



Variations of salinity during reproduction and development affect ontogenetic trajectories in a coastal amphibian

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Received: 19 July 2023 / Accepted: 2 January 2024

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Abstract

Although coastal ecosystems are naturally submitted to temporal variations of salinity, salinization has been increasing over time threatening coastal biodiversity. Species that exploit such habitats can thus be exposed to brackish water at different life stages. However, the impacts of variations of salinity on wildlife remain poorly understood. This is particularly true for coastal amphibians, due to the strong dependency of early life stages (embryos and larvae) on aquatic environments. In order to investigate the effect of salinity during egg laying and embryonic and larval development of coastal amphibians, we used a full-factorial design to expose reproductive adults, eggs, and larvae of coastal spined toads (*Bufo spinosus*) to fresh (0 g.l⁻¹) or brackish water (4 g.l⁻¹). At egg laying, we evaluated parental investment in reproduction. During embryonic and larval development, we assessed effects on survival, development, and growth. We highlighted strong effects of environmental salinity on reproduction (reduced egg laying time, marginally reduced egg size, and reduced investment in reproduction). Responses to salinity were highly dependent on the developmental stages of exposure (stronger effects when individuals were exposed during embryonic development). These effects carried over when exposure occurred at egg laying or during embryonic development, highlighting the importance of the environmental conditions during early life on ontogenetic trajectories. We also highlighted partial compensation when individuals were transferred back to freshwater. Whether the magnitude of these responses can allow coastal biodiversity to overcome the observed detrimental effects of salinization remain to be assessed.

Keywords Carry-over effects · Embryonic development · Freshwater · Recovery · Reproductive investment · Tadpoles

Introduction

Salinization of freshwater bodies is strongly increasing over time (Williams 2001). Coastal wetlands are particularly subjected to salinization because of salt water intrusions (Knighton et al. 1991; Visschers et al. 2022), reductions in freshwater flow resulting from dams and reservoirs, droughts and increased abstraction (Reid et al. 2019), rising sea levels

(Vermeer and Rahmstorf 2009; Church and White 2011; Bakker et al. 2017), and the predicted increase in frequency and intensity of marine surges (Nicholls et al. 1999; McLean et al. 2001; Knutson et al. 2010; Dettinger 2011; Trenberth et al. 2015; IPCC 2022). Due to their particular position between terrestrial and oceanic systems, coastal ecosystems are also naturally submitted to variations of salinity (Xue et al. 2013), and are thus known to experience temporal and spatial heterogeneity of salinity (Estévez et al. 2019; Ranjbar and Ehteshami 2019; Fu et al. 2021). Importantly, salinity can increase through time, but can also decrease when precipitations increase (Lorrain-Soligon et al. 2023c). However, the impacts of these short-term variations of salinity on wildlife remain poorly understood.

Salinization is expected to strongly affect coastal biodiversity (Amores et al. 2013; Debue et al. 2022). Indeed, most organisms need to maintain their internal homeostasis and rely on physiological compensatory mechanisms to regulate water and ion fluxes (Schultz and McCormick 2012;

Responsible Editor: Bruno Nunes

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Evans and Kültz 2020). Most species have specific ranges of tolerance, and crossing these limits of tolerance activates mechanisms that aim at regulating ionic and hydric fluxes (Evans 2009). These mechanisms include modulation of drinking and urination rates, regulation of the permeability of membranes to water, remodeling of cells and tissues, and increased expression and activity of ion transporters (Evans 2009; Kültz 2015; Rivera-Ingraham and Lignot 2017). However, these mechanisms are often metabolically costly and the associated energetic expenditures can affect energetic allocation to other functions (Herbert et al. 2015; Rivera-Ingraham and Lignot 2017). As such, salinity can influence critical functions such as foraging (Chuang et al. 2022), activity (Leite et al. 2022; Moniruzzaman et al. 2022), anti-predator responses (Hoover et al. 2013), and growth (Liu et al. 2022). In addition, elevated salinity can also increase the occurrence of malformations (Hieu et al. 2021), potentially leading to increased mortality (Cañedo-Argüelles et al. 2013; Moniruzzaman et al. 2022; Woodley et al. 2023). In adults, increased salinity can reduce egg production and fecundity (Pinder et al. 2005; Froneman 2023; Woodley et al. 2023), egg size (Lorrain-Soligon et al. 2023a), or the age at first reproduction (Woodley et al. 2023). In addition, increasing salinity is known to reduce sperm performance, motility, and velocity (Wilder and Welch 2014; Byrne et al. 2015, 2022; Green et al. 2021). Ultimately, salinization can alter community structure (Hart et al. 2003; Anufrieva and Shadrin 2018), across all trophic levels (Hintz and Relyea 2019).

This is especially true for coastal amphibians. Indeed, amphibians are particularly sensitive to salinity due to their highly permeable skin used for gas exchanges, and for ions and water transport (Shoemaker and Nagy 1984; Martin and Nagy 1997), and to their relatively low osmoregulatory abilities (Katz 1989). Early life stages (i.e., eggs and larvae) are particularly susceptible to environmental salinity (Albecker and McCoy 2017) because of their strong dependency on aquatic environment compared to adults (Wells 2007) and to their comparatively lower osmoregulatory abilities (Karraker and Gibbs 2011). Increasing salinity from various sources can lead to reduced thyroid hormones levels (Gomez-Mestre et al. 2004) and to changes in osmoregulatory hormones such as corticosterone and aldosterone (Tornabene et al. 2021b, 2022) in larval amphibians. Ultimately, this lead to decreased embryonic and larval survival (Christy and Dickman 2002; Chinathamby et al. 2006; Dougherty and Smith 2006; Sanzo and Hecnar 2006; Karraker et al. 2008; Bernabò et al. 2013; Jones et al. 2015), and to increased malformations (Gosner and Black 1957; Sanzo and Hecnar 2006; Haramura 2007; Karraker 2007; Hopkins et al. 2013; Brady 2013; Tornabene et al. 2021a), time to hatching (Haramura 2016; Tornabene et al. 2021a), hatching success (Tornabene et al. 2021a),

larval duration (Christy and Dickman 2002; Gomez-Mestre and Tejado 2003; Lukens and Wilcoxon 2020; Tornabene et al. 2021a), and to reduced growth and thus to induce smaller body size (Wu and Kam 2009; Bernabò et al. 2013; Wood and Welch 2015; Haramura 2016; Lukens and Wilcoxon 2020; Tornabene et al. 2021a, b).

