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1 **Spatial distribution and activity patterns as welfare indicators in response to water quality**  
2 **changes in European sea bass, *Dicentrarchus labrax***

3

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15

16 **Abstract**

17 In aquaculture, fish are exposed to unavoidable stressors that can be detrimental for their health and  
18 welfare. However, welfare in farmed fish can be difficult to assess, and, so far, no standardized test  
19 has been universally accepted as a welfare indicator. This work contributes to the establishment of  
20 behavioural welfare indicators in a marine teleost in response to different water quality acute stressors.  
21 Groups of ten fish were exposed to high Total Ammonia Nitrogen concentration (High TAN, 18 mg.L<sup>-1</sup>),  
22 Hyperoxia (200 % O<sub>2</sub> saturation), Hypoxia (20 % O<sub>2</sub> saturation), or control water quality (100% O<sub>2</sub>  
23 saturation and TAN < 2.5 mg.L<sup>-1</sup>) over 1 hour. Fish were then transferred in a novel environment for a  
24 group behaviour test under the same water quality conditions over 2 hours. Videos were recorded to  
25 assess thigmotaxis, activity and group cohesion. After this challenge, plasma cortisol concentration  
26 was measured in a subsample, while individual behavioural response was measured in the other fish  
27 using novel tank diving test. Prior to this study, the novel tank diving test was validated as a  
28 behavioural challenge indicative of anxiety state, by using nicotine as anxiolytic drug. Overall, all  
29 stress conditions induced a decrease in activity, thigmotaxis and group cohesion while only fish  
30 exposed to Hypoxia and High TAN conditions displayed elevated plasma cortisol concentrations. In  
31 *post*-stress condition, activity was still affected but normal behaviour was recovered within the 25  
32 minutes of the test duration. Our work suggests that the activity, thigmotaxis and group cohesion are  
33 good behavioural indicators of exposure to degraded water quality, and could be used as standardized  
34 measures to assess fish welfare.

35 **Keywords:** Fish; Welfare; Water quality; Behaviour; Stress.

## 36 1. Introduction

37 Fish production has expanded importantly during the last decades, both because of the world's  
38 diminishing natural wild resources and the increase in demand for fish products (FAO, 2018).  
39 Aquaculture represented 53 % of the total fish production (including non-food uses) in 2016 (FAO,  
40 2018) and is now recognized as a major food production industry. Thus, as well as for terrestrial  
41 farming industry, concerns about sustainability, environmental issues and animal welfare in  
42 aquaculture are increasing (Conte, 2004; Ashley, 2007; Martins et al., 2010; Martins et al., 2012;  
43 Hixson, 2014; FAO, 2018; Lembo et al., 2019). It is common that under aquaculture conditions, and in  
44 every fish husbandry system, variations of water quality variables such as temperature, pH, oxygen  
45 (O<sub>2</sub>), carbon dioxide (CO<sub>2</sub>) or Total Ammonia Nitrogen concentrations (TAN) occur. Such variations  
46 when they reach a certain threshold, depending on the species *preferendum*, could be considered as  
47 stress factors (stressor) and therefore deleterious for fish health and welfare. The exposure to stressors,  
48 such as degraded water quality may mobilize fish energy for coping with the stressor hereby  
49 decreasing the available energy allocated to growth and reproduction, or directly causes death if the  
50 magnitude of stress is too high (Barton, 2002; Sneddon et al., 2016). Therefore, it can finally affect  
51 fish production and has economic consequences for farmers (Conte, 2004; Lembo et al., 2019).

52 Exposure to stress factors triggers a cascade of biological events within an organism to cope with these  
53 factors. In fish, the hypothalamo-pituitary-interrenal axis (HPI) is involved in the production and  
54 release of cortisol into circulation acting as an activator of the physiological and behavioural responses  
55 (Sumpter, 1997; Sadoul and Vijayan, 2016; Schreck and Tort, 2016). Among the previously cited  
56 variables, oxygen and ammonia concentrations are known to activate the HPI axis when they vary,  
57 leading to the stimulation of cortisol release and the triggering of behavioural adaptive responses  
58 (Knoph and Olsen, 1994; van Raaij et al., 1996; Espmark and Baeverfjord, 2009).

