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

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28 the laboratory.

29

30 **Abstract**

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

52 **Key-words:** Carry-over effects, Embryonic development, Freshwater, Recovery,
53 Reproductive investment, Tadpoles

54 **1. INTRODUCTION**

55 Salinization of freshwater bodies is strongly increasing over time (Williams 2001).
56 Coastal wetlands are particularly subjected to salinization because of salt water
57 intrusions (Knighton et al. 1991; Visschers et al. 2022), reductions in freshwater flow
58 resulting from dams and reservoirs, droughts and increased abstraction (Reid et al.
59 2019), rising sea levels (Vermeer and Rahmstorf 2009; Church and White 2011; Bakker
60 et al. 2017), and the predicted increase in frequency and intensity of marine surges
61 (Nicholls et al. 1999; McLean et al. 2001; Knutson et al. 2010; Dettinger 2011; Trenberth
62 et al. 2015; IPCC 2022). Due to their particular position between terrestrial and oceanic
63 systems, coastal ecosystems are also naturally submitted to variations of salinity (Xue
64 et al. 2013), and are thus known to experience temporal and spatial heterogeneity of
65 salinity (Estévez et al. 2019; Ranjbar and Ehteshami 2019; Fu et al. 2021). Importantly,
66 salinity can increase through time, but can also decrease when precipitations increase
67 (Lorrain-Soligon et al. 2023c). However, the impacts of these short term variations of
68 salinity on wildlife remain poorly understood.

69

70 Salinization is expected to strongly affect coastal biodiversity (Amores et al. 2013;
71 Debue et al. 2022). Indeed, most organisms need to maintain their internal homeostasis
72 and rely on physiological compensatory mechanisms to regulate water and ion fluxes
73 (Schultz and McCormick 2012; Evans and Kültz 2020). Most species have specific
74 ranges of tolerance, and crossing these limits of tolerance activates mechanisms that
75 aim at regulating ionic and hydric fluxes (Evans 2009). These mechanisms include
76 modulation of drinking and urination rates, regulation of the permeability of

77 membranes to water, remodeling of cells and tissues, and increased expression and
78 activity of ion transporters (Evans 2009; Kültz 2015; Rivera-Ingraham and Lignot 2017).
79 However, these mechanisms are often metabolically costly and the associated
80 energetic expenditures can affect energetic allocation to other functions (Herbert et al.
81 2015; Rivera-Ingraham and Lignot 2017). As such, salinity can influence critical
82 functions such as foraging (Chuang et al. 2022), activity (Leite et al. 2022;
83 Moniruzzaman et al. 2022), anti-predator responses (Hoover et al. 2013) and growth
84 (Liu et al. 2022). In addition, elevated salinity can also increase the occurrence of
85 malformations (Hieu et al. 2021), potentially leading to increased mortality (Cañedo-
86 Argüelles et al. 2013; Moniruzzaman et al. 2022; Woodley et al. 2023). In adults,
87 increased salinity can reduce egg production and fecundity (Pinder et al. 2005;
88 Froneman 2023; Woodley et al. 2023), egg size (Lorrain-Soligon et al. 2023a), or the age
89 at first reproduction (Woodley et al. 2023). In addition, increasing salinity is known to
90 reduce sperm performance, motility and velocity (Wilder and Welch 2014; Byrne et al.
91 2015, 2022; Green et al. 2021). Ultimately, salinization can alter community structure
92 (Hart et al. 2003; Anufrieva and Shadrin 2018), across all trophic levels (Hintz and
93 Relyea 2019).

94

95 This is especially true for coastal amphibians. Indeed, amphibians are particularly
96 sensitive to salinity due to their highly permeable skin used for gas exchanges, and for
97 ions and water transport (Shoemaker and Nagy 1984; Martin and Nagy 1997), and to
98 their relatively low osmoregulatory abilities (Katz 1989). Early life stages (i.e. eggs and
99 larvae) are particularly susceptible to environmental salinity (Albecker and McCoy

100 2017) because of their strong dependency on aquatic environment compared to adults
101 (Wells 2007) and to their comparatively lower osmoregulatory abilities (Karraker and
102 Gibbs 2011). Increasing salinity from various sources can lead to reduced thyroid
103 hormones levels (Gomez-Mestre et al. 2004) and to changes in osmoregulatory
104 hormones such as corticosterone and aldosterone (Tornabene et al. 2021b, 2022) in
105 larval amphibians. Ultimately, this lead to decreased embryonic and larval survival
106 (Christy and Dickman 2002; Chinathamby et al. 2006; Dougherty and Smith 2006;
107 Sanzo and Hecnar 2006; Karraker et al. 2008; Bernabò et al. 2013; Jones et al. 2015), and
108 to increased malformations (Gosner and Black 1957; Sanzo and Hecnar 2006;
109 Haramura 2007; Karraker 2007; Hopkins et al. 2013; Brady 2013; Tornabene et al.
110 2021a), time to hatching (Haramura 2016; Tornabene et al. 2021a), hatching success
111 (Tornabene et al. 2021a), larval duration (Christy and Dickman 2002; Gomez-Mestre
112 and Tejedo 2003; Lukens and Wilcoxon 2020; Tornabene et al. 2021a), and to reduced
113 growth and thus to induce smaller body size (Wu and Kam 2009; Bernabò et al. 2013;
114 Wood and Welch 2015; Haramura 2016; Lukens and Wilcoxon 2020; Tornabene et al.
115 2021a, b).

116

117 Despite their sensitivity, amphibians are known to be very plastic in behavioral and
118 morphological traits (Hoverman and Relyea 2008), and their developmental traits can
119 vary in response to environmental cues (Chivers et al. 2001; Warkentin 2011). For
120 instance, tadpoles can modulate their growth rates according to the quality of
121 environmental conditions (Werner 1986; Vonesh and Warkentin 2006). Additionally,
122 it has been suggested that the influence of a given environmental factor on

123 development and growth of tadpoles is highly dependent on the stage and condition
124 during which they experience such factor (Denver et al. 2002). This can be highly
125 relevant for changes in salinity. For example, *Fejervarya limnocharis* and *Litoria ewingii*
126 tadpoles released from salinity stress at early larval stages reached a size at
127 metamorphosis similar to that of tadpoles maintained in low salinity throughout
128 development, indicating compensatory growth (Squires et al. 2010; Wu et al. 2012).
129 Yet, the efficiency of such compensatory mechanisms may vary if individuals are
130 exposed earlier or later during larval development (Hsu et al. 2018). Additionally,
131 whether parents can influence the tolerance of their offspring according to the salinity
132 in which they are laying eggs is unknown.