Despite their sensitivity, amphibians are known to be very plastic in behavioral and morphological traits (Hoverman and Relyea 2008), and their developmental traits can vary in response to environmental cues (Chivers et al. 2001; Warkentin 2011). For instance, tadpoles can modulate their growth rates according to the quality of environmental conditions (Werner 1986; Vonesh and Warkentin 2006). Additionally, it has been suggested that the influence of a given environmental factor on development and growth of tadpoles is highly dependent on the stage and condition during which they experience such factor (Denver et al. 2002). This can be highly relevant for changes in salinity. For example, *Fejervarya limnocharis* and *Litoria ewingii* tadpoles released from salinity stress at early larval stages reached a size at metamorphosis similar to that of tadpoles maintained in low salinity throughout development, indicating compensatory growth (Squires et al. 2010; Wu et al. 2012). Yet, the efficiency of such compensatory mechanisms may vary if individuals are exposed earlier or later during larval development (Hsu et al. 2018). Additionally, whether parents can influence the tolerance of their offspring according to the salinity in which they are laying eggs is unknown.

In order to investigate the effect of salinity during egg laying and embryonic and larval development of coastal amphibians, we used a full-factorial experimental design to expose reproductive adults, eggs, and larvae of coastal spined toads (*Bufo spinosus*, a species which coastal individuals are supposedly adapted to exposure to salinity) to fresh (0 g.l^{-1}) or brackish water (4 g.l^{-1} , Fig. 1). More specifically, reproductive pairs (amplexus) were exposed to fresh or brackish water prior to egg laying (Fig. 1). Following egg laying, we exposed eggs to either fresh or brackish water until hatching (Fig. 1). Following hatching, we exposed larvae to either fresh or brackish water (Fig. 1). At egg laying, we evaluated fecundity and parental investment in reproduction. During both embryonic and larval development, we assessed effects of each experimental treatment on survival (hatching rate, larval survival), development (duration of embryonic and larval development until Gosner stage 30 [Gosner 1960], malformation), and growth (body length). We predicted that (1) brackish water would impact reproductive investment and egg size, and alter fecundity because salinity can impact sperm motility (Byrne et al. 2015, 2022); (2) salt exposure would decrease embryonic survival, larval survival, and growth (Albecker and McCoy 2017; Lukens and Wilcoxon 2020; Tornabene et al. 2021a) as well as malformations (Gosner and Black 1957; Sanzo and Hecnar

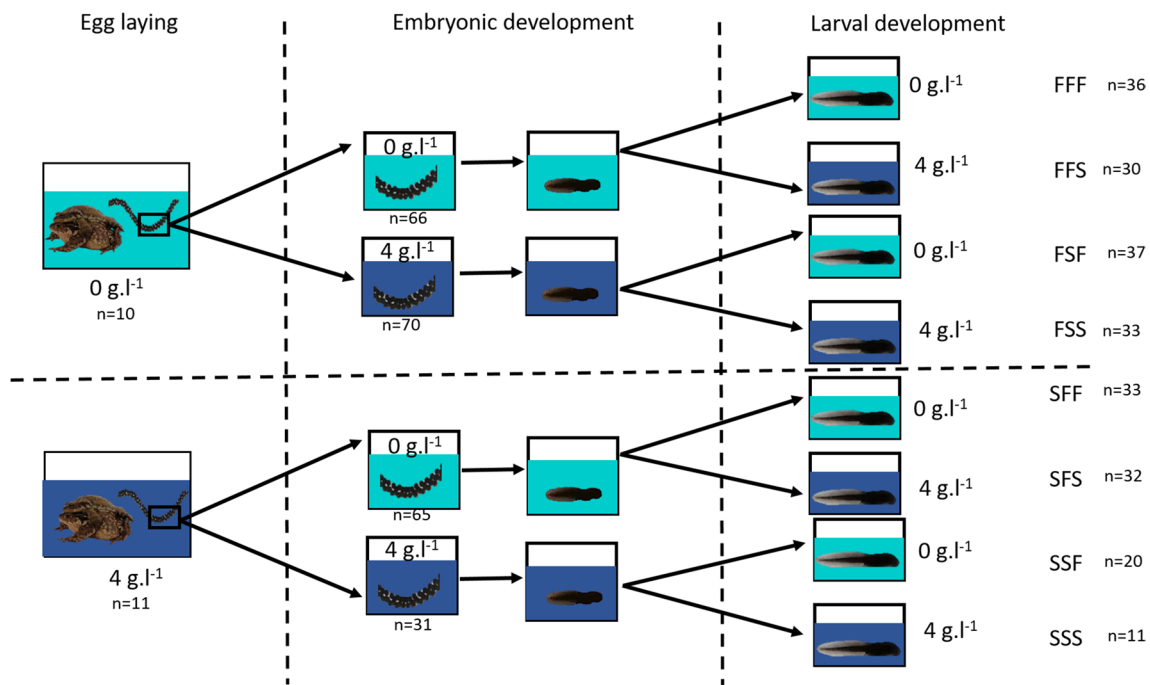


Fig. 1 Experimental design used to assess the effects of salinity during different stages of development in *Bufo spinosus*. Colors represent salinity treatments (light blue = fresh water [0 g.l⁻¹], dark blue = brackish water [4 g.l⁻¹])

2006; Haramura 2007; Karraker 2007; Hopkins et al. 2013; Brady 2013; Tornabene et al. 2021a), regardless of the stage of exposure. These effects should be alleviated in individuals transferred to freshwater, because of the resilience capacities of amphibians to salt exposure (Squires et al. 2010; Wu et al. 2012; Hsu et al. 2018; Lorrain-Soligon et al. 2022).

Methods

Study species

Spined toads (*Bufo spinosus*) are a common amphibian species found in western Europe (Speybroeck et al. 2018; Meek 2022). Breeding occurs in late winter, during which adults converge to reproductive ponds where they mate (amplexus) and lay their eggs constituted of 2 egg strings which can be constituted of up to 10,000 eggs (Miaud and Muratet 2018). Egg laying, and embryonic and larval developments all occur in ponds.