59 Thus, behavioural measurements have proven to be sensitive indicators of the complex existing  
60 biochemical and physiological changes that occur in response to stress (Schreck, 1990; Scherer, 1992;  
61 Schreck et al., 1997; Martins et al., 2012). Behaviours, such as changes in food-anticipatory activity,

62 feed intake, ventilation rate, individual and group swimming activity are commonly used as welfare  
63 indicators (Huntingford et al., 2006; Martins et al., 2012; Huntingford and Kadri, 2014; Carbonara et  
64 al., 2015; Carbonara et al., 2019). Group swimming behaviour is defined as the spatial distribution and  
65 swimming activity of the group of fish held within an aquaculture production unit and it covers shoal  
66 structure, the horizontal and vertical distribution of the group, their swimming speed and direction  
67 (Martins et al., 2012). For instance, exposure to negative *stimuli*, such as poor water quality, is known  
68 to lead to rapid escape movements (Stien et al., 2007; Bratland et al., 2010) or to alter group cohesion  
69 (Domenici et al., 2002; Espmark and Baeverfjord, 2009; Sadoul et al., 2014; Sadoul et al., 2017).  
70 Thus, group swimming behaviour appears to be a sensitive welfare indicator even if it is still lacking  
71 calibration efforts to be precisely translated into an operational welfare indicator; nevertheless some  
72 examples exist (Papandroulakis et al., 2014; Pettersen et al., 2014). Moreover, the appraisal of  
73 negative or positive stimuli and, hence, the psychological dimension of stress as defined for fish by  
74 Galhardo and Oliveira (2009) is seldom tackled in welfare research. There exists however a  
75 complementary measure which is the individual behavioural responses to novel environment and in  
76 particular the novel tank diving test which is worldwide used along with the measure of stereotypies,  
77 such as thigmotaxis to assess anxiety in zebrafish (*Danio rerio*) in ecotoxicology and pharmacology  
78 research (Levin et al., 2007; Egan et al., 2009; Vignet et al., 2014; Macaulay et al., 2015; Alfonso et  
79 al., 2019a). In further details, the novel tank diving test was validated as a tool for evaluating anxiety  
80 by using drugs, such as nicotine. Short exposure to nicotine is known to reduce anxiety in fish, through  
81 its action on nicotinic acetylcholine receptors as demonstrated by the use of specific inhibitors (Levin  
82 et al., 2007; Bencan & Levin, 2008). In the context of novel tank diving test, nicotine-exposure  
83 (bathing) has been shown to be anxiolytic by triggering change in fish space utilization, such as higher  
84 time spent in the top area of the novel tank which translate a relief from bottom dwelling behaviour  
85 that fish would express under predator threat for example. The novel tank diving test could thus be a  
86 helpful non-invasive tool to monitor farmed fish anxiety state *post* stress exposure hereby assessing  
87 psychological stress and contributing to the assessment of positive or negative emotions and, hence  
88 better welfare state determination.

89 Overall, the objectives of the present study were to further contribute to the establishment of  
90 behavioural welfare indicators including the psychological dimension of stress in a model marine  
91 teleost in response to different water quality stressors. Firstly, the novel tank diving test outcome was  
92 validated as a behavioural indicator of anxiety in European sea bass *Dicentrarchus labrax*, using  
93 nicotine as an anxiolytic reference drug. Secondly, behavioural responses of fish group in response to  
94 a novel environment under acute and severe water quality deterioration, including Total Ammonia  
95 Nitrogen (High TAN) increase (18 mg.L<sup>-1</sup>), Hyperoxia (200 % O<sub>2</sub> saturation) and Hypoxia (20 % O<sub>2</sub>  
96 saturation) were evaluated along with cortisol measurement. Finally, individual behaviour expressed  
97 following the same water quality exposures were assessed using the novel tank diving test translated  
98 from ecotoxicology studies.

## 99 **2. Material and methods**

100 Experiments were authorized by ethics committee agreement APAFIS#7098 and all procedures  
101 involving animals were in accordance with the ethical standards of the institution and followed the  
102 recommendations of Directive 2010/63/EU.

