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# Short- and medium-term exposure to salinity alters response to predation, activity and spatial movements in tadpoles

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1 **Short- and medium-term exposure to salinity alters response to**  
2 **predation, activity and spatial movements in tadpoles**

3 **Abbreviated title: Exposure to salinity alters tadpoles' behaviour**

4

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21

22 **Abstract**

23 Current environmental changes can drastically affect aquatic freshwater ecosystems.  
24 Salinization is one such change that affects freshwater species due to osmotic costs,  
25 which induce a wide variety of behavioural responses, including altered response to  
26 predation. This is particularly important in larval anurans, known to modulate their  
27 morphology, physiology and behaviour in response to salinity. Although these  
28 responses are known for long-term exposure, the effects of shorter exposure to salinity,  
29 relevant for coastal biodiversity as a result of seawater intrusions and precipitation  
30 variations, have rarely been assessed despite their importance in understanding the  
31 speed at which impacts on biodiversity occur. In this study, we experimentally  
32 acclimated larvae of spined toad (*Bufo spinosus*) during two weeks to freshwater (zero  
33 g/litre) or to moderate levels of salinity (four g/litre). The response to predation,  
34 activity and spatial movements of each individual was then assessed both at the  
35 salinity of acclimation and after a change in salinity. We demonstrated that medium-  
36 term exposure to salinity decreased activity, decreased growth and increase mortality.  
37 In contrast, sudden changes in salinity increased the escape distance of tadpoles in  
38 response to a predation stimulus but decreased activity. These results suggest that the  
39 exposure to both stressors (predation cues and varying salinities) lead to an increased  
40 activity presumably linked to a stress response. However, individuals exposed to a  
41 change in salinity (from zero to four g/litre salinity) or to moderate salinity for  
42 medium durations expressed similar behavioural responses, indicating that the  
43 detrimental effects of high salinity can occur rapidly (<one hour). Future studies  
44 should investigate the dynamics of osmolality in larval anurans exposed to brackish  
45 water in order to assess whether these behavioural shifts are linked with hydro-

46 mineral dysregulation, and the duration after which this hydro-mineral dysregulation  
47 occurs.

48 **Key-words:** Growth, Osmotic stress, Mortality, Movement complexity, Predation,  
49 Tadpoles

## 50 INTRODUCTION

51 Current environmental changes can dramatically affect freshwater ecosystems  
52 (Angeler et al., 2014; Jackson et al., 2016; Woodward et al., 2010). Salinization, the  
53 increase of salinity in water bodies, is one such change that affects many wetlands  
54 worldwide (Cunillera-Montcusí et al., 2022; Herbert et al., 2015; Kaushal et al., 2018).  
55 Salinization can result from natural (primary salinization) or human-induced  
56 processes (secondary salinization; Herbert et al., 2015). Secondary salinization is  
57 linked to the alteration of the hydrobiological cycle (Neubauer & Craft, 2009) in  
58 response to several different causes such as sea-level rise (Llovel et al., 2019; Nicholls  
59 & Cazenave, 2010; Werner & Simmons, 2009), increase in frequency and intensity of  
60 marine storms (IPCC, 2022), changes in precipitation and riverine flow (Herbert et al.,  
61 2015; Martínez-Megías & Rico, 2021; Neubauer & Craft, 2009), application of road de-  
62 icing salt (Denoël et al., 2010; Hall et al., 2017; Hintz & Relyea, 2019; Sanzo & Hecnar,  
63 2006), or water and land uses linked to anthropogenic activities (Alcérreca-Huerta et  
64 al., 2019; Ghalambor et al., 2021; Herbert et al., 2015; Leal Filho et al., 2022; Mulamba  
65 et al., 2019; Tweedley et al., 2019). All these processes have led to unprecedented rates  
66 of salinization worldwide (Cunillera-Montcusí et al., 2022; Hintz et al., 2022; Kaushal  
67 et al., 2018), which is currently threatening biodiversity (Herbert et al., 2015).

68

69 Freshwater species are, with some exceptions (Cañedo-Argüelles et al., 2013;  
70 Hopkins & Brodie, 2015; Walker et al., 2023), generally intolerant to relatively high  
71 salinity levels (Kumar & Afaq, 2022). Indeed, maintaining osmolality in saline  
72 environments is metabolically costly (Evans & Kültz, 2020; Lillywhite & Evans, 2021;

73 Peña-Villalobos et al., 2013; Pistole et al., 2008; Rivera-Ingraham & Lignot, 2017;  
74 Schultz & McCormick, 2012), and alter energetic allocation to other organismal  
75 functions such as gas exchange (Kidder III et al., 2006), growth (Alkhamis et al., 2022;  
76 Cañedo-Argüelles et al., 2013), energy acquisition (Alkhamis et al., 2022; Cañedo-  
77 Argüelles et al., 2013; Herbert et al., 2015; Pinder et al., 2005), and body reserves  
78 through changes in food conversion rate (Luz et al., 2008) and protein and lipid  
79 contents (Wang et al., 2022).

80

81         Because of these changes in energetic allocation, exposure to salinity also affects  
82 the behaviour of individuals. Indeed, in a large variety of taxa, chronic exposure to  
83 increased salinity has been shown to induce a wide variety of behavioural responses  
84 such as reduced or suppressed appetite (Ashley et al., 2021; Davenport & Ward, 1993),  
85 reduced food intake (Hannam et al., 2003; Luz et al., 2008), and reduced activity levels  
86 (Lorrain-Soligon, Bichet, et al., 2022; Wood & Welch, 2015; Zhou et al., 2022) all of  
87 which could strongly affect food acquisition. In addition, exposure to salinity has been  
88 shown to negatively affect self-maintenance behaviours (e.g., preening in birds,  
89 Hannam et al., 2003), to increase the frequency of specific behaviours (e.g., sneezing  
90 and head-shaking to expel the secretions of salt glands, Rocha et al., 2016), and to affect  
91 social interactions (Zhou et al., 2022). Ultimately, exposure to salinity can induce  
92 behavioural responses such as saltwater avoidance and increased freshwater-seeking  
93 behaviour (Ashley et al., 2021; Gutiérrez, 2014; Lorrain-Soligon, Robin, et al., 2022)  
94 which are expected to influence species distribution (Brischoux et al., 2012, 2021).