Captures and measurements

Amplectant pairs were captured on one coastal pond (salinity 3.1 g.l⁻¹ during captures, conductimeter YSI Professional Plus) situated in the Réserve Naturelle Nationale du Marais d'Yves (location: 46.04013, - 1.05363). This site can display seasonal variations of salinity ranging from 0.5 to 6

g.l⁻¹, as what is found in nearby ponds. Captures occurred at night (between 20 pm and 4 am) on 21/02/2023 and 22/02/2023. Twenty-one amplectant pairs 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, individuals were kept in a thermally controlled room with temperature set at 17 °C and controlled photoperiod (12 h dark–12 h light), temperature and photoperiod being set for the totality of the experiment. Pairs were transitorily (for measurements only) separated and all individuals were measured (snout-vent length, SVL) using a caliper (± 1 mm) and weighed using an electronic balance (± 0.1 g). Once measurements were completed, separated amplectant individuals were shortly reunited and pairs re-formed systematically as soon as the partners were brought back into contact.

Experimental procedures

Amplectant pairs, embryos, and larvae were exposed to either freshwater or brackish water (sea salt, NaCl), following a full factorial design (Fig. 1). At the end of the experiment, larval individuals were thus exposed to one of the eight following treatments: (1) FFF, clutch obtained in freshwater, embryonic development in freshwater, larval development in freshwater; (2) FSF, clutch obtained in freshwater, embryonic development in brackish water, larval development in freshwater; (3) FFS, clutch obtained in freshwater, embryonic development in

freshwater, larval development in brackish water; (4) FSS, clutch obtained in freshwater, embryonic development in brackish water, larval development in brackish water; (5) SFF, clutch obtained in brackish water, embryonic development in freshwater, larval development in freshwater; (6) SSF, clutch obtained in brackish water, embryonic development in brackish water, larval development in freshwater; (7) SFS, clutch obtained in brackish water, embryonic development in freshwater, larval development in brackish water; (8) SSS, clutch obtained in brackish water, embryonic development in brackish water, larval development in brackish water. Water of these treatments was changed every 7 days.

Tadpoles were fed ad libitum with thawed frozen organic spinach. Individuals were checked twice a day for their specific developmental stage, for mortality, and for abnormalities (which were checked visually).

Egg laying ($n = 21$)

Once the amplexant pairs were reunited, they were placed in a plastic container (35 * 55 * 26 cm) containing either freshwater (~ 20 l, salinity: 0.3 g.l⁻¹, 10 amplexant pairs) or brackish water (~ 20 l, salinity: 4.0 g.l⁻¹, 11 amplexant pairs) as well as branches for laying support. The two groups did not differ in size (freshwater: females = 74.7 mm ± 1.61 SE, males = 64.1 mm ± 1.00 SE; brackish water: females = 75.9 mm ± 2.08 SE, males = 62.7 mm ± 1.22 SE; differences between females in the two groups: estimate = 1.209, SE = 2.604, $t_{1,19} = 0.464$, p -value = 0.648; differences between males in the two groups: estimate = 1.373, SE = 1.601, $t_{1,19} = 0.857$, p -value = 0.402). Amplexant pairs were left in these tanks until egg laying. Individuals were checked every 2 h to compute time to egg laying (measured from their placement in a plastic container to the completion of egg laying). Adult individuals were then released at their site of capture. Adults were thus kept under laboratory conditions for a maximum of 7 days (maximal

time to egg laying 151 h), and the water of their treatment was left unchanged during this duration. They were unfed during this period, as adults anurans usually do not feed during the reproduction. Twelve pieces of each egg strings, containing 30 eggs each (i.e., a total of 360 eggs for each egg string) were selected randomly and kept for our experiment, and the remaining eggs (i.e., 100–4500) were released at the site of origin of their parents.

Embryonic development ($n = 232$)

The two treatments were applied in 2-l individual tanks (13 * 18 * 18 cm, $n = 232$, see Table 1) containing one of the two treatment (freshwater: 0 g.l⁻¹ salinity: 0.3 ± 0.0 g.l⁻¹; brackish water: 4 g.l⁻¹ salinity 4.0 ± 0.06 g.l⁻¹). Water was changed once a week. Each clutch was represented by 6 segments of 30 eggs in each of the experimental treatments. To prevent osmotic shock, segments for which the clutch was obtained in another salinity than which their embryonic development occur (i.e., clutch that were obtained at 0 g.l⁻¹ but for which embryonic development will occur at 4 g.l⁻¹ and clutch that were obtained at 4 g.l⁻¹ but for which embryonic development will occur at 0 g.l⁻¹) were gradually exposed to the salinity of the treatment, with an increase or a decrease of 1 g.l⁻¹ a day (Hsu et al. 2018). This way, segments were exposed to their final treatment over 4 days. Each segment were kept in the laboratory until tadpoles hatched and reached a free feeding stage (Gosner stage 25, hereafter GS25) (Gomez-Mestre and Tejedo 2003).

Larval development ($n = 232$)

Upon hatching (GS25), we selected one individual per segment (randomly, among alive and not malformed tadpoles of each segment), and these individuals were once again distributed in two treatments: freshwater (0 g.l⁻¹ salinity: 0.3 ± 0.0 g.l⁻¹) and brackish water (4 g.l⁻¹ salinity 4.0 ± 0.06 g.l⁻¹). For each clutch, three segments and thus three

Table 1 Number of surviving individuals in each treatment at each life stage

Embryonic treatment	Number of individuals in the treatment	Surviving individuals at GS25	Larval treatment	Number of individuals in the treatment	Surviving individuals at GS25	Surviving individuals at GS30
FF	66	66	FFF	36	36	30
			FFS	30	30	15
FS	102	70	FSF	53	37	14
			FSS	49	33	2
SF	66	65	SFF	33	33	22
			SFS	33	32	18
SS	83	31	SSF	46	20	7
			SSS	37	11	2

tadpoles were thus exposed to each treatment (FFF, FFS, FSF, FSS, SFF, SFS, SSF, and SSS). In some segments, no individuals survived until GS25. In these cases, individuals were replaced by other individuals from the same treatments but originating from different clutches in order to keep a total of 232 tadpoles that were followed during larval development (see Table 1 for the count of tadpoles in each treatment, and Appendix A for the contribution of each clutch). All other tadpoles were released at the site where their parents were captured.

Similarly to embryos, when individuals were transferred to a different treatment (i.e., tadpoles for which embryonic development occur at 0 g.l⁻¹ but larval development will occur at 4 g.l⁻¹ and tadpoles for which embryonic development occur at 4 g.l⁻¹ but larval development will occur at 0 g.l⁻¹), tadpoles were gradually exposed to the salinity of the treatment, with an increase or a decrease of 1 g.l⁻¹ a day to prevent osmotic shock (Hsu et al. 2018).