### 103 **2.1. Fish rearing**

104 Juvenile European sea bass were hatched and reared at Ifremer Palavas-les-flots research station  
105 (France, 34250) until 280 days *post* fertilization (dpf) according to sea bass rearing standard (Chatain,  
106 1994). They were then transferred to Ifremer L’Houmeau (France, 17137). No mortality occurred  
107 during the transfer between the two facilities. They were then randomly separated in groups of 100  
108 fish into 4 tanks of 400 L (90x90x50 cm). Tanks shared a recirculating system with a flow rate of 4 m<sup>3</sup>  
109 per hour and water was renewed at a rate of 20 % per day. Water temperature was maintained at 21.5  
110 ± 1°C, oxygen around 100 % saturation, and salinity and pH were respectively set to 20.5 ± 1 and 8.3.  
111 The light regime was 13:11 L/D. Total Ammonia Nitrogen (TAN) concentration was < 2.5 mg.L<sup>-1</sup>  
112 (equivalent to 0.1 mg.L<sup>-1</sup> [NH<sub>3</sub>]). The fish were hand-fed using commercial diet from Le Gouessant  
113 (France) once a day each morning at 9:00 at 1 % of biomass. Fish were reared in L’Houmeau for 3  
114 months before the first experiment.

## 2.2. Translating and validating the novel tank diving test in European sea bass

The novel tank diving test assessing position choice along the vertical dimension has been previously validated as a metric of adaptation to a novel environment and a proxy of the anxiety level of individual fish (Levin et al., 2007; Egan et al., 2009). The first objective of this work was to adapt the test protocol (observation duration, tank size relative to fish size) to European sea bass, and then to validate the measure of anxiety using nicotine as a reference anxiolytic drug.

Two groups of fish (385 dpf, n=24 per group) were randomly selected and transferred from the home rearing tank to two 50 L tanks into the experimental room. After a 1-hour acclimation period, control fish (n=24) were transferred one by one in a 3L tank with the same water quality for 8 min (24.5 x 15 x 13.5 cm, AquaBox 3; Aqua Schwarz GmbH). The same protocol was followed for the nicotine-bathed fish (n=24), except they were bathed one at a time during five min in a 3L tank containing 5 mg.L<sup>-1</sup> nicotine solution (Pestanal®, Sigma Aldrich) then placed in normal plain water for the next three minutes. After a 1-hour acclimation period, control fish (n=24) were transferred one by one in a 3L tank (24.5 x 15 x 13.5 cm, AquaBox 3; Aqua Schwarz GmbH) filled with normal plain water for five min and then transferred to a second 3L tank (same water quality) for an extra three min. The same protocol was followed for the nicotine-bathed fish (n=24), except they were first bathed one at a time during 5 min in a 3L tank containing 5 mg.L<sup>-1</sup> nicotine solution (Pestanal®, Sigma Aldrich) added to normal water and then transferred in plain water for the next three minutes. This two steps bathing protocol was applied to ensure correct elimination of nicotine (at least in the gills) and to ensure similar handling for both conditions. For both treatment, directly after the bathing period, the individual fish was gently transferred in a novel tank containing normal plain water (29 x 21 x 17 cm, 10 L trapezoid tank from Aquatic Habitat. Inc.), and a video was recorded in side view during 25 minutes. For space occupancy analysis, the tank was virtually separated into two areas according to Egan et al. (2009): top area including one half of the volume and bottom area including the other half. Time spent in top area (s) and latency to enter top area (s), variables which are both indicative of anxiety level, were measured. In addition, distance travelled (cm) and number of transitions between

141 areas, indicative of fish activity level were also measured. Variables were recorded in each frame and  
142 they were summed over periods of 5 min or over the whole test duration (*i.e.* 25 min).

### 143 **2.3. Exposure to stress condition and water quality characterization**

144 In the morning prior to the experiment (*i.e.* exposure to stress condition), a group of 10 fish was gently  
145 randomly caught from the rearing tank and transferred to the behavioural room where they were  
146 maintained in a tank (70 L, height 48 cm, diameter 49.5 cm) filled with 60 L of the same water as in  
147 their home tank (**Figure 1**). Fish were kept under standard condition during 1 h for tank acclimation  
148 and then, during the next hour, one of the four following conditions was applied *i.e.* Control, High  
149 Total Ammonia Nitrogen (TAN) concentration, Hyperoxia and Hypoxia. Experiments were performed  
150 in triplicates (n=10 fish x 3 run per condition).

