.l-1	Coastal-Inland	-0.134	0.245	-0.547	0.585
	2 g.l-1	Coastal-Inland	-0.09	0.189	-0.475	0.635
	4 g.l-1	Coastal-Inland	NA	NA	NA	NA
Mean jump distance	0 g.l-1	Coastal-Inland	-0.922	0.538	-1.712	0.094
	2 g.l-1	Coastal-Inland	-0.347	0.324	-1.073	0.292
	4 g.l-1	Coastal-Inland	NA	NA	NA	NA

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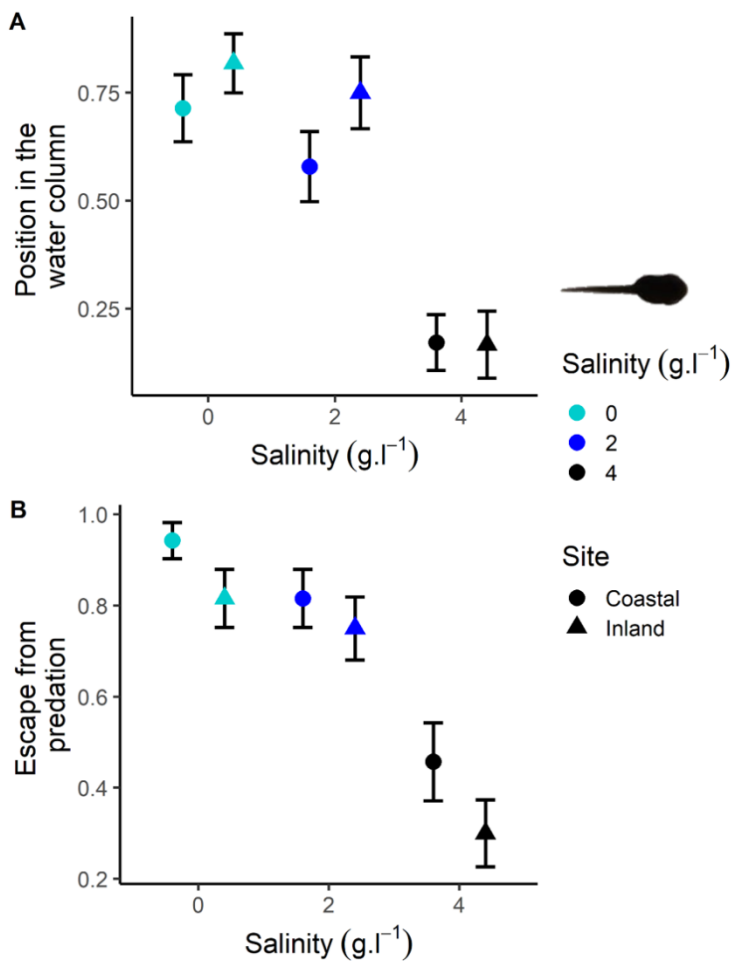
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41 Appendix H - (A) Time to hatching and (B) morphology score at GS25, for the two
42 sites (Coastal or Inland) and the three treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE.



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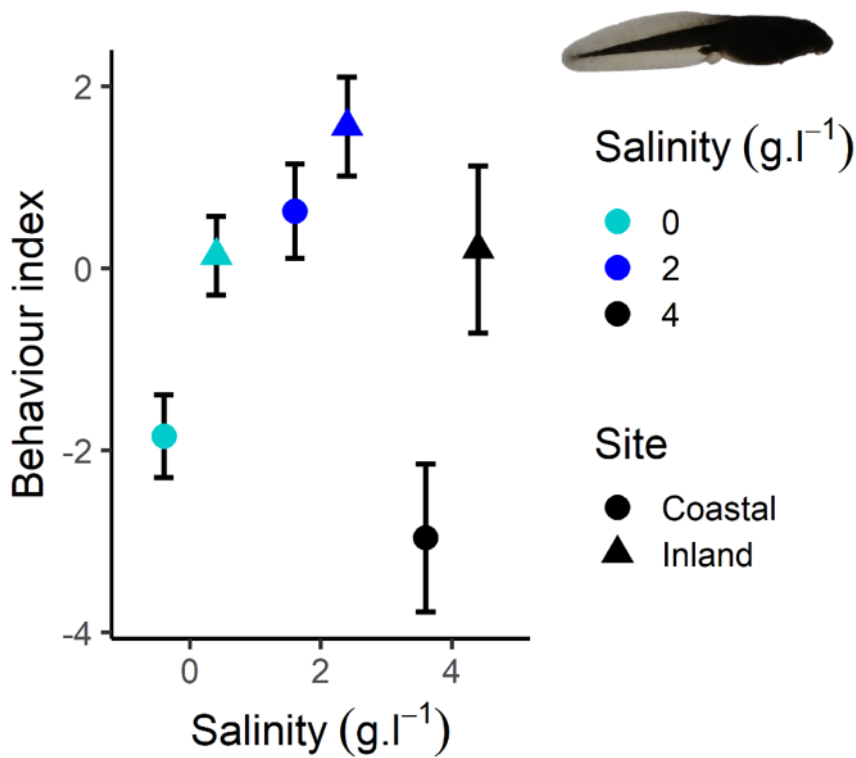
44 Appendix I - Behavioural metrics that have been recorded on individuals at Gosner
45 stage 25 (hatching). (A) Position in the water column (0: if individuals stay at the
46 bottom of their aquarium, 1: if individuals have moved within the water column) and
47 (B) escape from predation, evaluated by stimulating individuals (0: if individuals did
48 not escape, 1 if they try to escape), for the two sites (Coastal or Inland) and the three
49 treatments (0, 2 and 4 g.l⁻¹). Mean \pm SE.



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52 Appendix J - Behaviour score, extracted for the first axis of the PCA between all the
53 different metrics variables for Gosner stage 37 (DFA, DFAC, total distance, total
54 distance corrected by individual body size, mean speed, mean speed corrected by
55 individuals body size, total duration of activity), for the two sites (Coastal or Inland)
56 and the three treatments (0, 2 and 4 g.l⁻¹). Mean ± SE.



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58