Larval development was monitored until G30 (Gosner 1960), in order to have approximately the same time considered for embryonic development and larval development, and because GS30 is a pivotal stage when somatic growth decreases and significant morphological changes occur (Cheron et al. 2021). At the end of this experimental procedure, individuals were released at the site where their parents were captured.

Measurements

Reproductive effort ($n = 21$)

Once egg laying was completed, individuals were again weighed to calculate variations in body mass. Additionally, each clutch was weighed using an electronic balance (± 0.1 g). Moreover, in order to assess fecundity, each egg string was placed in a container (35 × 20 × 25 cm) containing 2 cm of dechlorinated tap water and a scale (graph paper). A picture was taken from above in order to measure the total length of the egg string using ImageJ software (Schneider et al. 2012). For each clutch, we randomly selected six 10-cm segments and individually counted the number of eggs within each segment. The mean number of eggs per 10-cm segment was calculated and used to assess fecundity (number of eggs) for each clutch based on the length of the egg strings. Finally, on a subsample of 100 randomly selected eggs in each egg strings, we measured egg diameter.

Embryonic development ($n = 232$)

At GS 25, hatching success (proportion of eggs that hatched, on the segment of 30 eggs) and deformation rates (proportion of larvae that hatched but were malformed, on

the total number of hatched individuals) were calculated. Additionally, for each segments, we described general activity of all hatchlings as being, on a scale of 1 to 5, the proportion of live individuals that were active; 1 = 0–20%, 2 = 20–40%, 3 = 40–60%, 4 = 60–80%, 5 = 80–100% over a 1-min period.

Larval development ($n = 232$)

At GS25 and GS30, we measured total length of individuals. Each tadpole was put into a Petri dish with the water from its own tank and photographed. Individuals were then measured using the software ImageJ (Schneider et al. 2012). We also measured activity following a scale ranging from 1 to 5 (from 1 if the individual is amorphous to 5 if the individual move energetically, following Lorrain-Soligon et al. (2022)). The tadpoles were not stimulated to record these observations.

Statistical analyses

We computed linear models (LMs) or linear mixed models (LMMs) and generalized linear mixed models (GLMMs) using the lme4 (Bates et al. 2015) and lmerTest (Kuznetsova et al. 2017) packages. For all test computed, models accuracy was tested using the check_model function from the performance package (Lüdtke et al. 2020). When models did not fit the data, the response variable was log + 1 transformed. Binomial model accuracy was also tested by checking overdispersion. Significance level was set at 0.05; differences at a p -value level < 0.1 were considered marginal. For all comparisons studied, mean \pm SE of each group are given in Appendix B.

Reproductive effort

We evaluated the effect of salinity (clutches laid in freshwater [0 g.l⁻¹] or in brackish water [4 g.l⁻¹]) on clutch length, clutch size (number of eggs), clutch mass, egg diameter, laying time, and variation of body mass (for females and males). For these analyses, we set linear models (LMs), with salinity as an explanatory variable. We also tested for the relationships between egg diameter and clutch size in each salinity by setting a LM with egg diameter as a dependent variable, and clutch size, salinity, and their interaction as explanatory variables. Finally, we tested for the effect of the interaction between female mass and salinity on clutch size and clutch mass, using LMs. For these models, best variables were retained using a top-down selection (using p -values), and only the retained variables are presented.

Embryonic development

We evaluated the effect of salinity during spawning (clutch laid in freshwater [0 g.l^{-1}] or in brackish water [4 g.l^{-1}]), and during embryonic development (individuals developing in freshwater [0 g.l^{-1}] or brackish water [4 g.l^{-1}]).

We computed these effects on time to hatching (log + 1 transformed) and individual total size with linear mixed models (LMMs), and on hatching success, malformation rate, general activity, and tadpole activity (both rated on a scale from 1 to 5) using binomial generalized linear mixed models (GLMMs). For all these models, we included salinity during spawning, salinity during embryonic development, and their interaction as explanatory variables, and the clutch as a random effect (as multiple individuals originated from the same clutch). Best variables were retained using a top-down selection (using p -values), and only the retained variables are presented.

Larval development

We evaluated the effect of salinity at different stages of exposure. We computed the effect of salinity during spawning (clutches laid in freshwater [0 g.l^{-1}] or in brackish water [4 g.l^{-1}]), salinity during embryonic development (eggs developing in freshwater [0 g.l^{-1}] or brackish water [4 g.l^{-1}]), and salinity during larval development (individuals developing in freshwater [0 g.l^{-1}] or brackish water [4 g.l^{-1}]). We computed these effects on development duration between S25 and S30, growth rate between GS25 and GS30, and total size (log + 1 transformed) at GS30 with LMMs, and on mortality and activity (rated on a scale from 1 to 5) using binomial GLMMs. For all these models, we included salinity during spawning, salinity during embryonic development, salinity during larval development and their interaction as explanatory variables, and the clutch as a random effect (as multiple individuals originated from the same clutch). Best variables were retained using a top-down selection (using p -values), and only the retained variables are presented. At GS30, only two individuals were malformed (in FFS and FSF) and thus statistics were not computed. All data analysis were performed using R 3.6.3 (R Core Team 2020) and Rstudio v1.1.419.

Results

Reproductive effort

Clutches produced in freshwater or in brackish water were similar for length (estimate = 70.05, SE = 263.58, $t_{1,19} = 0.27$, p -value = 0.793) and number of eggs (estimate = 182.60, SE = 405.40, $t_{1,19} = 0.45$, p -value = 0.658).

However, clutches produced in freshwater were heavier (estimate = 28.21, SE = 13.07, $t_{1,19} = 2.16$, p -value = 0.044), and their eggs were marginally larger (estimate = 0.11, SE = 0.06, $t_{1,19} = 1.80$, p -value = 0.088, Fig. 2A). Laying time was shorter for individuals that laid eggs in brackish water compared to those that laid eggs in freshwater (estimate = -31.77, SE = 14.84, $t_{1,19} = -2.14$, p -value = 0.046, Fig. 2B).