95

96           Importantly, exposure to salinity can also interact with - and thus influence the  
97 responses to - other environmental stressors (Velasco et al., 2019). This seems  
98 particularly relevant for environmental stressors which can ultimately affect  
99 organismal fitness such as predation. Indeed, increased salinity has been shown to  
100 reduce the intensity of anti-predator responses (Hoover et al., 2013). Such alteration  
101 of anti-predator responses can thus strongly affect survival (Liu & Steiner, 2017). While  
102 organisms tend to avoid both saltwater and predator cues (Tietze & Gerald, 2016),  
103 salinity avoidance appears more important than predator avoidance when options are  
104 restricted (i.e., selection of freshwater with predation cues rather than saltwater  
105 without predation cues, Tietze & Gerald, 2016). Nonetheless, the combined effects of  
106 salinity and predation remains poorly studied, despite some evidences that predator-  
107 induced stress can induce stronger effects than other environmental stressors (Relyea  
108 & Mills, 2001).

109

110           Amphibians are particularly sensitive to salinity due to their highly permeable  
111 skin serving as a surface for respiration, and for ion and water transport (Martin &  
112 Nagy, 1997; Shoemaker & Nagy, 1984), due to their limited ability to maintain  
113 normosmolality (Katz, 1989), and due to larval dependence to the aquatic environment  
114 (Wells, 2007). As such, larval anurans are particularly well suited to study the  
115 behavioural responses to salinity exposure. Indeed, salinity may depresses thyroid  
116 hormones in tadpoles (Gomez-Mestre et al., 2004), and lead to changes in  
117 osmoregulatory hormones such as corticosterone and aldosterone (Tornabene,  
118 Hossack, et al., 2021; Tornabene, Crespi, et al., 2022). Exposure to salinity has also been

119 shown to reduce development (Gomez-Mestre et al., 2004; Haramura, 2016; Hopkins  
120 et al., 2013; Lukens & Wilcoxon, 2020), and growth (Haramura, 2016; Lukens &  
121 Wilcoxon, 2020; Tornabene, Breuner, et al., 2021; Wood & Welch, 2015; Wu & Kam,  
122 2009). In addition, larvae exposed to higher salinity express reduced activity and  
123 foraging behaviour (Chuang et al., 2022; Hall et al., 2017; Sanzo & Hecnar, 2006; Wood  
124 & Welch, 2015), lowered responses to stimuli (Karraker, 2007), lowered swimming  
125 performance (Haramura, 2016), erratic movements (Tornabene, Breuner, et al., 2021),  
126 and reduced speed and distance (Denoël et al., 2010), all of which can affect their  
127 susceptibility to predation. Importantly, while these effects have been widely studied  
128 in individuals reared for their entire development in brackish water, few studies have  
129 examined the responses of individuals exposed for short events of increasing salinity  
130 (but see LoPiccolo, 2022). Yet, such studies are important in order to investigate the  
131 effects of salinity variations and particularly sudden changes in salinity, which is  
132 particularly relevant in coastal environments where salinity can rapidly increase due  
133 to marine submersions (Lorrain-Soligon et al., 2021), sea-water intrusion (Gopinath et  
134 al., 2015; Ranjbar & Ehteshami, 2019), as well as changes in precipitation and riverine  
135 flow (Herbert et al., 2015; Martínez-Megías & Rico, 2021; Neubauer & Craft, 2009), and  
136 in freshwater environments due to road-deicing salt (Brady, 2012; Brady et al., 2022;  
137 Denoël et al., 2010), but also potentially rapidly decrease due to changes in  
138 precipitation regimes (Lorrain-Soligon, Robin, et al., 2023). Importantly, some of these  
139 processes induce changes in salinity occurring within very short temporal scales  
140 (within an hour, e.g. marine submersions and road-deicing salt application), while



141 others might induce changes in salinity across longer time scales (few days, e.g.  
142 changes in precipitations and riverine flows).

143

144 In this study, to better understand the effects of salinity variation on amphibian  
145 behavior, we experimentally acclimated larvae of spined toad (*Bufo spinosus*) to  
146 freshwater (0 g/litre) or to moderate levels of salinity (4 g/litre) for 2 weeks. We then  
147 evaluated the response to predation (escape distance in response to a stimuli) and  
148 different metrics of behaviour (mean speed, total distance, active time, position  
149 relative to the center of an arena, and behavioural complexity) both at the salinity  
150 experienced during acclimation and after a sudden change of salinity. At the beginning  
151 and at the end of acclimation, individuals were also measured to compare growth  
152 rates. We predicted that 2 weeks exposure to salinity should decrease growth rate and  
153 increase mortality due to the sensitivity of larval anurans to salinity (Albecker &  
154 McCoy, 2017; Gomez-Mestre et al., 2004). Additionally, we hypothesized exposure to  
155 salinity for 2 weeks would decrease performance (Denoël et al., 2010; Hall et al., 2017;  
156 Haramura, 2016; Karraker, 2007; Sanzo & Hecnar, 2006; Wood & Welch, 2015), which  
157 might decrease their responsiveness to predation. A sudden change of salinity during  
158 behavioural tests, as compared to a longer exposure to salinity, should increase  
159 activity due to osmotic stress but with no costs of osmoregulation (LoPiccolo, 2022).

160

161

162 **METHODS**

163

164 **Study species and captures**

165 The spined toad (*Bufo spinosus*) is a widespread amphibian species found in western  
166 Europe (Meek, 2022; Speybroeck et al., 2018). Breeding occurs in late winter, during  
167 which adults converge to reproductive ponds where they pair (amplexus) and lay their  
168 eggs. Egg laying, embryonic and larval developments all occur in ponds. 75 tadpoles  
169 naïve from salt exposure were captured from 20 to 24 April 2023 in a small pond  
170 (0.4248°W, 46.1459°N), where salinity was measured to be 0.0 g/litre using a  
171 conductimeter (YSI Professional Plus) at 15 cm depth. Sampling was focused  
172 specifically on a pond situated relatively far from the coastline and roads in order to  
173 assess the effect of salinity and its variation on individuals naïve to salt exposure. At  
174 least 18 different amplexant pairs were observed in this pond prior to sampling,  
175 suggesting that tadpoles involved in this experiment did not originate from the same  
176 family. Sampling was carried out along the banks of the pond, using transparent  
177 plastic boxes (14\*7\*6 cm). Tadpoles ranged from Gosner stage 30 to Gosner stage 33  
178 (Gosner, 1960), during which no significant change in behaviour and morphology  
179 occurs (Cheron et al., 2021).