133

134 In order to investigate the effect of salinity during egg-laying and embryonic and larval
135 development of coastal amphibians, we used a full-factorial experimental design to
136 expose reproductive adults, eggs and larvae of coastal spined toads (*Bufo spinosus*, a
137 species which coastal individuals are supposedly adapted to exposure to salinity) to
138 fresh (0 g.l⁻¹) or brackish water (4 g.l⁻¹, Figure 1). More specifically, reproductive pairs
139 (amplexus) were exposed to fresh or brackish water prior to egg-laying (Figure 1).
140 Following egg laying, we exposed eggs to either fresh or brackish water until hatching
141 (Figure 1). Following hatching, we exposed larvae to either fresh or brackish water
142 (Figure 1). At egg laying, we evaluated fecundity and parental investment in
143 reproduction. During both embryonic and larval development, we assessed effects of
144 each experimental treatment on survival (hatching rate, larval survival), development
145 (duration of embryonic and larval development until Gosner stage 30 [Gosner 1960],

146 malformation), and growth (body length). We predicted that (1) Brackish water would
147 impact reproductive investment and egg size, and alter fecundity because salinity can
148 impact sperm motility (Byrne et al. 2015, 2022). (2) Salt exposure would decrease
149 embryonic survival, larval survival and growth (Albecker and McCoy 2017; Lukens
150 and Wilcoxon 2020; Tornabene et al. 2021a) as well as malformations (Gosner and Black
151 1957; Sanzo and Hecnar 2006; Haramura 2007; Karraker 2007; Hopkins et al. 2013;
152 Brady 2013; Tornabene et al. 2021a), regardless of the stage of exposure. These effects
153 should be alleviated in individuals transferred to freshwater, because of the resilience
154 capacities of amphibians to salt exposure (Squires et al. 2010; Wu et al. 2012; Hsu et al.
155 2018; Lorrain-Soligon et al. 2022).

156 **2. METHODS**

157 **2.1. Study species**

158 Spined toads (*Bufo spinosus*) are a common amphibian species found in western Europe
159 (Speybroeck et al. 2018; Meek 2022). Breeding occurs in late winter, during which
160 adults converge to reproductive ponds where they mate (amplexus) and lay their eggs
161 constituted of 2 eggs strings which can be constituted of up to 10000 eggs (Miaud and
162 Muratet 2018). Egg laying, embryonic and larval developments all occur in ponds.

163

164 **2.2. Captures and measurements**

165 Amplectant pairs were captured on one coastal pond (salinity 3.1 g.l⁻¹ during captures,
166 conductimeter YSI Professional Plus) situated in the Réserve Naturelle Nationale du
167 Marais d'Yves (location: 46.04013, -1.05363). This site can display seasonal variations
168 of salinity ranging from 0.5 g.l⁻¹ to 6 g.l⁻¹, as what is found in nearby ponds. Captures
169 occurred at night (between 20 pm and 4 am) on 21/02/2023 and 22/02/2023. Twenty
170 one amplectant pairs were captured by hand, placed in a transport box (14*16*9cm),
171 and brought to the laboratory immediately after field sessions.

172 At the laboratory, individuals were kept in a thermally controlled room with
173 temperature set at 17 °C and controlled photoperiod (12 h dark-12 h light),
174 temperature and photoperiod being set for the totality of the experiment. Pairs were
175 transitorily (for measurements only) separated and all individuals were measured
176 (snout-vent length, SVL) using a caliper (\pm 1 mm) and weighed using an electronic
177 balance (\pm 0.1g). Once measurements were completed, separated amplectant

178 individuals were shortly reunited and pairs re-formed systematically as soon as the
179 partners were brought back into contact.

180

181 **2.3. Experimental procedures**

182 Amplectant pairs, embryos and larvae were exposed to either freshwater or brackish
183 water (sea salt, NaCl), following a full factorial design (Figure 1). At the end of the
184 experiment, larval individuals were thus exposed to one of the eight following
185 treatments: (1) FFF: clutch obtained in freshwater, embryonic development in
186 freshwater, larval development in freshwater; (2) FSF: clutch obtained in freshwater,
187 embryonic development in brackish water, larval development in freshwater; (3) FFS:
188 clutch obtained in freshwater, embryonic development in freshwater, larval
189 development in brackish water; (4) FSS: clutch obtained in freshwater, embryonic
190 development in brackish water, larval development in brackish water; (5) SFF: clutch
191 obtained in brackish water, embryonic development in freshwater, larval
192 development in freshwater; (6) SSF: clutch obtained in brackish water, embryonic
193 development in brackish water, larval development in freshwater; (7) SFS: clutch
194 obtained in brackish water, embryonic development in freshwater, larval
195 development in brackish water; (8) SSS: clutch obtained in brackish water, embryonic
196 development in brackish water, larval development in brackish water. Water of these
197 treatments was changed every seven days.

198

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

202

203 2.3.1. Egg laying (n=21)

204 Once the amplexant pairs were reunited, they were placed in a plastic container
205 (35*55*26 cm) containing either freshwater (~20L, salinity: 0.3 g.l⁻¹, 10 amplexant pairs)
206 or brackish water (~20L, salinity: 4.0 g.l⁻¹, 11 amplexant pairs) as well as branches for
207 laying support. The two groups did not differ in size (freshwater: females= 74.7 mm ±
208 1.61 SE, males= 64.1 mm ± 1.00 SE; brackish water: females= 75.9 mm ± 2.08 SE, males=
209 62.7 mm ± 1.22 SE; differences between females in the two groups: Estimate=1.209,
210 SE=2.604, t_{1,19}=0.464, p-value=0.648; differences between males in the two groups:
211 Estimate=1.373, SE=1.601, t_{1,19}=0.857, p-value=0.402). Amplexant pairs were left in
212 these tanks until egg laying. Individuals were checked every two hours to compute
213 time to egg laying (measured from their placement in a plastic container to the
214 completion of egg laying). Adult individuals were then released at their site of capture.
215 Adults were thus kept under laboratory conditions for a maximum of seven days
216 (maximal time to egg laying 151 hours), and the water of their treatment was left
217 unchanged during this duration. They were unfed during this period, as adults
218 anurans usually don't feed during the reproduction. Twelve pieces of each egg strings,
219 containing 30 eggs each (i.e. a total of 360 eggs for each egg string) were selected
220 randomly and kept for our experiment, and the remaining eggs (i.e. 100– 4500) were
221 released at the site of origin of their parents.

222

223 *2.3.2. Embryonic development (n=232)*

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

237

238 *2.3.3. Larval development (n=232)*

239 Upon hatching (GS25), we selected 1 individual per segment (randomly, among alive
240 and not malformed tadpoles of each segment), and these individuals were once again
241 distributed in two treatments: freshwater (0 g.l⁻¹ salinity: 0.3 ± 0.0 g.l⁻¹), and brackish
242 water (4 g.l⁻¹ salinity 4.0 ± 0.06 g.l⁻¹). For each clutch, three segments and thus three
243 tadpoles were thus exposed to each treatment (FFF, FFS, FSF, FSS, SFF, SFS, SSF, SSS).
244 In some segments, no individuals survived until GS25. In these cases, individuals were

245 replaced by other individuals from the same treatments but originating from different
246 clutches in order to keep a total of 232 tadpoles that were followed during larval
247 development (see table 1 for the count of tadpoles in each treatment, and Appendix A
248 for the contribution of each clutch). All other tadpoles were released at the site where
249 their parents were captured.