Egg diameter was related to the interaction between clutch size and salinity (estimate < 0.001, SE < 0.001, $t_{3,17} = 2.65$, p -value = 0.017, Fig. 2C). Egg diameter was not influenced by clutch size in clutches laid in freshwater (estimate < 0.001, SE < 0.001, $t_{1,8} = 1.21$, p -value = 0.260, Fig. 2C), but increased with increasing clutch size in clutches

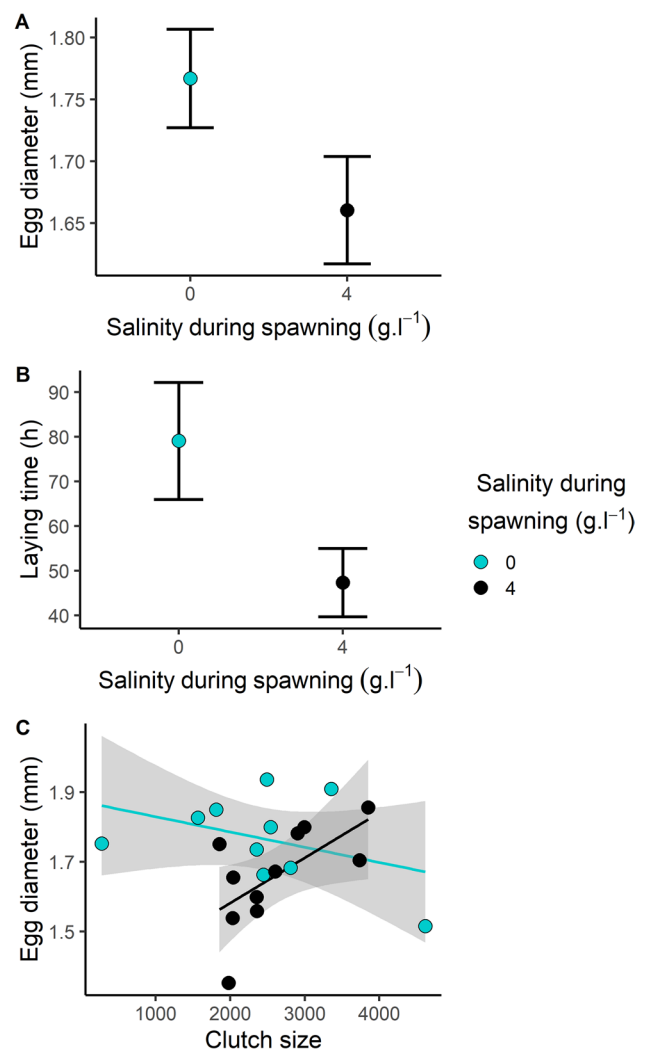


Fig. 2 Effects of salinity during spawning on reproductive parameters. **A** Egg diameter, **B** clutch size (number of eggs), and **C** relationships between clutch size and egg diameter, for clutches produced in freshwater (0 g.l^{-1}) or brackish water (4 g.l^{-1}). Data are presented as mean \pm SE. See Fig. 1 for sample size

laid in brackish water (estimate < 0.001, SE < 0.001, $t_{1,9} = 2.40$, p -value = 0.040, Fig. 2C).

Interestingly, females spawning at 0 g.l^{-1} ($-13.56\% \pm 2.23 \text{ SE}$) and 4 g.l^{-1} ($-12.21\% \pm 1.36 \text{ SE}$) lost similar mass during egg laying (estimate = 1.35, SE = 2.56, $t_{1,19} = 0.53$, p -value = 0.604). Males reproducing at 0 g.l^{-1} lost mass ($-1.63\% \pm 2.19 \text{ SE}$) while males reproducing at 4 g.l^{-1} gained mass ($3.79\% \pm 1.27 \text{ SE}$) during egg laying (estimate = 5.41, SE = 2.47, $t_{1,19} = 2.19$, p -value = 0.041).

Clutch size increased with female mass (estimate = 40.876, SE = 10.303, $t_{1,19} = 3.967$, p -value < 0.001). When testing for the interaction between females body mass and salinity, clutch mass increased with female body mass (estimate = 1.211, SE = 0.362, $t_{1,19} = 3.347$, p -value = 0.004) and was higher in the freshwater treatment compared to the brackish one (estimate = 25.192, SE = 10.579, $t_{1,19} = 2.831$, p -value = 0.029).

Embryonic development

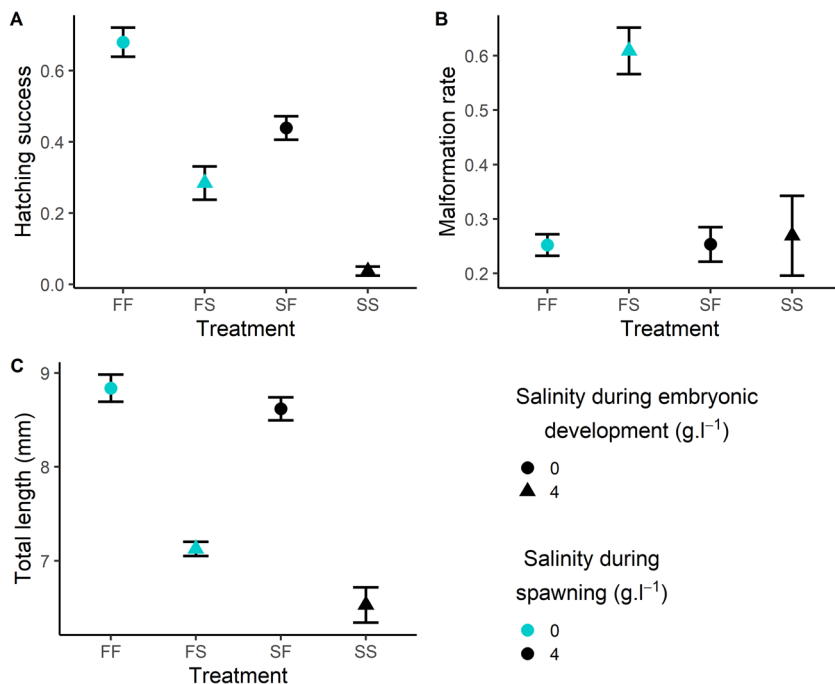
Hatching success was higher for clutches produced in freshwater (estimate = 0.480, SE = 0.194, $z = 2.649$, p -value = 0.014), and higher in embryos that developed in freshwater (estimate = 0.972, SE = 0.064, $z = 15.103$, p -value < 0.001). There was an interaction between the salinity during spawning and the salinity during embryonic development (estimate = -1.493 , SE = 0.141, $z = -10.580$, p -value < 0.001, Fig. 3A, Appendix C), hatching success being higher for individuals originating from a clutch laid in freshwater, and that developed in freshwater.