180

181 **Treatments**

182 Immediately after capture, individuals were brought to the laboratory (thermally  
183 controlled room with temperature set at 17 °C and controlled photoperiod 12 h dark-  
184 12 h light).

185 Individuals were acclimated for 4 days in an individual tank (13\*18\*18cm) with  
186 water from the pond of origin. After these 4 days, individuals were randomly  
187 attributed to two treatments to which they were acclimated during 2 weeks in  
188 individual 2 L tanks (13\*18\*18cm) in which they remained during the whole duration  
189 of the experiment: control (0 g/litre salinity:  $0.3 \pm 0.0$  g/litre,  $N=31$ ), and moderate  
190 salinity (brackish water, 4 g/litre salinity  $4.0 \pm 0.06$  g/litre,  $N=44$ ). Number of  
191 individuals was higher in the moderate salinity to take into account expected mortality  
192 in this experimental group (Albecker & McCoy, 2017; Bernabò et al., 2013;  
193 Chinathamby et al., 2006; Christy & Dickman, 2002; B. Jones et al., 2015; Karraker et  
194 al., 2008; Rios-López, 2008; Sanzo & Hecnar, 2006) and maintain a sufficient sample  
195 size for final analyses. While 4 g/litre salinity might be elevated for some amphibian  
196 species (Hopkins & Brodie, 2015), amplexus and larvae of *Bufo spinosus* have been  
197 found up to 6 g/litre in the field (LLS pers obs., Lorrain-Soligon, Périssette, et al., 2023).  
198 Salinity treatments were obtained by dissolution of sea-salt (NaCl). Our goal was to  
199 mimic the range of salinity found in coastal environments where the species is known  
200 to reproduce (Lorrain-Soligon, Périssette, et al., 2023). For the moderate salinity group,  
201 to reach final salinity, we progressively increased the salinity of the water at a rate of  
202 1 g/litre a day to prevent osmotic shock, and because natural salinity fluctuations do  
203 not exceed  $\pm 2$  g/litre a day (Hsu et al., 2018). In the same time, we progressively  
204 replaced pond water by dechlorinated (aged) tap water. During the remaining

205 procedure, water was changed once a week and tadpoles were fed ad libitum with  
206 organic spinach. Individuals were checked twice a day for mortality.

207

## 208 **Tadpole size**

209 Upon capture, individuals were individually placed in a petri dish, positioned upon a  
210 scale (graph paper), containing 2 cm of water. A picture was taken from above in order  
211 to measure total length. Measurement were performed using the software ImageJ  
212 (Schneider et al., 2012). Total length did not vary between the two treatments at the  
213 beginning of exposure (0 g/litre: 17.19 mm  $\pm$  0.32 SE; 4 g/litre: 17.88 mm  $\pm$  0.31 SE;  
214 linear model: Estimate=0.689, SE=0.485,  $t_{1.65}$ =1.419,  $P$ =0.161). After 2 weeks,  
215 individuals were measured once again following the same protocol in order to assess  
216 growth rates which were computed as the difference between size at the end of the  
217 experiment and size at the beginning of the experiment divided by size at the  
218 beginning of the experiment.

219

## 220 **Tadpole behaviour**

221 We performed a comprehensive set of measures of behaviour at the end of the two-  
222 week exposure to 2 treatments: 0 g/litre and 4 g/litre (see Figure 1). The tests were  
223 performed across 6 days, and all individuals performed the tests in the morning  
224 (between 9 a.m and 12 p.m, 6 tadpoles at a time, 3 from each acclimation treatment),  
225 under controlled light intensity.

226 These measures were performed at the salinity at which individuals were acclimated  
227 during two weeks (see “Medium-term responses to salinity” below); but also  
228 following a sudden change of salinity (see “Responses to salinity variations” below).

229

### 230 Medium-term responses to salinity

231

232 First, to investigate the response to predation, we assessed swimming distance in  
233 response to a stimulus. Tadpoles were placed at the start of a 70 cm half pipe closed at  
234 the two extremities and filled with the salinity corresponding to the acclimation  
235 treatment (Figure 1). Tadpoles were gently touched and the escape distance was  
236 measured. We measured 6 successive escape distances. These tests lasted for < 1 min  
237 and individuals were then transferred for the second set of behavioural tests (see  
238 below). For analyses (see below), we used the mean distance (calculated from the 6  
239 trials, or when individuals computed at least 3 successful trials).

240

241 Second, on the same day and immediately after the first test, to assess activity  
242 and spatial movements, tadpoles were placed in an individual arena (Petri dish,  
243 diameter 13.5 cm, water level 1.5 cm) filled with the salinity corresponding to the  
244 acclimation treatment (Figure 1). Tadpoles were left acclimated for 20 min, and then  
245 filmed for 35 min, using a camera placed above the arena (GoPro HERO; GoPro, San

246 Mateo, CA, USA). These trials thus lasted for approximatively one hour. Videos were  
247 later analyzed to extract behavioural data (see “Videos analyses” below).

248

#### 249 Responses to salinity variations

250

251 The following day, we switched salinity exposure for tadpoles, using the same  
252 protocols as previously described (Figure 1). Tadpoles acclimated at 0 g/litre  
253 performed this second set of tests at 4 g/litre, while individuals acclimated at 4g/litre  
254 performed this second set of tests at 0g/litre (Figure 1). To ensure that the order of the  
255 successive tests did not influence behaviour, we performed a previous trial on a  
256 subsample of 10 individuals that performed a test at 0 g/litre first, and a second test at  
257 0 g/litre the day after. We then computed mean escape distance (see paragraph  
258 below). We found that escape distance did not differ between day 1 and day 2 when  
259 individuals were exposed to the same treatment (linear mixed model: Estimate=-0.163,  
260 SE=1.849,  $t_{1.109}=-0.088$ ,  $P=0.930$ ).

261

262 At the end of these experiments, individuals were released at their site of capture.  
263 Individuals in the moderate salinity treatment were progressively acclimated to  
264 freshwater before release, to avoid osmotic shock.