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

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

262

263 **2.4. Measurements**

264 *2.4.1. Reproductive effort (n=21)*

265 Once egg laying was completed, individuals were again weighed to calculate
266 variations in body mass. Additionally, each clutch was weighed using an electronic
267 balance ($\pm 0.1g$). Moreover, in order to assess fecundity, each egg string was placed in

268 a container (35 × 20 × 25 cm) containing 2 cm of dechlorinated tap water and a scale
269 (graph paper). A picture was taken from above in order to measure the total length of
270 the egg string using ImageJ software (Schneider et al. 2012). For each clutch, we
271 randomly selected six 10-cm segments and individually counted the number of eggs
272 within each segment. The mean number of eggs per 10-cm segment was calculated and
273 used to assess fecundity (number of eggs) for each clutch based on the length of the
274 egg strings. Finally, on a subsample of 100 randomly selected eggs in each egg strings,
275 we measured egg diameter.

276

277 *2.4.2. Embryonic development (n=232)*

278 At GS 25, hatching success (proportion of eggs that hatched, on the segment of 30 eggs)
279 and deformation rates (proportion of larvae that hatched but were malformed, on the
280 total number of hatched individuals) were calculated. Additionally, for each segments,
281 we described general activity of all hatchlings as being, on a scale of 1 to 5, the
282 proportion of live individuals that were active; 1=0-20%, 2=20-40%, 3=40-60%, 4=60-
283 80%, 5=80-100%) over a 1-min period.

284

285 *2.4.3. Larval development (n=232)*

286 At GS25 and GS30, we measured total length of individuals. Each tadpole was put into
287 a Petri dish with the water from its own tank, and photographed. Individuals were
288 than measured using the software ImageJ (Schneider et al. 2012). We also measured
289 activity following a scale ranging from 1 to 5 (from 1 if the individual is amorphous,

290 to 5 if the individual move energetically, following Lorrain-Soligon et al. [2022]). The
291 tadpoles were not stimulated to record these observations.

292

293

294 **2.5. Statistical analyses**

295 We computed Linear Models (LMs) or Linear Mixed Models (LMMs) and Generalized
296 Linear Mixed Models (GLMMs) using the lme4 (Bates et al. 2015) and lmerTest
297 (Kunzetsova et al. 2017) packages. For all test computed, models accuracy was tested
298 using the check_model function from the performance package (Lüdecke et al. 2020).
299 When models did not fit the data, the response variable was log+1 transformed.
300 Binomials models accuracy was also tested by checking overdispersion. Significance
301 level was set at 0.05, differences at a p-value level <0.1 were considered marginal. For
302 all comparisons studied, mean \pm SE of each group are given in Appendix B.

303

304 **2.5.1. Reproductive effort**

305 We evaluated the effect of salinity (clutches laid in freshwater [0 g.l⁻¹] or in brackish
306 water [4 g.l⁻¹]) on clutch length, clutch size (number of eggs), clutch mass, egg
307 diameter, laying time, variation of body mass (for females and males). For these
308 analyses, we set linear models (LMs), with salinity as an explanatory variable. We also
309 tested for the relationships between egg diameter and clutch size in each salinity by
310 setting a LM with egg diameter as a dependent variable, and clutch size, salinity and
311 their interaction as explanatory variables. Finally, we tested for the effect of the
312 interaction between female mass and salinity on clutch size and clutch mass, using

313 LMs. For these models, best variables were retained using a top-down selection (using
314 p-values), and only the retained variables are presented.

315

316 *2.5.2. Embryonic development*

317 We evaluated the effect of salinity during spawning (clutch laid in freshwater [0 g.l⁻¹]
318 or in brackish water [4 g.l⁻¹]), and during embryonic development (individuals
319 developing in freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]).

320

321 We computed these effects on time to hatching (log+1 transformed) and individuals
322 total size with Linear Mixed models (LMMs), and on hatching success, malformation
323 rate, general activity and tadpole activity (both rated on a scale from 1 to 5) using
324 binomial Generalized Linear Mixed Models (GLMMS). For all these models, we
325 included salinity during spawning, salinity during embryonic development and their
326 interaction as explanatory variables, and the clutch as a random effect (as multiple
327 individuals originated from the same clutch). Best variables were retained using a top-
328 down selection (using p-values), and only the retained variables are presented.

329

330 *2.5.3. Larval development*

331 We evaluated the effect of salinity at different stages of exposure. We computed the
332 effect of salinity during spawning (clutches laid in freshwater [0 g.l⁻¹] or in brackish
333 water [4 g.l⁻¹]), salinity during embryonic development (eggs developing in freshwater
334 [0 g.l⁻¹] or brackish water [4 g.l⁻¹]), and salinity during larval development (individuals

335 developing in freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]). We computed these effects
336 on development duration between S25 and S30, growth rate between GS25 and GS30,
337 and total size (log+1 transformed) at GS30 with LMMs, and on mortality and activity
338 (rated on a scale from 1 to 5) using binomial GLMMs. For all these models, we
339 included salinity during spawning, salinity during embryonic development, salinity
340 during larval development and their interaction as explanatory variables, and the
341 clutch as a random effect (as multiple individuals originated from the same clutch).
342 Best variables were retained using a top-down selection (using p-values), and only the
343 retained variables are presented. At GS30, only 2 individuals were malformed (in FFS
344 and FSF) and thus statistics were not computed. All data analysis were performed
345 using R 3.6.3 (R Core Team 2020) and Rstudio v1.1.419.

346 **3. RESULTS**

347 **3.1. Reproductive effort**

348 Clutches produced in freshwater or in brackish water were similar for length
349 (Estimate=70.05, SE= 263.58, $t_{1,19}=0.27$, p-value=0.793) and number of eggs
350 (Estimate=182.60, SE= 405.40, $t_{1,19}=0.45$, p-value=0.658). However, clutches produced
351 in freshwater were heavier (Estimate=28.21, SE= 13.07, $t_{1,19}=2.16$, p-value=0.044), and
352 their eggs were marginally larger (Estimate=0.11, SE= 0.06, $t_{1,19}=1.80$, p-value=0.088,
353 Figure 2A). Laying time was shorter for individuals that laid eggs in brackish water
354 compared to those that laid eggs in freshwater (Estimate=-31.77, SE= 14.84, $t_{1,19}=-2.14$,
355 p-value=0.046, Figure 2B).

356

357 Egg diameter was related to the interaction between clutch size and salinity
358 (Estimate<0.001, SE<0.001, $t_{3,17}=2.65$, p-value=0.017, Figure 2C). Egg diameter was not
359 influenced by clutch size in clutches laid in freshwater (Estimate<0.001, SE<0.001,
360 $t_{1,8}=1.21$, p-value=0.260, Figure 2C), but increased with increasing clutch size in
361 clutches laid in brackish water (Estimate<0.001, SE<0.001, $t_{1,9}=2.40$, p-value=0.040,
362 Figure 2C).