Malformation rate was higher in embryos which developed in brackish water (estimate = 0.893, SE = 0.114, $z = 7.840$, p -value < 0.001), but not related to salinity during spawning (estimate = 0.086, SE = 0.242, $z = 0.357$, p -value = 0.721). There was an interaction between the salinity during spawning and the salinity during embryonic development (estimate = -1.059 , SE = 0.301, $z = -3.524$, p -value < 0.001, Fig. 3B, Appendix C), malformation rate being higher for individuals originating from a clutch laid in freshwater, and that developed in brackish water.

Time to hatching was higher in embryos which developed in brackish water (estimate = 0.107, SE = 0.013, $t_{1,221} = 8.387$, p -value < 0.001), but not related to spawning in brackish water (estimate = 0.004, SE = 0.025, $t_{1,27} = 0.168$, p -value = 0.868). There was an interaction between the salinity during spawning and the salinity during embryonic development (longer time to hatching for individuals that were laid in freshwater but developed in brackish water, estimate = -0.065 , SE = 0.021, $t_{1,218} = -3.104$, p -value = 0.002, Appendix C).

General activity (estimate = 1.164, SE = 0.173, $z = 6.735$, p -value < 0.001), tadpole activity (estimate = 1.141, SE = 0.117, $z = 9.706$, p -value < 0.001), and total length (estimate = -2.157 , SE = 0.0.139, $t_{1,228} = -15.540$, p -value < 0.001, Fig. 3C) were all influenced by salinity experienced during embryonic development solely, and were higher for embryos that developed in freshwater.

Fig. 3 Effects of salinity during spawning and embryonic development on different aspects of embryonic development assessed upon hatching (Gosner stage 25). **A** Hatching success, **B** malformation rate, and **C** total length (mm). FF, spawning in freshwater [0 g.l^{-1}], embryonic development in freshwater; FS, spawning in freshwater, embryo in brackish water [4 g.l^{-1}]; SF, spawning in brackish water, embryo in freshwater; SS, spawning in brackish water, embryo in brackish water. Data are presented as mean \pm SE. See Fig. 1 for sample size



Larval development

Mortality at GS30 was influenced by salinity to which individuals were exposed during embryonic development (higher for individuals which embryonic development occurred in brackish water [FSF, FSS, SSF, and SSS], estimate = 3.405, SE = 0.005, $z = 676.3$, p -value < 0.001, Fig. 4A) and during larval development (higher for larvae developing in brackish water [FFS, FSS, SFS, and SSS], estimate = 1.421, SE = 0.005, $z = 282.5$, p -value < 0.001, Fig. 4A), but not their interaction.

Development duration between S25 and S30 varied with salinity during embryonic development (longer for individuals which embryonic development occurred in brackish water [particularly FSS, SSF, and SSS], estimate = 1.409, SE = 0.635, $t_{1,101} = 2.218$, p -value = 0.029, Fig. 4B), and there was an interaction between salinity during embryonic development and salinity during larval development (estimate = 3.307, SE = 1.095, $t_{1,91} = 3.020$, p -value = 0.003, Fig. 4B, Appendix D), as well as between salinity during spawning and salinity during larval development (estimate = -2.914, SE = 0.768, $t_{1,94} = -3.793$, p -value < 0.001, Fig. 4B, Appendix D).

Growth rate between GS25 and GS30 was only influenced by salinity during embryonic development, and was higher for individuals which embryonic development occurred in brackish water (particularly FSF for individuals laid in

freshwater, and SSF and SSS for individuals laid in brackish water, estimate = 0.081, SE = 0.036, $t_{1,110} = 2.240$, p -value = 0.027, Fig. 4C).

Total length was influenced by salinity experienced during embryonic development solely, decreasing for individuals which embryonic development occurred in brackish water (estimate = -0.033, SE = 0.012, $t_{1,110} = -2.653$, p -value = 0.009, Fig. 4D). Conversely, activity (estimate = -0.350, SE = 0.147, $z = -2.387$, p -value = 0.017) was influenced by salinity during larval development solely, decreasing for larvae that developed in brackish water (particularly FFS, FFS, and SFS).

Discussion

Our study demonstrated that environmental salinity can negatively affect reproduction, as well as embryonic and larval development in a coastal anuran. Importantly, our full-factorial design allowed us to highlight that early exposure to brackish water did not induce increased tolerance during later exposure (i.e., tadpoles spawned in brackish water did not perform better in brackish water). However, individuals returned to freshwater after being exposed to brackish water expressed partial recovery, indicating resilience to salinity stress.

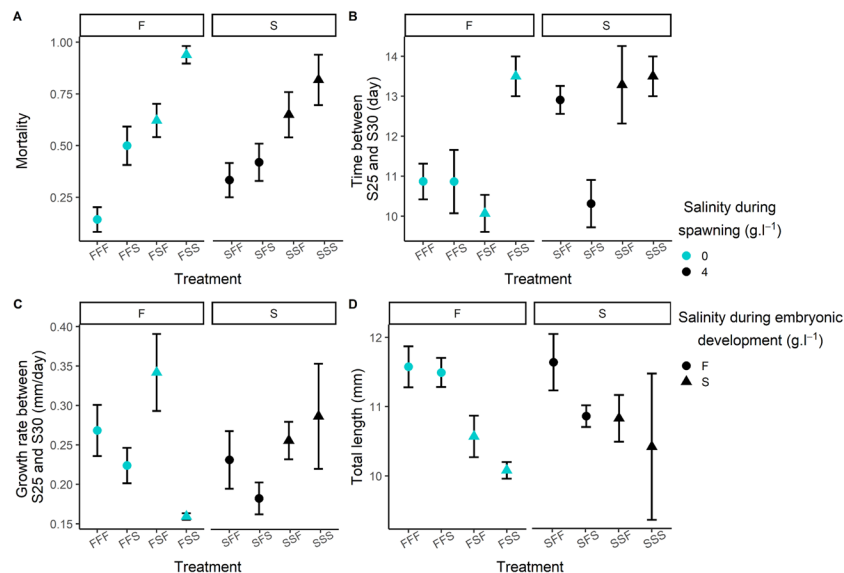


Fig. 4 Effects of salinity during spawning, embryonic development, and larval development on different parameters of larval development measured at Gosner stage 30. **A** Mortality rate, **B** duration between GS25 and GS30, **C** growth between GS25 and GS30, and **D** total length (mm). FFF, spawning in freshwater [0 g.l⁻¹], embryonic development in freshwater, larval development in freshwater; FFS, spawning in freshwater, embryo in brackish water [4 g.l⁻¹], larvae in freshwater; FSS, spawning in freshwater, embryo in freshwater, larvae in brackish water; FSS, spawning in freshwater, embryo in brackish water, larvae in brackish water; SFF, spawning in brackish water, embryo in freshwater, larvae in freshwater; SFS, spawning in brackish water, embryo in freshwater, larvae in brackish water; SSS, spawning in brackish water, embryo in brackish water, larvae in brackish water. Data are presented as mean \pm SE. Treatments and sample size are illustrated in Fig. 1