265

#### 266 Videos analyses

267 Videos were analyzed with the software ToxTrac (Rodriguez et al., 2018). We  
268 used a white background in order to create a contrast that allowed the software to  
269 detect and monitor tadpoles. The successive positions of a tadpole (defined from the  
270 x- and y- calibrated coordinates within the Petri dish) were then extracted per frame,  
271 and the distance moved between two consecutive frames was calculated. Using  
272 'Tracking RealSpace' data, we determined the total duration of activity; the total  
273 distance travelled (relative to total length), mean swimming speed (relative to total  
274 length), and frequency of a tadpole being within 50mm of the center of the arena (based  
275 on the central point of the tadpole). Indeed, tadpoles can be more visible to predators  
276 in open areas (see Denoël et al., 2012 and references therein). We thus expected that  
277 swimming at the center of the aquarium could thus be considered as a "risky"  
278 behaviour.

279

280 Additionally, we evaluated DFA (Detrended fluctuation analysis) and DFAC  
281 (corrected detrended fluctuation analysis) using the *fractal* package (version 2.0-4,  
282 Constantine & Percival, 2017). DFA is a robust method used to estimate the degree to  
283 which time series are long-range dependent and self-affine (Cannon et al., 1997), which  
284 is now currently used in the study of animal behaviour (Alados & Huffman, 2000;  
285 MacIntosh et al., 2011) including larval amphibians (Cheron et al., 2021, 2023).  
286 Following MacIntosh et al. (2013), to compute DFA, we coded behavioural sequences  
287 as binary time series  $[z(i)]$ : an immobile individual is coded as -1, while a mobile  
288 individual (an individual is considered moving if it moves more than 0.25 mm.s<sup>-1</sup>) is  
289 coded as 1, at 1 s intervals to length  $N$  (for 35 minutes). How DFA and DFAC were

290 computed is thoroughly described in MacIntosh et al. (2013) and Peng et al. (1995).  
291 DFA is inversely related to the fractal dimension, a classical index of structural  
292 complexity (Mandelbrot, 1982), and thus smaller values reflect greater complexity.

293

#### 294 **Ethical Note**

295 The only ethical concern regarding the study system is the exposure to salinity.  
296 However, individuals were exposed to environmental salinity levels (4 g/litre, while  
297 tadpoles of this species can be found up to 6 g/litre, LLS pers obs., Lorrain-Soligon,  
298 Périsset, et al., 2023), and individuals were progressively acclimated to these salinity  
299 levels to avoid osmotic shock. Additionally, individuals were kept in optimal  
300 conditions, with large water volumes (2 L) and food provided *ad libitum*. We did not  
301 perform invasive sampling, and we handled individuals only for transfer in  
302 behavioural tests. They were kept under laboratory conditions for 2 weeks, and were  
303 released at their site of capture at the end of the experiments. This work was approved  
304 by the French authorities under permit APAFIS #33592-2021102610033982.

305

#### 306 **Statistical analyses**

307 All measures of distances or speed were corrected by individual total length, and were  
308 thus expressed relative to size (i.e. number of time individuals moved their total  
309 length) to provide a more meaningful measure of locomotor performance than  
310 absolute speed (Van Damme & Van Dooren, 1999). For all models computed, model  
311 accuracy was tested using the `check_model` function from the *performance* package  
312 allowing to test for residuals distribution and normality (Lüdtke et al., 2020). For LMs



313 (Linear Models) and LMMs (Linear Mixed Models), when models did not fit the data,  
314 the response variable was  $\log_{10}+1$  transformed. Binomial model accuracy was also  
315 tested by checking overdispersion.

316

317 First, we assessed differences of growth between treatments by setting a Linear  
318 Model (LM) and mortality (as a binary component: 0 [mortality] or 1 [survival]) by  
319 setting binomial Generalized Linear Models (GLMs) with acclimation treatment (0 or  
320 4 g/litre) as an explanatory variable. Second, we assessed differences in mean escape  
321 distance during the predation test using a Linear Mixed Model (LMM). Lastly, we  
322 assessed differences in total duration of activity ( $\log_{10}+1$  transformed), total distance  
323 travelled ( $\log_{10}+1$  transformed), mean swimming speed ( $\log_{10}+1$  transformed), and  
324 frequency of positioning at less than 50mm to the center of the arena ( $\log_{10}+1$   
325 transformed) using Linear Mixed Models (LMMs) and behavioural complexity (DFA)  
326 using GLMMs with Gamma distribution, due to a skewed distribution (Dobson &  
327 Barnett, 2018). For all, we used the interaction between salinity acclimation treatment  
328 (0 or 4 g/litre) and salinity behavioural test treatment (0 or 4 g/litre) as explanatory  
329 variables, and individual as a random effect. For these models, best variables were  
330 retained using a top-down selection, and only the retained variables are presented.

331

332

333 All data analysis were performed using R 3.6.3 (R Core Team, 2020) and Rstudio  
334 v1.1.419.

335 **RESULTS**

336 **Mortality and tadpole size**

337 During acclimation (2 weeks), mortality was higher in individuals kept in brackish  
338 water compared to freshwater (4 individuals died in freshwater, mortality rate=16%,  
339 and 29 individuals died in brackish water, mortality rate=66%, Estimate=2.569,  
340 SE=0.623,  $z_{1.73}=4.123$ ,  $P<0.001$ ). Growth rate (as a proportion of the initial size of  
341 individuals) was higher in freshwater compared to brackish water (freshwater:  $0.212$   
342  $\pm 0.007$  SE; saltwater:  $0.061 \pm 0.008$  SE; Estimate [proportion of the initial total  
343 length]= $0.152$ , SE= $0.021$ ,  $t_{1.46}=7.393$ ,  $P<0.001$ ).

344

345 **Tadpole behaviour**

346 Responses to predation stimuli

347 Mean escape distance (number of time an individual travelled its total length)  
348 was influenced by salinity during trial (Estimate= $5.220$ , SE= $1.577$ ,  $t_{1.59}=3.309$ ,  $P=0.002$ ,  
349 Figure 2) and the interaction between acclimation treatment and salinity during trial  
350 (Estimate= $-7.597$ , SE= $2.213$ ,  $t_{1.59}=-3.433$ ,  $P=0.001$ , Figure 2, Table A1), but not by salinity  
351 during acclimation solely (Estimate= $2.433$ , SE= $2.230$ ,  $t_{1.94}=1.091$ ,  $P=0.278$ ). Mean  
352 escape distance was higher for individuals acclimated in freshwater and that  
353 performed the test in brackish water (Figure 2).