363

364 Interestingly, females spawning at 0 g.l⁻¹ (-13.56 % ± 2.23 SE) and 4 g.l⁻¹ (-12.21 % ± 1.36
365 SE) lost similar mass during egg laying (Estimate=1.35, SE=2.56, $t_{1,19}=0.53$, p-
366 value=0.604). Males reproducing at 0 g.l⁻¹ lost mass (-1.63 % ± 2.19 SE) while males
367 reproducing at 4 g.l⁻¹ gained mass (3.79% ± 1.27 SE) during egg laying (Estimate=5.41,
368 SE=2.47, $t_{1,19}=2.19$, p-value=0.041).

369

370 Clutch size increased with female mass (Estimate=40.876, SE=10.303, $t_{1.19}=3.967$, p-
371 value<0.001). When testing for the interaction between females body mass and
372 salinity, clutch mass increased with female body mass (Estimate=1.211, SE=0.362,
373 $t_{1.19}=3.347$, p-value=0.004) and was higher in the freshwater treatment compared to the
374 brackish one (Estimate=25.192, SE=10.579, $t_{1.19}=2.831$, p-value=0.029).

375

376 *3.2. Embryonic development*

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

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

391 Time to hatching was higher in embryos which developed in brackish water
392 (Estimate=0.107, SE=0.013, $t_{1.221}=8.387$, p-value<0.001), but not related to spawning in

393 brackish water (Estimate=0.004, SE=0.025, $t_{1.27}=0.168$, p-value=0.868). There was an
394 interaction between the salinity during spawning and the salinity during embryonic
395 development (longer time to hatching for individuals that were laid in freshwater but
396 developed in brackish water, Estimate=-0.065, SE=0.021, $t_{1.218}=-3.104$, p-value=0.002,
397 Appendix C).

398 General activity (Estimate=1.164, SE=0.173, $z=6.735$, p-value<0.001), tadpole activity
399 (Estimate=1.141, SE=0.117, $z=9.706$, p-value<0.001) and total length (Estimate=-2.157,
400 SE=0.0.139, $t_{1.228}=-15.540$, p-value<0.001, Figure 3C) were all influenced by salinity
401 experienced during embryonic development solely, and were higher for embryos that
402 developed in freshwater.

403

404

405 ***3.3. Larval development***

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

412 Development duration between S25 and S30 varied with salinity during embryonic
413 development (longer for individuals which embryonic development occurred in
414 brackish water [particularly FSS, SSF and SSS], Estimate=1.409, SE=0.635, $t_{1.101}=2.218$,

415 p-value=0.029, Figure 4B), and there was an interaction between salinity during
416 embryonic development and salinity during larval development (Estimate=3.307,
417 SE=1.095, $t_{1,91}=3.020$, p-value=0.003, Figure 4B, Appendix D), as well as between
418 salinity during spawning and salinity during larval development (Estimate=-2.914,
419 SE=0.768, $t_{1,94}=-3.793$, p-value<0.001, Figure 4B, Appendix D).

420 Growth rate between GS25 and GS30 was only influenced by salinity during
421 embryonic development, and was higher for individuals which embryonic
422 development occurred in brackish water (particularly FSF for individuals laid in
423 freshwater, and SSF and SSS for individuals laid in brackishwater, Estimate=0.081,
424 SE=0.036, $t_{1,110}=2.240$, p-value=0.027, Figure 4C).

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

431 4. DISCUSSION

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

439

440 *4.1. Reproduction*

441 Clutch size did not differ between freshwater and brackish water treatment, and clutch
442 size increased with female size and female mass loss for both treatments, with no
443 difference between the two treatments. Such results further suggest that clutch size
444 might depend on energetic investment that occurred prior to egg laying (Sinervo and
445 DeNardo 1996; Nilsson and Svensson 1997; Lorrain-Soligon et al. 2023a). Interestingly,
446 we found that salinity influenced some reproductive parameters in adults, suggesting
447 a rapid modulation of reproductive effort based on the salinity to which reproductive
448 adults were exposed. Indeed, reproductive adults exposed to brackish water laid their
449 eggs more rapidly than those kept in fresh water. Two opposing hypotheses can
450 explain such result. First, females may lay their eggs more rapidly in brackish water
451 in order to evade an osmotically constraining environment. Conversely, because adult
452 individuals originated from salt-exposed coastal environment, they may consider
453 brackish water as a favourable environment if they are locally adapted to such

454 conditions and if there are less competitors in this environment (Hopkins and Brodie
455 2015; Lorrain-Soligon et al. 2023b); but delay laying in freshwater because they
456 consider this environment as suboptimal. This later hypothesis seems relatively
457 unlikely as our first hypothesis is further supported by the fact that, in brackish water,
458 eggs were smaller but not fewer. This result suggests that females produced eggs of
459 comparatively lower quality in brackish water, as eggs produced in the freshwater
460 treatment are marginally larger, and larger eggs often have higher hatching success
461 and offspring fitness (Xu et al. 2019; Renoirt et al. 2022). The number of undeveloped
462 eggs (presumably unfertilized given the negative effect of salinity on sperm, (Wilder
463 and Welch 2014; Green et al. 2021)) was also higher in brackish water.

464

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

476 investigated through the evaluation of metabolic rates in reproducing adults exposed
477 to different salinities.

478

479 The fact that clutch mass was higher in the freshwater treatment is likely linked to
480 water absorption due to osmotic exchanges with the environment (Venturino et al.
481 2003; Karraker and Gibbs 2011; López-Alcaide and Macip-Ríos 2011), or due to higher
482 investment in glycoproteins surrounding the eggs (i.e. egg jellies, see above). More
483 importantly, we found a positive correlation between egg size and clutch size for
484 clutches laid in brackish water but not for clutches laid in freshwater, while fecundity
485 was expected to negatively trade off with egg size (Lasne et al., 2018; Smith & Fretwell,
486 1974). Based on the fractional egg hypothesis (Ricklefs 1968; Nussbaum 1981; Ford and
487 Seigel 2010), the negative relation between fecundity and egg size is expected because
488 species with large clutch sizes and small eggs (typical of Bufonid toads, Wells, 2007)
489 should add additional offspring should extra energy become available (Ricklefs 1968;
490 Nussbaum 1981). In our study, we found that increased clutch size is concomitant
491 with increased egg size in clutches laid in brackish water suggesting that complex
492 interactions between prior energetic investment in reproduction and current spawning
493 conditions govern reproductive effort.

494

495 Lastly, reproducing males have lost mass in freshwater, but gained mass in brackish
496 water. Such result could be linked to reduced ejaculate volume, with males in
497 freshwater producing more sperm than their counterparts exposed to brackish water.