151 For the control condition, fish were maintained under the same standard condition than in the rearing  
152 room (*i.e.*, at  $21.5 \pm 1^\circ\text{C}$ , 100 %  $\text{O}_2$  saturation and  $\text{TAN} < 2.5 \text{ mg.L}^{-1}$ ). For high TAN condition, 8.5 g  
153 of ammonium chloride  $\text{NH}_4\text{Cl}$  (Fluka 09711, Sigma Aldrich) dissolved in 0.5 L of water was added  
154 twice at 30 min interval to reach a targeted TAN concentration of  $18 \text{ mg.L}^{-1}$  which corresponds to 1.6  
155  $\text{mg.L}^{-1}$  of  $\text{NH}_3$ . Three water samples (50 mL) were taken to quantify *a posteriori* the TAN  
156 concentration: (i) before adding ammonium chloride, (ii) at the beginning and (iii) at the end of the  
157 novel environment in group. Samples were stored at  $-22^\circ\text{C}$  before further analysis. Then, TAN  
158 concentration was quantified using a spectrophotometer with continuous flow (Alliance Integral  
159 Futura, Frépillon, France) and analytical method is described below.

160 Samples were filtered using GF/F  $0.7\mu\text{m}$  filter (Whatman, Maidstone, United Kingdom). TAN  
161 concentration was quantified using a spectrophotometer with continuous flow (Alliance Integral  
162 Futura, Frépillon, France) using colorimetric method. The solutions used for the calibration analyses  
163 that were performed the same day came from stock solutions from  $0.1\text{g.L}^{-1}$  to  $1\text{g.L}^{-1}$  of ammoniacal  
164 nitrogen ( $\text{NH}_4^+$ ) stocked at a temperature of  $8^\circ\text{C}$ . The calibration curve of ammoniacal nitrogen  
165 showed a high  $R^2$  validating the procedure ( $\text{NH}_4^+=1.439 \times \text{OD} - 0.002$ ;  $R^2 = 0.99$ , where OD is the  
166 optical density measured using the spectrophotometer).



167 The NH<sub>3</sub> concentration was determined using the following equation as described by Johansson and  
168 Wedborg (1980):

$$169 \quad [NH_3] = \frac{[NH_4^+]}{K_1 \times [H^+]}$$

170 Where  $[H^+] = 10^{-pH}$  and  $\log K_1 = -0.467 + 0.00113 \times \text{salinity} \times 2887.9/\text{temperature (K)}$  according to  
171 Johansson and Wedborg (1980).

172 For Hypoxia condition, oxygen concentration was slowly lowered to reach 20 % of O<sub>2</sub> saturation using  
173 nitrogen bubbling. For Hyperoxia condition, oxygen concentration was slowly increased to reach 200  
174 % of O<sub>2</sub> saturation using oxygen bubbling. Oxygen concentration was monitored every 5 min during  
175 the entire experiment using an Oxygen probe (Oxi 3310, WTW, Xylem Analytics Germany Sales  
176 GmbH & Co. KG). Oxygen concentration, NH<sub>4</sub><sup>+</sup> and NH<sub>3</sub> concentrations recorded over the  
177 experimental duration are presented below.

178 For control condition, water oxygen concentration was maintained at 100% saturation (at 21.5 ± 1°C)  
179 and TAN concentration was < 2.5 mg.L<sup>-1</sup> during both bathing and novel environment in group. For  
180 High TAN condition, oxygen concentration was above 100 % and TAN concentrations were 15.3 ±  
181 4.2 and 18.8 ± 7.6 mg.L<sup>-1</sup> at the beginning and at the end of the test respectively, corresponding to  
182 UIA-N concentrations of 1.34 ± 0.37 and 1.64 ± 0.67 mg.L<sup>-1</sup>. For both Hyperoxia and Hypoxia  
183 conditions, TAN concentration was maintained at less than 2.5 mg.L<sup>-1</sup>. For the hyperoxia condition,  
184 oxygen concentration increased slowly during the bathing period to reach 208 ± 5.6 % of saturation,  
185 while for the hypoxia it slowly decreased to reach 21.3 ± 3.8 % of saturation before the start of the  
186 novel environment challenge in group. All conditions were then maintained during two hours until the  
187 end of the group test (**Figure 2**). In the high TAN condition, the concentration of NH<sub>3</sub> represents half  
188 of the LC50 concentration reported for European sea bass after 96 h of exposure under similar  
189 hydrological conditions (i.e. temperature=17.5°C, salinity=34, and pH=8.15) than in our experiment  
190 (Person-Le Ruyet et al., 1995).