354

355

356 Medium-term responses to salinity and responses to salinity variations

357 Total distance ( $\log_{10}+1$  transformed) was influenced by salinity during  
358 acclimation (Estimate=-0.855, SE=0.137,  $t_{1,113}=-6.230$ ,  $P<0.001$ , Figure 3a) and the  
359 interaction between acclimation treatment and salinity during trial (Estimate=0.464,  
360 SE=0.177,  $t_{1,58}=2.623$ ,  $P=0.011$ , Figure 3a, Table A1), but not by salinity during trial  
361 solely (Estimate=-0.191, SE=0.125,  $t_{1,58}=-1.529$ ,  $P=0.132$ ). Overall, total distance was  
362 higher for individuals that were acclimated in freshwater, and that performed the test  
363 in freshwater.

364

365 Mean active speed ( $\log_{10}+1$  transformed) was influenced by salinity during  
366 acclimation solely (Estimate=-0.149, SE=0.034,  $t_{1,58}=-4.396$ ,  $P<0.001$ , Figure 3b), and  
367 was lower for individuals raised in brackish water compared to individuals raised in  
368 freshwater. Total active time ( $\log_{10}+1$  transformed) was influenced by salinity during  
369 acclimation (Estimate=-0.672, SE=0.118,  $t_{1,114}=-5.690$ ,  $P<0.001$ , Figure 3c), marginally  
370 influenced by salinity during trial (Estimate=-0.206, SE=0.109,  $t_{1,58}=-1.888$ ,  $P=0.064$ ,  
371 Figure 3c), and influenced by the interaction between acclimation treatment and  
372 salinity during trial (Estimate=0.429, SE=0.154,  $t_{1,58}=2.780$ ,  $P=0.007$ , Figure 3c, Table  
373 A1). Overall, total active time was higher for individuals that were acclimated in  
374 freshwater, and that performed the test in freshwater. Frequency of positioning at less  
375 than 50 mm from the center of the arena ( $\log_{10}+1$  transformed) was influenced by  
376 salinity during acclimation solely (Estimate=0.478, SE=0.118,  $t_{1,58}=4.046$ ,  $P<0.001$ ,  
377 Figure 3d), and was higher for individuals raised in brackish water compared to  
378 individuals raised in freshwater.

379

380           DFAc was influenced by salinity during acclimation (Estimate=-0.035,  
381 SE=0.009,  $t=-4.021$ ,  $P<0.001$ , Figure 3c), salinity during trial (Estimate=-0.019, SE=0.005,  
382  $t=-3.712$ ,  $P<0.001$ , Figure 3c), and influenced by the interaction between acclimation  
383 treatment and salinity during trial (Estimate=-0.029, SE=0.007,  $t=4.090$ ,  $P<0.001$ , Figure  
384 3c, Table A1). Overall, DFac were the lowest (higher behavioural complexity) for  
385 individuals that were acclimated in freshwater, and that performed the test in  
386 freshwater, and the highest (lower behavioural complexity) for individuals that were  
387 acclimated in brackish water, and that performed the test in freshwater.

388

389 **DISCUSSION**

390

391 In this study, we demonstrated that larval spined toads naive from salt exposure  
392 altered their behaviour in response to salinity. We showed that a 2 week-long exposure  
393 to moderate levels of salinity (4 g/litre) decreased growth rate, increased mortality  
394 and decreased escape distance (and thus reaction to predation), mean speed and  
395 behavioural complexity. However, when individuals were acclimated in freshwater  
396 and transferred to brackish water, escape distance increased, but mean speed and  
397 behavioural complexity decreased.

398

399 First, exposure to salinity during 2 weeks induced lower growth rates and  
400 higher mortality as expected from previous investigations (Albecker & McCoy, 2017;  
401 Gomez-Mestre et al., 2004; Tornabene, Breuner, et al., 2022). Additionally, we  
402 highlighted that such exposure to salinity negatively influenced behavioural  
403 performance (Denoël et al., 2010; Hall et al., 2017; Haramura, 2016; Karraker, 2007;  
404 Sanzo & Hecnar, 2006; Wood & Welch, 2015). Specifically, individuals exposed to  
405 salinity exhibited lower escape distance, shorter total distance travelled, slower  
406 swimming speed, lower activity time, decreased behavioural complexity, and were  
407 more often positioned closer to the center of the arena. In natural environments, such  
408 alterations of key behavioural traits may led to reduced foraging (Bartumeus, 2007;  
409 Viswanathan et al., 2008), because such performance should be adaptive in  
410 unpredictable environments where they can enhance the probability of resource

411 encounters (Bartumeus, 2007; Viswanathan et al., 2008) and prevent predators from  
412 anticipating the prey future position (Gazzola et al., 2021; Jones et al., 2011; Richardson  
413 et al., 2018). In addition, these alterations can also induce a higher risk of predation  
414 (Chovanec, 1992; Denoël et al., 2012; Eterovick et al., 2010; Laurila, 2000), as remaining  
415 closer to the edges is considered as less risky and spatial avoidance is expected in  
416 tadpoles exposed to a predation threat (Relyea, 2001). The effects we found on growth,  
417 mortality and behaviour are probably linked to higher costs of osmoregulation  
418 (Gomez-Mestre et al., 2004), mediated by modulations of specific hormonal mediators  
419 such as corticosterone and aldosterone (Tornabene, Crespi, et al., 2022; Tornabene,  
420 Hossack, et al., 2021). Importantly, we show that these responses can be expressed over  
421 a relatively short temporal scale (2 weeks) which is shorter than those used in previous  
422 investigations (Denoël et al., 2010; Gomez- Mestre et al., 2004; Tornabene, Hossack, et  
423 al., 2021). This suggests that the energetic cost of osmoregulation can appear relatively  
424 rapidly at a larval stages (Gosner stages 30-33, see also Lorrain-Soligon et al., 2024)  
425 considered to be comparatively less sensitive than embryo or earlier larval  
426 developmental stages (Albecker & McCoy, 2017; Chinathamby et al., 2006; Uchiyama  
427 & Yoshizawa, 1992). Future studies are required to investigate whether increasing  
428 salinity increases metabolic rates in larval anurans, as suggested in other species (Peña-  
429 Villalobos et al., 2013; Pistole et al., 2008).