vae in brackish water; FSS, spawning in freshwater, embryo in brackish water, larvae in brackish water; SFF, spawning in brackish water, embryo in freshwater, larvae in freshwater; SSF, spawning in brackish water, embryo in brackish water, larvae in freshwater; SFS, spawning in brackish water, embryo in freshwater, larvae in brackish water; SSS, spawning in brackish water, embryo in brackish water, larvae in brackish water. Data are presented as mean \pm SE. Treatments and sample size are illustrated in Fig. 1

Reproduction

Clutch size did not differ between freshwater and brackish water treatment, and clutch size increased with female size and female mass loss for both treatments, with no difference between the two treatments. Such results further suggest that clutch size might depend on energetic investment that occurred prior to egg laying (Sinervo and DeNardo 1996; Nilsson and Svensson 1997; Lorrain-Soligon et al. 2023a). Interestingly, we found that salinity influenced some reproductive parameters in adults, suggesting a rapid modulation of reproductive effort based on the salinity to which reproductive adults were exposed. Indeed, reproductive adults exposed to brackish water laid their eggs more rapidly than those kept in fresh water. Two opposing hypotheses can explain such result. First, females may lay their eggs more rapidly in brackish water in order to evade an osmotically constraining environment. Conversely, because adult individuals originated from salt-exposed coastal environment, they may consider brackish water as a favorable environment if they are locally adapted to such conditions and if there are less competitors in this environment (Hopkins and Brodie 2015; Lorrain-Soligon et al. 2023b), but delay laying in freshwater because they consider this environment as suboptimal. This later hypothesis seems relatively unlikely as our first hypothesis is further supported by the fact that, in brackish water, eggs were smaller but not fewer. This result suggests that females produced eggs of comparatively lower quality in brackish water, as eggs produced in the freshwater treatment are marginally larger, and larger eggs often have higher hatching success and offspring fitness (Xu et al. 2019; Renoirt et al. 2023). The number of undeveloped eggs (presumably unfertilized given the negative effect of salinity on sperm (Wilder and Welch 2014; Green et al. 2021)) was also higher in brackish water.

Interestingly, despite the marginal effect of salinity on egg size, females from both treatments lost a similar amount of mass. In combination with smaller eggs, this result could be linked to a relatively larger investment in egg jellies (which are critical to egg development and protection (Bonnell and Chandler 1996; Yurewicz et al. 1975)) in females exposed to brackish water, a hypothesis that is not supported by clutch mass which was higher for females exposed to freshwater. However, investigations focusing on investment in egg protection (egg jellies) in response to environmental salinity remain too scarce. More likely, the fact that females exposed to brackish water have lost a similar amount of mass than their counterpart exposed to freshwater but laid smaller eggs might be linked to direct costs of osmoregulation in adult amphibians exposed to brackish water (Lorrain-Soligon et al. 2022). These variations should be investigated through the evaluation of metabolic rates in reproducing adults exposed to different salinities.

The fact that clutch mass was higher in the freshwater treatment is likely linked to water absorption due to osmotic exchanges with the environment (Venturino et al. 2003; Karaker and Gibbs 2011; López-Alcaide and Macip-Ríos 2011), or due to higher investment in glycoproteins surrounding the eggs (i.e., egg jellies, see above). More importantly, we found a positive correlation between egg size and clutch size for clutches laid in brackish water but not for clutches laid in freshwater, while fecundity was expected to negatively trade off with egg size (Lasne et al. 2018; Smith and Fretwell 1974). Based on the fractional egg hypothesis (Ricklefs 1968; Nussbaum 1981; Ford and Seigel 2010), the negative relation between fecundity and egg size is expected because species with large clutch sizes and small eggs (typical of Bufonid toads (Wells 2007)) should add additional offspring should extra energy become available (Ricklefs 1968; Nussbaum 1981). In our study, we found that increased clutch size is concomitant with increased egg size in clutches laid in brackish water, suggesting that complex interactions between prior energetic investment in reproduction and current spawning conditions govern reproductive effort.

Lastly, reproducing males have lost mass in freshwater, but gained mass in brackish water. Such result could be linked to reduced ejaculate volume, with males in freshwater producing more sperm than their counterparts exposed to brackish water. This hypothesis seems corroborated by the reduced proportion of fertilized eggs in the brackish treatment. However, we did not evaluate sperm volume in each treatment, which might need to be investigated. However, this gain of mass in males can also be linked to the fact that they can be osmotically imbalanced (Hall et al. 2020; Tornabene et al. 2021b).

Overall, the effects of salinity we found on reproductive adults and clutches clearly affect the investment in reproduction, which suggest that reproductive pond selection in heterogeneous environment may allow to circumvent such environmental constraint (Albecker and McCoy 2017). However, if coastal salinization continues to increase (Herbert et al. 2015; Singh 2021; Cunillera-Montcusí et al. 2022) and individuals are constrained to reproduce in brackish water, this could affect the quality of subsequent offspring development, and thereby negatively influence coastal population persistence.