191 After the initial bathing period, the fish group was challenged in a novel environment with the same  
192 water quality (see section 2.4.1). After 2 hours, fish from two replicates were then monitored  
193 individually into novel tank diving test in plain system water (see section 2.4.2, n=20 for control, n=20

194 for High TAN and n=18 for Hyperoxia and Hypoxia). The third replicate was used to quantify plasma  
195 cortisol concentration following the challenge in group (n=10 per condition, see section 2.5). Fish  
196 were randomly selected for blood sampling or for performing novel tank diving test. After blood  
197 sampling (n=9 or 10 fish per condition) or novel tank diving test, all fish were measured for weight (g,  
198 to the nearest mg) and standard length (cm, to the nearest mm) under the same anaesthesia conditions  
199 (see section 2.5).

## 200 **2.4. Behavioural procedures**

201 All behavioural experiments were performed in a dedicated room where environmental parameters  
202 were identical to rearing conditions. All videos were recorded at 25 frame.s<sup>-1</sup> with an analogue camera  
203 ICD-48E (Ikegami) and a 2.1-13.5 lens (Fujinon) linked to a computer with an acquisition card and  
204 EthoVision XT 10.0 software (Noldus, The Netherlands). Data extraction and analyses were  
205 performed using EthoVision XT 13.1 software. Swaps between individuals during novel environment  
206 in group were manually corrected using the track editor module (Noldus, The Netherlands).

### 207 *2.4.1. Novel environment in group*

208 After the bathing period, the entire group of fish (n=10) was gently transferred into a novel arena (110  
209 cm x 110 cm x 6 cm, 70 L). After 1 min, video recording started for 2 hours in top view. For each  
210 condition, water parameters were maintained similar to those obtained at the end of the bathing period  
211 for the respective condition following the same procedure explained in section 2.2.2.

212 For space occupancy, the visualization of heat maps was produced for each 5-min period using  
213 Ethovision XT 13 (Noldus The Netherlands). For further analysis, the arena was separated into two  
214 areas: Centre area including one half of the volume and periphery area including the other half; time  
215 spent in periphery (s), indicative of thigmotaxis behaviour (Ferrari et al., 2014), was recorded.  
216 Distance travelled by each fish (cm), indicative of individual fish activity, and the interindividual  
217 distances (cm), indicative of group cohesion (Buske and Gerlai, 2011), were also recorded. Variables  
218 were recorded for each frame and they were summed (for Distance travelled) or averaged (for time

219 spent in periphery and interindividual distances) over period of 5 min every 30 minutes (*i.e.*, four  
220 sampling periods: 0-5 min, 30-35 min, 60-65 min and 90-95 min).

#### 221 2.4.2. *Novel tank diving test post-stress condition*

222 After novel environment challenge in group, fish were gently transferred individually into a novel tank  
223 (same as the one used for method validation with nicotine, see above) containing plain system water.  
224 Fish swimming activity and vertical position was monitored during 25 min to assess anxiety state and  
225 behavioural recovery following stressful conditions exposure. For space occupancy and fish  
226 swimming activity analyses, the same procedure as presented before in section 2.2 was followed.

### 227 2.5. Cortisol measurement

228 Immediately after the end of the observation period in the novel environment challenge, fish were  
229 gently caught and transferred into a 10 L tank which contained 500  $\mu\text{L.L}^{-1}$  of a benzocaine stock  
230 solution (50  $\text{g.L}^{-1}$  in 100 % ethanol; Benzocaine Sigma-Aldrich, Saint-Quentin Fallavier, France).  
231 Blood samples were obtained within 3 minutes from the venous sinus with heparinised syringes.  
232 Thereafter, blood was centrifuged (5 min at 4000 g) to obtain plasma samples which were stored at -  
233 22°C until further analyses. Plasma cortisol concentration was determined by ELISA (RE52061, IBL  
234 International, Hamburg, Germany) using a Synergy-HT (BioTek Instruments, Winooski, VT, USA)  
235 following manufacturer instructions.

236 At the end of the experiment, all fish were euthanized and sexed using an overdose of benzocaine (1  
237  $\text{mL.L}^{-1}$  from the stock solution described above), following the recommendations of Directive  
238 2010/63/EU.