430

431         Beside those effects during the acclimation phase of tadpoles to salinity, our  
432 experimental design allowed us to highlight different behavioural responses following  
433 a sudden change in salinity. First, we found that immediately after an increase of

434 salinity, individuals reared in freshwater increased their escape distance in response  
435 to a predation stimuli. Conversely, individuals reared in brackish water did not  
436 modulate their behaviour during short-term exposure to either freshwater or  
437 moderate level of salinity (4 g/litre), suggesting that a sudden decrease in water  
438 salinity did not trigger osmotic stress responses in these individuals, and that the costs  
439 of osmoregulation for individuals reared in brackish water (Gomez-Mestre et al., 2004)  
440 exceed the osmotic shock (or physiological relief) from being transferred to freshwater.  
441 These results suggest that, when exposed to a rapid increase in salinity, larval anurans  
442 can be more prompt to react to a predation threat, highlighting a higher response to  
443 predation cues in high salinity treatment for individuals reared in freshwater (Hoover  
444 et al., 2013; Troyer & Turner, 2015), presumably due to the presence of both stressors.  
445 Whether such response persists over longer time scales needs to be investigated.

446

447         Second, sudden exposure to salinity for individuals reared in freshwater  
448 induced shorter distance travelled, slower mean speed, and decreased behavioural  
449 complexity. These individuals thus expressed similar responses than those exposed  
450 during longer durations (Denoël et al., 2010; Hall et al., 2017; Haramura, 2016;  
451 Karraker, 2007; Sanzo & Hecnar, 2006; Wood & Welch, 2015), indicating that elevated  
452 salinity can have detrimental effects on larval amphibians after a very short exposure.  
453 Interestingly, and similar to our results on response to predation, individuals reared  
454 in brackish water did not express different behaviour in response to exposure to fresh  
455 or brackish water. Our results thus point out that a relatively short exposure to salinity  
456 (2 weeks) can lead to irreversible effects on key behavioural traits, even when tadpoles

457 were transferred to freshwater, which was expected to induce a physiological relief (or  
458 an additional stressor leading to an osmotic shock). As a result, even if salinity happen  
459 to decrease in saline ponds as a result of increased precipitation (Lorrain-Soligon,  
460 Robin, et al., 2023), this could not be sufficient to dampen the effect of an elevated  
461 salinity during earlier larval development. Future studies should investigate the  
462 dynamics of osmolality in larval anurans exposed to brackish water in order to assess  
463 whether these behavioural shifts are linked with hydro-mineral dysregulation as  
464 shown in other studies (Brady et al., 2022; Denoël et al., 2010; Lorrain-Soligon, Bichet,  
465 et al., 2022; Lorrain-Soligon, Robin, et al., 2022), and the duration after which this  
466 hydro-mineral dysregulation occurs.

467

## 468 CONCLUSION

469 While chronic exposure to salinity has long been shown to alter the behaviour of larval  
470 anurans, we demonstrated that this effect can occur after very short exposure to  
471 salinity (1 hour), and is maintained for longer exposure (2 weeks). This strongly  
472 suggests that osmolality and associated hydro mineral dysregulation following  
473 exposure to salinity can be very rapid, which might threaten coastal biodiversity as a  
474 result of rapid salinity fluctuations.



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479

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484

485 **Data archiving statement**

486 The data that supports the findings of this study are available in the supplementary  
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488

489 **Conflict of interest statement**

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

491

492 **Ethics statement**

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908



909 **Caption to figures**

910 **Figure 1** - Experimental design used in this study. 1) Tadpoles were acclimated for 2  
911 weeks in individual 2 L tanks to control (0 g/litre salinity) or moderate salinity  
912 (brackish water, 4 g/litre salinity). 2) Their response to predation was assessed  
913 measuring individuals swimming distance in a pipe when stimulated. 3) Behavioural  
914 responses of individuals to salinity were measured in an arena filled with water of the  
915 test salinity. Tadpoles were left acclimated for 20 min, and then filmed for 35 min. For  
916 the 2 and 3 steps, tadpoles acclimated at 0 g/litre performed the test at 0 g/litre first,  
917 and the test at 4 g/litre the day after, and conversely for tadpoles acclimated at 4  
918 g/litre, in order to investigate the effects of sudden change in salinity. Results were  
919 always analyzed as an interaction between salinity of the acclimation (1) and salinity  
920 of the trial (2 or 3).

921

922 **Figure 2** - Mean escape distance (relative to total size) in response to a predation  
923 stimulus, according to salinity during acclimation (2 weeks in freshwater [0 g/litre] or  
924 brackish water [4 g/litre]) and salinity during the trial (freshwater [0 g/litre] or  
925 brackish water [4 g/litre]).

926

927 **Figure 3** - (a) Total distance (relative to total size), (b) mean active speed (relative to  
928 total size), (c) total active time, and (d) frequency of positioning at less than 50 mm  
929 from the center of the arena, according to salinity during acclimation (2 weeks in

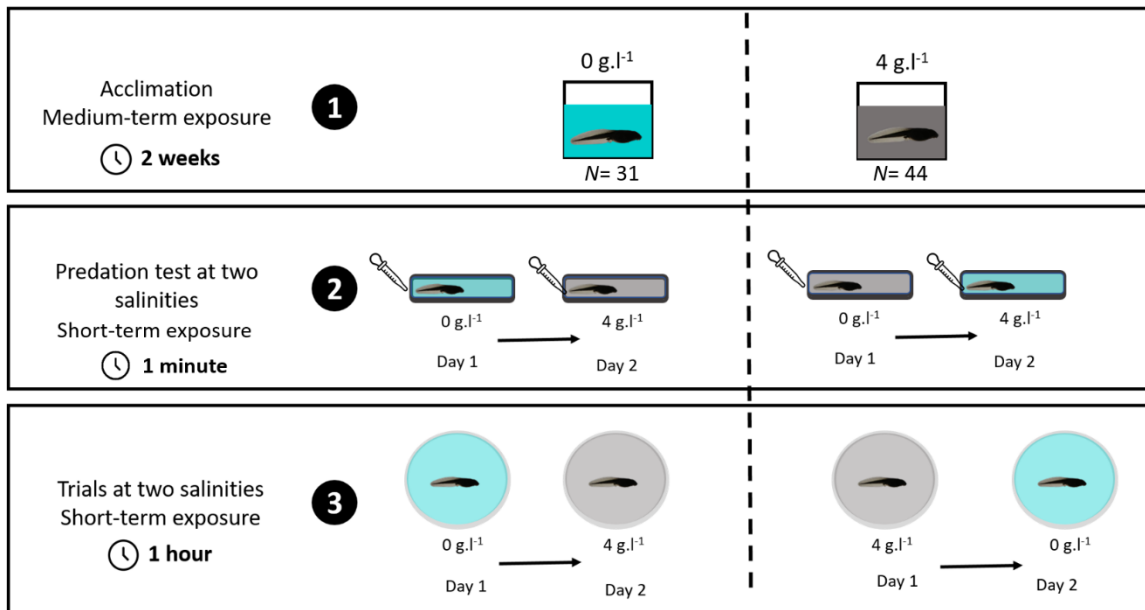
930 freshwater [0 g/litre] or brackish water [4 g/litre]) and salinity during the trial  
931 (freshwater [0 g/litre] or brackish water [4 g/litre]).