498 This hypothesis seems corroborated by the reduced proportion of fertilized eggs in the
499 brackish treatment. However, we did not evaluate sperm volume in each treatment,
500 which might need to be investigated. However, this gain of mass in males can also be
501 linked to the fact that they can be osmotically imbalanced (Hall et al. 2020; Tornabene
502 et al. 2021b).

503

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

512

513 *4.2. Embryonic development*

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

520 hatching, individuals in the 4 g.l⁻¹ treatment were smaller (irrespective of the salinity
521 in which they were spawned) (Wu and Kam 2009; Wood and Welch 2015; Haramura
522 2016; Lukens and Wilcoxon 2020; Tornabene et al. 2021a), which is probably linked to
523 higher costs of osmoregulation (Gomez-Mestre et al. 2004). Individuals in the 4 g.l⁻¹
524 treatment also presented lower activity. Interestingly, hatching success was always
525 lower for individuals originating from a clutch that was spawned in brackish water
526 irrespective of the salinity in which the remaining embryonic development occurred.
527 Additionally, individuals that were laid in freshwater but developed in brackish water
528 had a higher malformation rate compared to individuals in any other treatments. As
529 individuals developing in the SF and SS groups (that were laid in brackish water)
530 presented a higher mortality rate, this could indicate selective mortality affecting
531 individuals susceptible to salinity, while more tolerant - surviving - individuals
532 displayed lower malformation rates. This hypothesis is further supported by the fact
533 that higher malformation rates were found in individuals spawned in freshwater that
534 developed in brackish water (Gosner and Black 1957; Hopkins et al. 2013). Taken
535 together, these results indicate long lasting effects of the salinity in which the clutch
536 was produced (and thus long-lasting effect of very early exposure to salinity during
537 embryonic development). Our results thus emphasize that embryos are sensitive to
538 change in salinity during their development, which can occur through changes of
539 temperatures and precipitations in coastal environments (Lorrain-Soligon et al. 2023c),
540 which may carry-over during later larval development and potentially during post-
541 metamorphic stages (Traversari 2021; Dahrouge and Rittenhouse 2022), affecting
542 overall amphibian populations.

543

544 *4.3. Larval development*

545 Overall, development duration was longer for individuals that were spawned in
546 brackish water, indicating long lasting, carry-over effects of the salinity in which adults
547 lay eggs, and accumulating effects during larval development (see Tornabene et al.
548 2021a). Individuals that experienced salinity during either embryonic or larval
549 development (FSF, FFS, SSF and SFS) or during both stages (FSS and SSS) expressed
550 higher mortality (see also Squires et al. 2010; Wu et al. 2012; Albecker and McCoy 2017;
551 Hsu et al. 2018). Additionally, individuals that developed in brackish water during
552 embryonic development were smaller (see also Squires et al. 2010), while there was no
553 effects of salinity experienced during larval development on total length. These
554 responses remain consistent regardless of the salinity in which clutches were spawned.
555 This further suggest that salinity experienced during embryonic development is more
556 detrimental than salinity experienced during larval development, which is in line with
557 the idea that early developmental stages (embryos) are more sensitive to salinity than
558 later developmental stages (larvae) (Albecker and McCoy 2017). This is likely due to
559 the development of internal gills (the main organs responsible for ion and water
560 balance in tadpoles, Uchiyama and Yoshizawa 1992) which improve the
561 osmoregulation ability at this developmental stage. However, in the context of
562 accumulating effects, larvae can be found to be more sensitive than embryos
563 (Tornabene et al. 2021a), which highlight that this effect might be context dependent.

564

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

571

572

573 Importantly, if individuals exposed to freshwater during larval development after
574 being exposed to brackish water during their embryonic development (FSF and SSF),
575 were smaller compared to those that were exposed to freshwater during most of their
576 development (FFF and SFF), they expressed a higher growth rate. This result suggest
577 partial compensatory responses, rather than full compensation or over-compensation
578 (Ali et al. 2003). Compensatory growth may act as an adaptive mechanism because it
579 increases the chance of an organism attaining a critical size at a given developmental
580 stage and (i.e., metamorphosis, Wilbur and Collins 1973), which can ultimately
581 influence fitness (Dmitriew 2011). It is a likely mechanisms in response to osmotic
582 stress in coastal organisms (Hsu et al. 2018), allowing to overcome the negative effects
583 of salinity by expressing catch-up growth when the salt stress ceases. Interestingly,
584 some individuals were able to express compensatory growth, but not all, which may
585 indicate differences in genotype or bacterium composition between individuals that
586 may lead to local adaptation (Albecker et al. 2019, 2021; Gabriel et al. 2021).

587 Importantly, recent research suggests that, although compensatory growth can
588 produce rapid benefits, it is also associated with delayed costs during later stages of
589 development and/or adult life (Metcalfe and Monaghan 2001). Such costs include
590 increased risk of predation, decreased resistance to starvation, decreased protein
591 maintenance and increased muscle lesions, reduction in locomotor performance,
592 reduced age at sexual maturation and higher telomere attrition rate (Metcalfe and
593 Monaghan 2001). Indeed, accelerated growth is known to induce accumulated cellular
594 damages which ultimately may impair fitness later in life (Mangel and Munch 2005),
595 which could thus affect adults amphibians living in coastal environments

596

597 **5. CONCLUSION**

598 Reproduction, embryonic and larval development in amphibians are all affected by
599 environmental salinity. Salinity in which individuals were spawned or salinity
600 experienced during embryonic development can also carry-over during subsequent
601 developmental stages, highlighting the importance of the environmental conditions
602 during early development on ontogenetic trajectories. Our study focused on a salt-
603 exposed – presumably salt tolerant -coastal anuran, highlighting that temporal and
604 spatial variations of salinity might outperform local adaptation, potentially
605 threatening coastal populations if salinity happens to increase. Importantly, we also
606 highlighted partial compensation when individuals were transferred to freshwater,
607 which can be a useful response in coastal environments. This partial compensation
608 (which will still depend on the response of each life stage to these fluctuating levels of
609 salinity) might be helpful for individuals in order to express resilience when

610 freshwater become available, and might help to set-up conservation measures. Indeed,
611 our results might indicate that, following a massive salinization, individuals may be
612 transferred to less impacted adjacent ponds, which might induce survival of the
613 populations given individuals' resilience. This might be applicable by practitioners
614 following a brutal marine submersion, focusing preferentially on embryos, that are
615 shown to be more sensitive. Whether such approach reveals cost-effective in response
616 to ongoing global changes remain to be considered.

617

618

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- 1007

1008 **Statements & Declarations**

1009 **Ethical approval**

1010 This work was approved by the French authorities under permits

1011 DREAL/2020D/8041 (animals capture) and APAFIS #33592-2021102610033982

1012 (animals husbandry).