Embryonic development

We show that embryonic development duration increased for individuals that were exposed to 4 g.l⁻¹ salinity during embryonic development, but only when they were spawned in freshwater. Increased development time in response to salinity has been described in amphibians (see Christy and Dickman 2002; Sanzo and Hecnar 2006; Wu and Kam 2009; Wijethunga et al. 2016; Lukens and Wilcoxon 2020), an effect which can be due to increasing energy expenditure in osmotically stressful environments. At hatching, individuals in the

4 g.l⁻¹ treatment were smaller (irrespective of the salinity in which they were spawned) (Wu and Kam 2009; Wood and Welch 2015; Haramura 2016; Lukens and Wilcoxon 2020; Tornabene et al. 2021a), which is probably linked to higher costs of osmoregulation (Gomez-Mestre et al. 2004). Individuals in the 4 g.l⁻¹ treatment also presented lower activity. Interestingly, hatching success was always lower for individuals originating from a clutch that was spawned in brackish water irrespective of the salinity in which the remaining embryonic development occurred. Additionally, individuals that were laid in freshwater but developed in brackish water had a higher malformation rate compared to individuals in any other treatments. As individuals developing in the SF and SS groups (that were laid in brackish water) presented a higher mortality rate, this could indicate selective mortality affecting individuals susceptible to salinity, while more tolerant—surviving—individuals displayed lower malformation rates. This hypothesis is further supported by the fact that higher malformation rates were found in individuals spawned in freshwater that developed in brackish water (Gosner and Black 1957; Hopkins et al. 2013). Taken together, these results indicate long-lasting effects of the salinity in which the clutch was produced (and thus long-lasting effect of very early exposure to salinity during embryonic development). Our results thus emphasize that embryos are sensitive to change in salinity during their development, which can occur through changes of temperatures and precipitations in coastal environments (Lorain-Soligon et al. 2023c), which may carry over during later larval development and potentially during post-metamorphic stages (Traversari 2021; Dahrouge and Rittenhouse 2022), affecting overall amphibian populations.

Larval development

Overall, development duration was longer for individuals that were spawned in brackish water, indicating long-lasting, carry-over effects of the salinity in which adults lay eggs, and accumulating effects during larval development (see Tornabene et al. 2021a). Individuals that experienced salinity during either embryonic or larval development (FSF, FFS, SSF, and SFS) or during both stages (FSS and SSS) expressed higher mortality (see also Squires et al. 2010; Wu et al. 2012; Albecker and McCoy 2017; Hsu et al. 2018). Additionally, individuals that developed in brackish water during embryonic development were smaller (see also Squires et al. 2010), while there was no effects of salinity experienced during larval development on total length. These responses remain consistent regardless of the salinity in which clutches were spawned. This further suggest that salinity experienced during embryonic development is more detrimental than salinity experienced during larval development, which is in line with the idea that early developmental stages (embryos) are more sensitive to salinity than later developmental stages (larvae) (Albecker and McCoy

2017). This is likely due to the development of internal gills (the main organs responsible for ion and water balance in tadpoles (Uchiyama and Yoshizawa 1992)) which improve the osmoregulation ability at this developmental stage. However, in the context of accumulating effects, larvae can be found to be more sensitive than embryos (Tornabene et al. 2021a), which highlight that this effect might be context dependent.

The detrimental effects of salinity on growth could be linked to changes in activity we found. Indeed, larvae exposed to higher salinity have been shown to express reduced activity and foraging behaviors (Hall et al. 2017; Tornabene et al. 2021a), lower responses to stimuli (Sanzo and Hecnar 2006; Karraker 2007), lower swimming performance (Haramura 2016), erratic movements (Tornabene et al. 2021a), and reduced speed and distance (Denoël et al. 2010).

Importantly, if individuals exposed to freshwater during larval development after being exposed to brackish water during their embryonic development (FSF and SSF) were smaller compared to those that were exposed to freshwater during most of their development (FFF and SFF), they expressed a higher growth rate. This result suggest partial compensatory responses, rather than full compensation or over-compensation (Ali et al. 2003). Compensatory growth may act as an adaptive mechanism because it increases the chance of an organism attaining a critical size at a given developmental stage and (i.e., metamorphosis (Wilbur and Collins 1973)), which can ultimately influence fitness (Dmitriev 2011). It is a likely mechanisms in response to osmotic stress in coastal organisms (Hsu et al. 2018), allowing to overcome the negative effects of salinity by expressing catch-up growth when the salt stress ceases. Interestingly, some individuals were able to express compensatory growth, but not all, which may indicate differences in genotype or bacterium composition between individuals that may lead to local adaptation (Albecker et al. 2019, 2021; Gabriel et al. 2021). Importantly, recent research suggests that, although compensatory growth can produce rapid benefits, it is also associated with delayed costs during later stages of development and/or adult life (Metcalf and Monaghan 2001). Such costs include increased risk of predation, decreased resistance to starvation, decreased protein maintenance, and increased muscle lesions, reduction in locomotor performance, reduced age at sexual maturation, and higher telomere attrition rate (Metcalf and Monaghan 2001). Indeed, accelerated growth is known to induce accumulated cellular damages which ultimately may impair fitness later in life (Mangel and Munch 2005), which could thus affect adult amphibians living in coastal environments.

Conclusion

Reproduction and embryonic and larval development in amphibians are all affected by environmental salinity. Salinity in which individuals were spawned or salinity experienced during embryonic development can also carry over during

subsequent developmental stages, highlighting the importance of the environmental conditions during early development on ontogenetic trajectories. Our study focused on a salt-exposed—presumably salt-tolerant—coastal anuran, highlighting that temporal and spatial variations of salinity might outperform local adaptation, potentially threatening coastal populations if salinity happens to increase. Importantly, we also highlighted partial compensation when individuals were transferred to freshwater, which can be a useful response in coastal environments. This partial compensation (which will still depend on the response of each life stage to these fluctuating levels of salinity) might be helpful for individuals in order to express resilience when freshwater become available, and might help to set-up conservation measures. Indeed, our results might indicate that, following a massive salinization, individuals may be transferred to less-impacted adjacent ponds, which might induce survival of the populations given individuals' resilience. This might be applicable by practitioners following a brutal marine submersion, focusing preferentially on embryos, that are shown to be more sensitive. Whether such approach reveals cost-effective in response to ongoing global changes remain to be considered.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11356-024-31886-1>.

Acknowledgements The authors would like to thank all the staff of the Marais d'Yves National Reserve (Thomas Herault, Karine Vennel, Camille Chave, and Alexandra Kratz) for their help capturing amplexant toads, as well as Mathieu Plateau for its help bringing them to the laboratory.

Author contribution LLS and FB have conceptualized the study. LLS, FR, and MJ participated to field prospection. LLS and TB participated to data collection. LLS analyzed the data. LLS and FB wrote the initial draft. All authors have reviewed and edited the manuscript and approved the final version.

Funding Funding was provided by the CNRS, La Rochelle Université, the LPO, the Agence de l'Eau Adour-Garonne, the Conseil Départemental de la Charente-Maritime, the ANR PAMPAS (ANR-18-CE32-0006), the Beauval Nature association, and the Contrat de plan Etat-région Econat.

Data availability Data supporting the findings of this study are made available as a supplementary material.

Declarations

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

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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