932

933 **Figure 4** - DFAc (corrected detrended fluctuation analysis, index of behavioural  
934 complexity), according to salinity during acclimation (2 weeks in freshwater [0 g/litre]  
935 or brackish water [4 g/litre]) and to salinity during the trial (freshwater [0 g/litre] or  
936 brackish water [4 g/litre]). DFA is inversely related to the fractal dimension, a classical  
937 index of structural complexity (Mandelbrot, 1982), and thus smaller values reflect  
938 greater complexity.

939

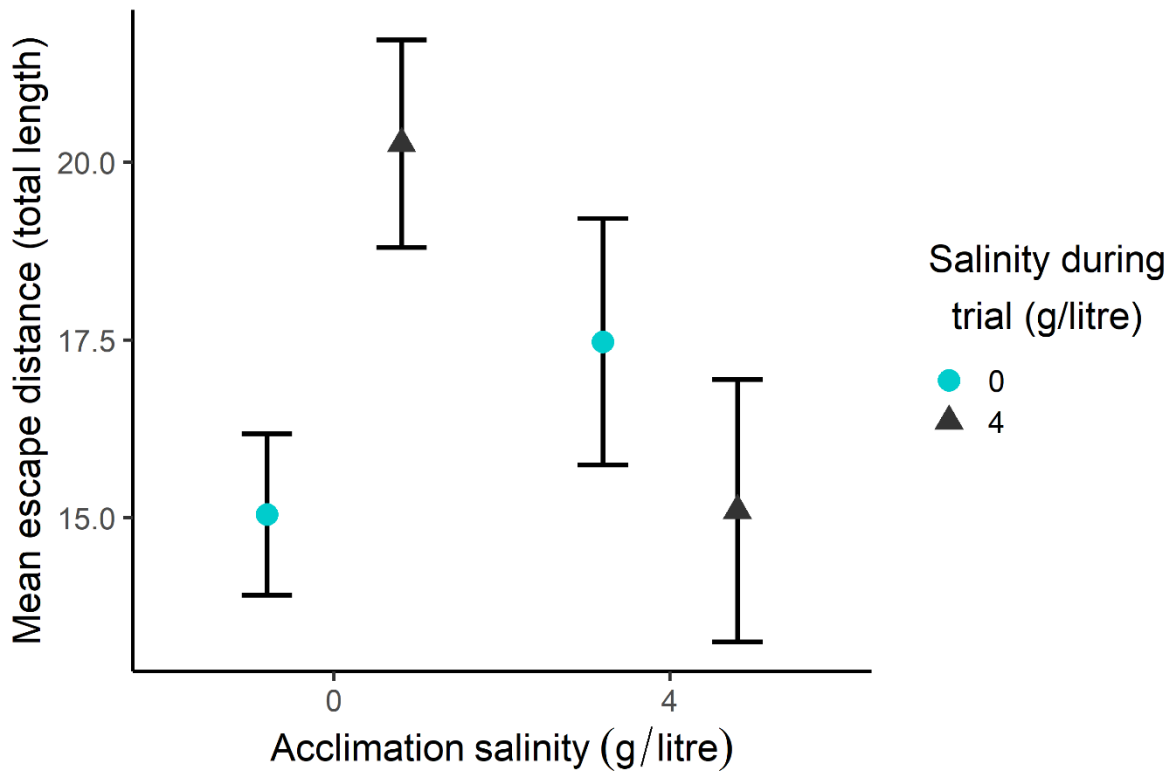
940 Figure 1



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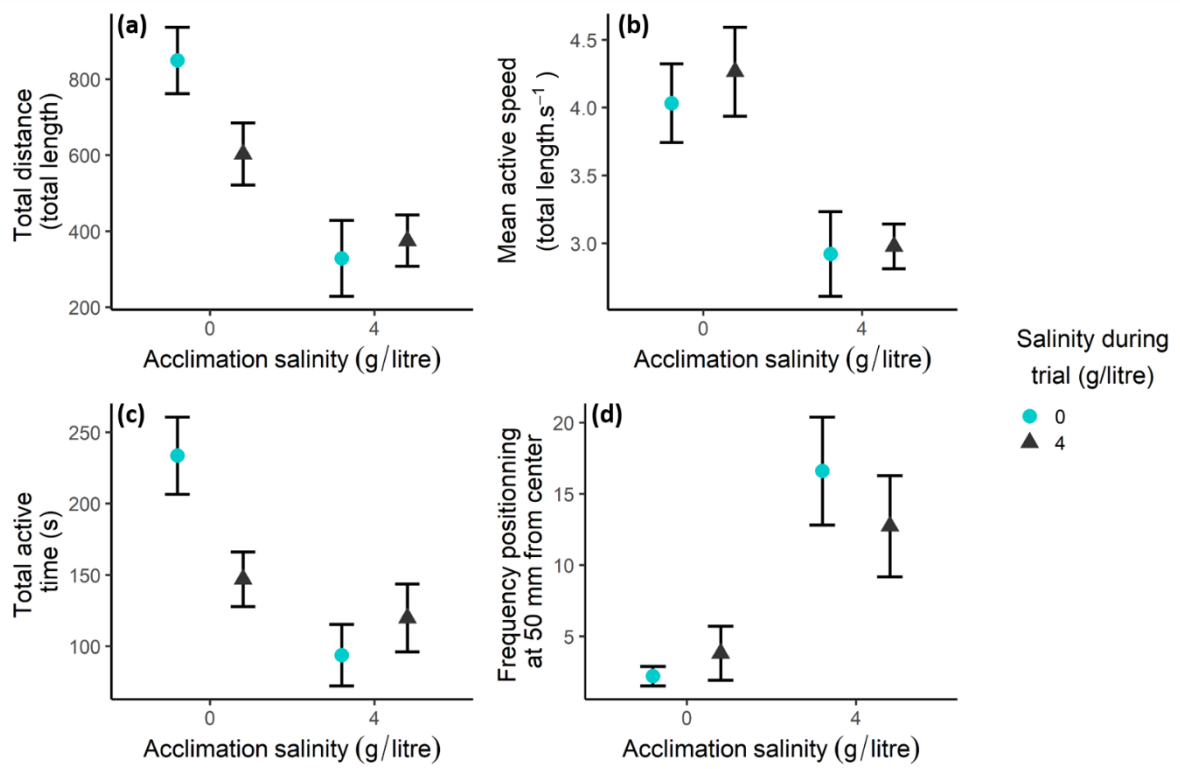
943 Figure 2