1013

1014 **Consent to participate**

1015 Not applicable.

1016

1017 **Consent to publish**

1018 Not applicable.

1019

1020 **Authors contribution**

1021 LLS and FB have conceptualized the study. LLS, FR and MJ participated to field

1022 prospection. LLS and TB participated to data collection. LLS analyzed the data. LLS

1023 and FB wrote the initial draft. All authors have reviewed and edited the manuscript

1024 and approved the final version.

1025

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1030 plan Etat-région Econat.

1031

1032 **Competing interest**

1033 The authors declare that they have no conflict of interest.

1034

1035 **Data archiving statement**

1036 Data supporting the findings of this study are made available as a supplementary
1037 material.

1038

1039

1040 Caption to figures

1041 Figure 1 – Experimental design used to assess the effects of salinity during different
1042 stages of development in *Bufo spinosus*. Colours represent salinity treatments (light
1043 blue = fresh water [0 g.l⁻¹], dark blue = brackish water [4 g.l⁻¹]).

1044

1045 Figure 2 – Effects of salinity during spawning on reproductive parameters. (A) Egg
1046 diameter, (B) clutch size (number of eggs), (C) relationships between clutch size and
1047 egg diameter, for clutches produced in freshwater (0 g.l⁻¹) or brackish water (4 g.l⁻¹).
1048 Data are presented as mean ± SE. See Figure 1 for sample size.

1049

1050 Figure 3 – Effects of salinity during spawning and embryonic development on
1051 different aspects of embryonic development assessed upon hatching (Gosner stage 25).
1052 (A) Hatching success, (B) malformation rate, and (C) total length (mm). FF: spawning
1053 in freshwater [0 g.l⁻¹], embryonic development in freshwater; FS: spawning in
1054 freshwater, embryo in brackish water [4 g.l⁻¹]; SF: spawning in brackish water, embryo
1055 in freshwater; SS: spawning in brackish water, embryo in brackish water. Data are
1056 presented as mean ± SE. See Figure 1 for sample size.

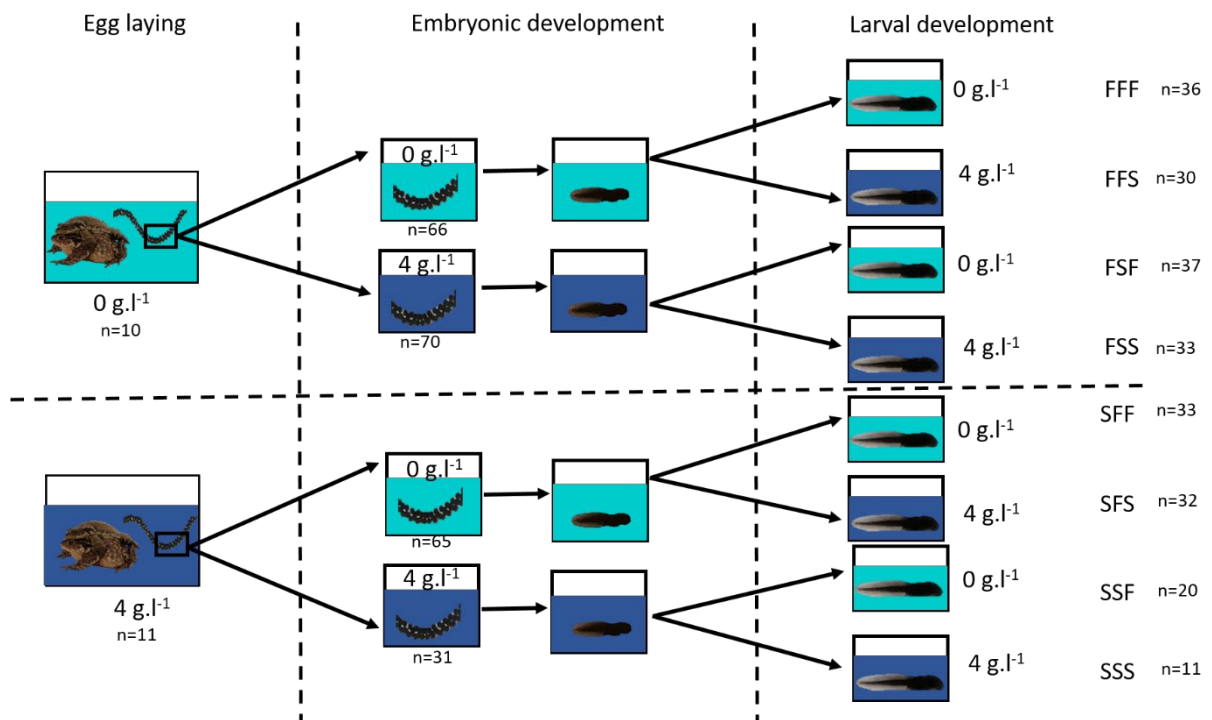
1057

1058 Figure 4 – Effects of salinity during spawning, embryonic development and larval
1059 development on different parameters of larval development measured at Gosner stage
1060 30. (A) Mortality rate, (B) duration between GS25 and GS30, (C) growth between GS25
1061 and GS30, and (D) total length (mm). FFF: spawning in freshwater [0 g.l⁻¹], embryonic
1062 development in freshwater, larval development in freshwater; FSF: spawning in
1063 freshwater, embryo in brackish water [4 g.l⁻¹], larvae in freshwater; FFS: spawning in
1064 freshwater, embryo in freshwater, larvae in brackish water; FSS: spawning in
1065 freshwater, embryo in brackish water, larvae in brackish water; SFF: spawning in
1066 brackish water, embryo in freshwater, larvae in freshwater; SSF: spawning in brackish
1067 water, embryo in brackish water, larvae in freshwater; SFS: spawning in brackish
1068 water, embryo in freshwater, larvae in brackish water; SSS: spawning in brackish
1069 water, embryo in brackish water, larvae in brackish water. Data are presented as mean
1070 ± SE. Treatments and sample size are illustrated in Figure 1.