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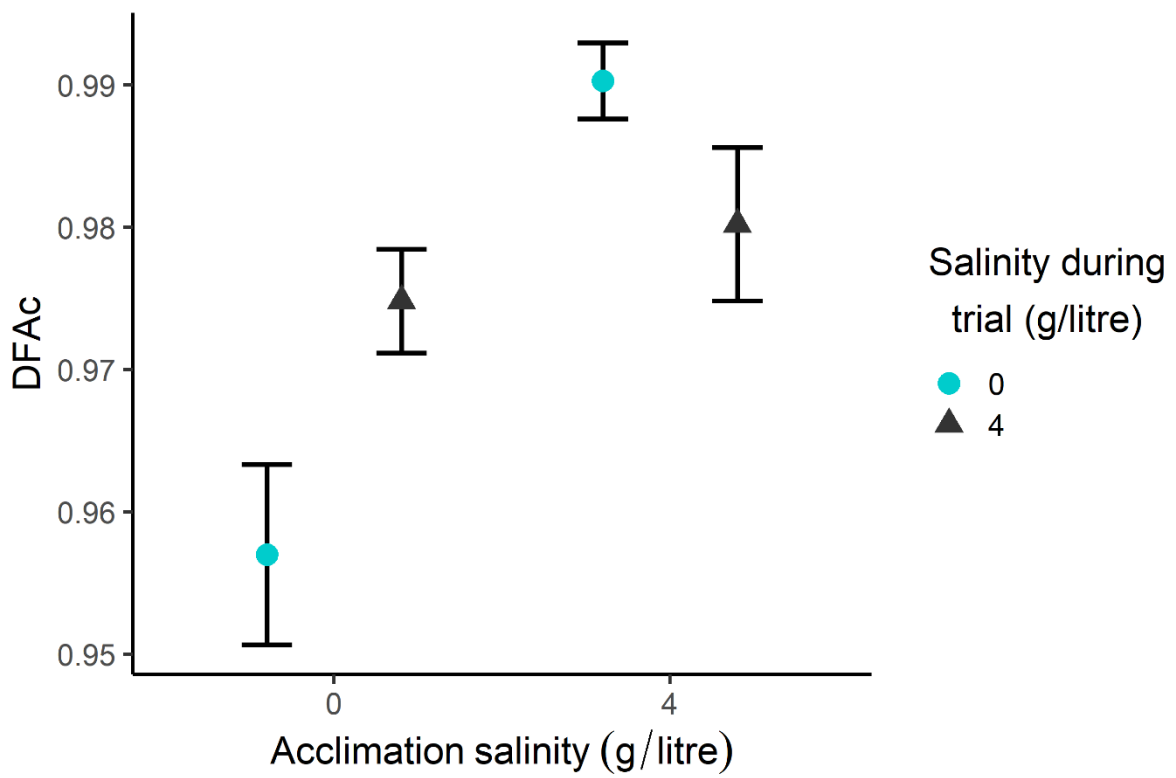
946 Figure 3



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949 Figure 4



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952

953 **Table A1 - Post-hoc analyses.** Differences considered are the differences in mean  
 954 escape distance, total distance, total active time, and DFAC, according to salinity  
 955 during acclimation (2 weeks in freshwater [0 g/litre] or brackish water [4 g/litre]) and  
 956 to salinity during the trial (freshwater [0 g/litre] or brackish water [4 g/litre]).

Variable	Comparisons	Estimate	SE	df	t/z	P	
Mean escape distance	Test 0 Acclimation 0	Acclimation 0 - Acclimation 4	-2.43	2.23	93.8	-1.091	0.278
	Test 4 Acclimation 4	Acclimation 0 - Acclimation 4	5.16	2.23	93.8	2.316	0.023
	Acclimation 0	Test 0 - Test 4	-5.22	1.58	59	-3.309	0.002
	Acclimation 4	Test 0 - Test 4	2.38	1.55	59	1.532	0.131
Total distance	Test 0 Acclimation 0	Acclimation 0 - Acclimation 4	0.855	0.137	113	6.23	<0.001
	Test 4 Acclimation 4	Acclimation 0 - Acclimation 4	0.391	0.137	113	2.848	0.005
	Acclimation 0	Test 0 - Test 4	0.191	0.125	58	1.529	0.132
	Acclimation 4	Test 0 - Test 4	-0.273	0.125	58	-2.181	0.033
Total active time	Test 0 Acclimation 0	Acclimation 0 - Acclimation 4	0.672	0.118	114	5.69	<0.001
	Test 4 Acclimation 4	Acclimation 0 - Acclimation 4	0.243	0.118	114	2.056	0.042
	Acclimation 0	Test 0 - Test 4	0.206	0.109	58	1.888	0.064
	Acclimation 4	Test 0 - Test 4	-0.223	0.109	58	-2.043	0.046
DFAC	Test 0 Acclimation 0	Acclimation 0 - Acclimation 4	0.035	0.009	Inf	4.021	<0.001
	Test 4 Acclimation 4	Acclimation 0 - Acclimation 4	0.006	0.009	Inf	0.664	0.507
	Acclimation 0	Test 0 - Test 4	0.019	0.005	Inf	3.712	<0.001
	Acclimation 4	Test 0 - Test 4	-0.010	0.005	Inf	-2.056	0.040

957