1071

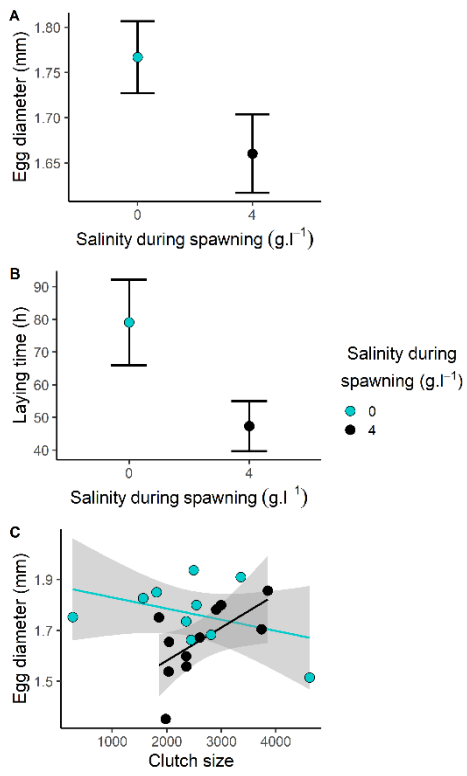
1072

1073 Figure 1



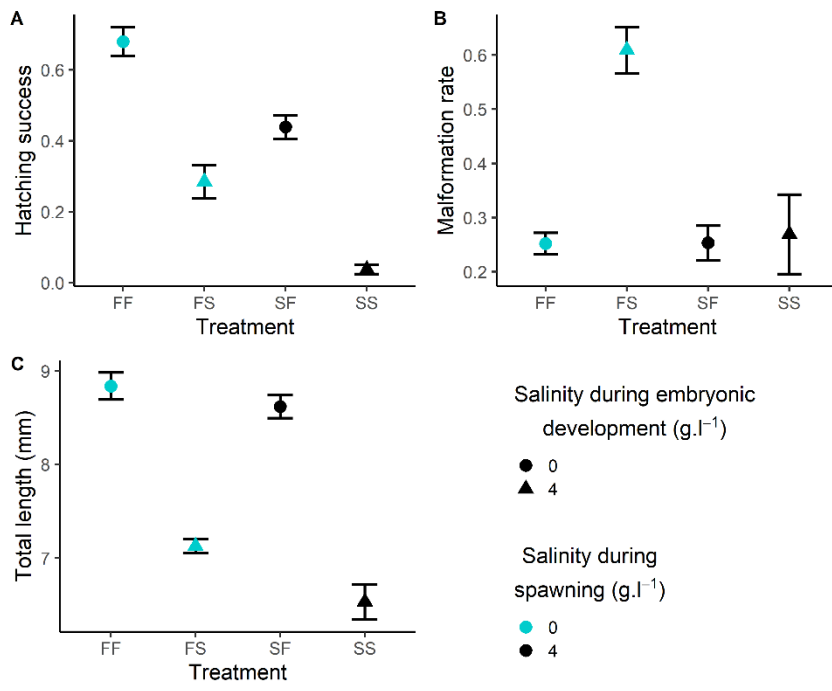
1074

1075 Figure 2

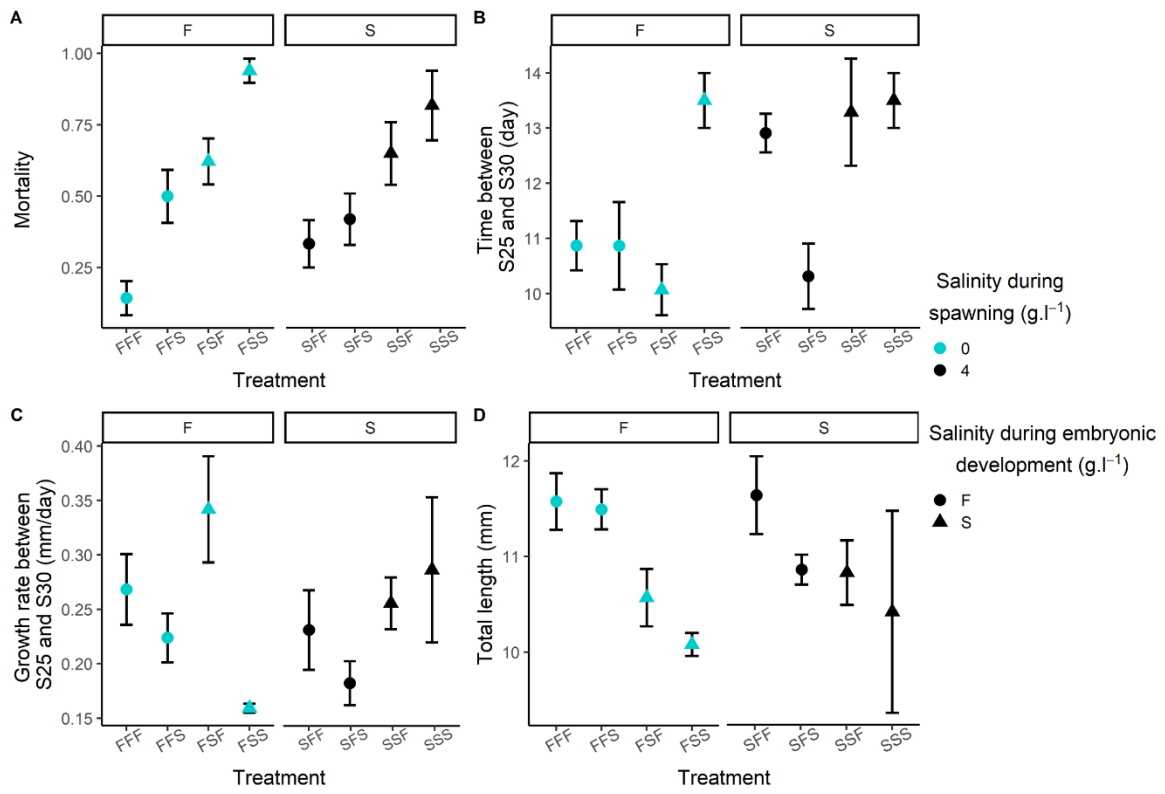


1076

1077 Figure 3



1078



1081 Caption to tables

1082 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

1083

1084 **Caption to Appendices**

1085 Appendix A - Contributions of clutches to each treatment (number of tadpoles
1086 originating from each clutch in each treatment)

1087 Appendix B - Mean \pm SE values for all studied variables and treatments during egg
1088 laying, embryonic development and larval development.

1089 Appendix C - Post-hoc analyses for the differences found at Gosner stage 25 (GS25)
1090 with the interaction between salinity during spawning (clutches laid in freshwater [0
1091 g.l⁻¹] or brackish water [4 g.l⁻¹]) and salinity during embryonic development
1092 (freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]), for hatching success, malformation rate,
1093 and time to hatching.

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1095 Appendix D - Post-hoc analyses for the differences found for duration between Gosner
1096 stage 25 (GS25) and Gosner stage 30 (GS30) with the interaction between salinity
1097 during embryonic development (freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]) and
1098 salinity during larval development (freshwater [0 g.l⁻¹] or brackish water [4 g.l⁻¹]), and
1099 the interaction between salinity during spawning (clutches laid in freshwater [0 g.l⁻¹]
1100 or brackish water [4 g.l⁻¹]) and salinity during larval development (freshwater [0 g.l⁻¹]
1101 or brackish water [4 g.l⁻¹]).

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Treatment	Clutch	Number of tadpoles
FFF	F01	3
	F02	9
	F03	3
	F04	3
	F05	3
	F06	3
	F07	3
	F08	3
	F09	3
	F10	3
FFS	F01	3
	F02	3
	F03	3
	F04	3
	F05	3
	F06	3
	F07	3
	F08	3
	F09	3
	F10	3
FSF	F01	3
	F02	1
	F05	3
	F07	21
	F08	5
	F09	4
FSS	F01	5
	F02	1
	F03	3
	F06	1
	F07	16
	F08	5
	F09	2
SFF	S01	3
	S02	3
	S03	3
	S04	3
	S05	3
	S06	3
	S07	3
	S08	3
	S09	3
	S10	3

	S11	3
	S01	3
	S02	3
	S03	3
	S04	3
	S05	3
SFS	S06	3
	S07	2
	S08	3
	S09	3
	S10	3
	S11	3
	S05	1
SSF	S10	8
	S11	11
	S02	2
	S06	3
SSS	S07	1
	S10	4
	S11	1

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Period	Parameter	Group	Mean	SE
Egg laying	Length (cm)	F	1608.2097	185.0155628
		S	1678.259455	186.6813434
	Number of eggs	F	2427.04	358.3985591
		S	2609.6	208.7763819
	Mass (g)	F	92.82	10.71980307
		S	64.60909091	7.777607187
	Eggs diameter (mm)	F	1.76688	0.039757389
		S	1.660572727	0.043287962
	Laying time (h)	F	79.1	13.10847902
		S	47.33272727	7.662623464
Females loss in mass (%)	F	-13.55765323	2.230276924	
	S	-12.20855975	1.364961958	
Males loss in mass (%)	F	-1.627788792	2.18901737	
	S	3.788011248	1.265685304	
Embryonic development	Hatching success	FF	0.68	0.039014277
		FS	0.2844444444	0.043299898
		SF	0.439393939	0.032995599
		SS	0.037373737	0.018445671
	Malformation rate	FF	0.252273761	0.018871836
		FS	0.609116088	0.026959964
		SF	0.253329084	0.031584961
		SS	0.269217687	0.04932672
	Time to hatching (days)	FF	17.73333333	0.150101261
		FS	18.81428571	0.223183124
		SF	17.81538462	0.191014885
		SS	19.06451613	0.270147247
	Total length (mm)	FF	8.839393939	0.144306052
		FS	7.127058824	0.075127201
		SF	8.619230769	0.122971289
		SS	6.529354839	0.188660779
General activity (scale on 1 to 5)	FF	3.766666667	0.140376386	
	FS	1.071428571	0.031346695	
	SF	3.415384615	0.163924683	
	SS	1.214285714	0.076478688	
Larval development	Mortality	FFF	0.142857143	0.059172634
		FFS	0.5	0.092847669
		FSF	0.735849057	0.073173841
		FSS	0.959183673	0.034800726
		SFF	0.333333333	0.083333333
		SFS	0.4375	0.089098306
		SSF	0.847826087	0.081204698
		SSS	0.945945946	0.069119496
		FFF	10.87096774	1.811827957
		FFS	10.86666667	1.983972819

Duration between S25 and S30 (days)	FSF	10.07142857	1.655732658
	FSS	13.5	2.350048355
	SFF	12.90909091	2.247184286
	SFS	10.31578947	1.823591173
	SSF	13.28571429	2.970776027
	SSS	13.5	4.070403152
	Growth rate between S25 and S30	FFF	0.268393183
FFS		0.22382476	0.015880458
FSF		0.341870161	0.029992171
FSS		0.159178572	0.001028693
SFF		0.231039881	0.029893008
SFS		0.182156128	0.015580051
SSF		0.2555141	0.014131725
SSS		0.286321429	0.028431838
Total length (mm)	FFF	11.57616129	0.275041152
	FFS	11.493	0.148745132
	FSF	10.57214286	0.18434435
	FSS	10.0815	0.029665049
	SFF	11.64254545	0.331575428
	SFS	10.86410526	0.119641317
	SSF	10.83157143	0.198921964
	SSS	10.4225	0.450066712
Activity (scale of 1 to 5)	FFF	3.774193548	0.170563757
	FFS	2.533333333	0.23771398
	FSF	3.642857143	0.189178582
	FSS	1.5	0.123091491
	SFF	3.681818182	0.164587583
	SFS	2.842105263	0.251820854
	SSF	4.285714286	0.109108945
	SSS	3	0

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1109 Appendix C

Gosner stage	Variable	Contrast	Estimate	SE	z/t	p-value
S25	Hatching success	Embryonic 0 Spawning0-Spawning4	0.48	0.194	2.469	0.014
		Embryonic 4 Spawning0-Spawning4	1.97	0.227	8.697	<0.001
		Spawning 0 Embryonic0-Embryonic4	0.972	0.064	15.103	<0.001
		Spawning 4 Embryonic0-Embryonic4	2.466	0.126	19.625	<0.001
	Malformation rate	Embryonic 0 Spawning0-Spawning4	0.087	0.242	0.357	0.721
		Embryonic 4 Spawning0-Spawning4	1.146	0.361	3.169	0.002
		Spawning 0 Embryonic0-Embryonic4	-0.893	0.114	-7.84	<0.001
		Spawning 4 Embryonic0-Embryonic4	0.166	0.278	0.598	0.55
	Time to hatching	Embryonic 0 Spawning0-Spawning4	-0.004	0.025	-0.168	0.868
		Embryonic 4 Spawning0-Spawning4	0.061	0.029	2.118	0.042
		Spawning 0 Embryonic0-Embryonic4	-0.107	0.013	-8.438	<0.001
		Spawning 4 Embryonic0-Embryonic4	-0.042	0.017	-2.544	0.012

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			Estimate	SE	t value	p-value
		Embryonic0 - Embryonic4	-1.40912	0.63540	-2.2177	0.028809
		Spawning0 - Spawning4	-0.87456	0.81189	-1.0772	0.294830
		Larval0 - Larval4	-0.53475	0.55331	-0.9665	0.336348
		Embryonic0:Larval0 Embryonic4:Larval0	0.24447	0.58293	0.4194	0.675807
		Embryonic0:Larval0 Embryonic0:Larval4	1.11884	0.42624	2.6249	0.010097
		Embryonic0:Larval0 Embryonic4:Larval4	-1.94387	1.03537	-1.8775	0.063455
Duation		Embryonic4:Larval0 Embryonic0:Larval4	0.87437	0.58972	1.4827	0.141317
between		Embryonic4:Larval0 Embryonic4:Larval4	-2.18834	1.01502	-2.1560	0.033720
S25 and S30		Embryonic0:Larval4 Embryonic4:Larval4	-3.06271	1.03309	-2.9646	0.003827
		Spawning0:Larval0 - Spawning4:Larval0	-2.33170	0.84095	-2.7727	0.011038
		Spawning0:Larval0 - Spawning0:Larval4	-1.99189	0.66563	-2.9925	0.003549
		Spawning0:Larval0 - Spawning4:Larval4	-1.40932	0.96120	-1.4662	0.151424
		Spawning4:Larval0 - Spawning0:Larval4	0.33981	1.00336	0.3387	0.736590
		Spawning4:Larval0 - Spawning4:Larval4	0.92238	0.68144	1.3536	0.179157
		Spawning0:Larval4 - Spawning4:Larval4	0.58257	0.95197	0.6120	0.544697