### 239 2.6. Statistical analyses

240 Statistical analyses were performed using Statistica 9.0 software (StatSoft, USA). All statistical  
241 analyses were carried out at a 5 % level of significance and values are represented as mean  $\pm$  SEM  
242 except where otherwise mentioned. Normality and homoscedasticity were tested *a priori* using  
243 Shapiro-Wilks test and when sample sizes were too small non parametric statistics were used.

244 Fish weight and length were compared between conditions using one-way ANOVA to ensure no bias  
245 existed for behavioural and physiological responses interpretation.

246 For validation of the Novel tank diving test, time spent in top area and distance travelled were  
247 compared between fixed factors (Nicotine bathed *vs.* Control) with a repeated-measures ANOVA  
248 (with 5 periods, *i.e.* 5-min, 10-min, 15-min, 20-min and 25-min) and a Tukey HSD *post-hoc* test. A  
249 Mann-Whitney U-test was also performed to compare between conditions, total time spent in top area,  
250 latency to enter top area, total distance travelled by fish and number of transitions between top and  
251 bottom areas.

252 For Novel environment challenge in group, distance travelled, interindividual distances and time spent  
253 in periphery area were compared between conditions with a Factorial ANOVA (with 4 periods, *i.e.* 0-  
254 5-min, 30-35-min, 60-65min, 90-95min) followed by Tukey HSD *post-hoc* test.

255 For novel tank diving test *post-stress* condition, time spent in top area and distance travelled were  
256 compared between conditions with a repeated-measures ANOVA (with 5 periods, *i.e.* 5-min, 10-min,  
257 15-min, 20-min and 25-min) and a Tukey HSD *post-hoc* test. A One-way ANOVA followed by Tukey  
258 HSD *post-hoc* test was performed to compare between conditions the total time spent in top area,  
259 latency to enter top area, total distance travelled by fish and number of transitions

260 Finally, since sex had no significant effect on plasma cortisol concentration, a Mann-Whitney U-test  
261 was performed to compare cortisol values between control and each condition.

### 262 **3. Results**

263 Overall, 168 fish were used for the different experiments: 48 for the validation of the novel tank diving  
264 test (body weight:  $50.8 \pm 0.6$  g, standard length:  $15.4 \pm 0.9$  cm,  $n=24$  per group); 120 for the stress  
265 condition exposures ( $51 \pm 1$  g,  $15.3 \pm 0.2$  cm,  $n=30$  per group), batches were homogeneous between  
266 conditions ( $F=1.5$ ,  $df=3$ ,  $p=0.23$  and  $F=2$ ,  $df=3$ ,  $p=0.13$  for body weight and standard length  
267 respectively).

#### 268 **3.1. Validation of novel tank diving test in European sea bass**

269 Irrespective of the condition (Nicotine bathed vs. Control) fish progressively explored the top area of  
270 the novel tank ( $F=6.0$ ,  $df=4$ ,  $p<0.001$ ). Nicotine bathed-fish spent significantly more time in the top  
271 area than control ones during each 5-min period of the test ( $F=31.8$ ,  $df=1$ ,  $p<0.001$ ; **Figure 3.A**) as  
272 well as over the total duration of the test ( $Z=4.8$ ,  $p<0.001$ ; **Figure 3.B**). Fish also progressively  
273 travelled more distance during the experiment whatever the condition ( $F=13.3$ ,  $df=4$ ,  $p<0.001$ ) but  
274 nicotine bathed-fish travelled significantly more distance than control ones during each 5-min period  
275 of the test ( $F=15.9$ ,  $df=1$ ,  $p<0.001$ ; **Figure 3.C**) as well as over the total duration of the test ( $Z=3.5$ ,  
276  $p<0.001$ ; **Figure 3.D**). Nicotine bathed-fish swam more between the two areas and entered quicker in  
277 the top area than control ones ( $Z=4.3$ ,  $p<0.001$  and  $Z=-5.0$ ,  $p<0.001$  respectively; **Figure 3.E,F**).

### 278 3.2. Novel environment in group

279 Heatmaps, representing the mean location frequency of fish from different conditions and during  
280 different periods of the novel environment challenge in group, are plotted in **Figure 4**. For control  
281 condition, fish responded to the group test by staying mainly in the periphery of the arena during the  
282 first five minutes of the test. Progressively, they explored the centre of the arena. On the contrary,  
283 other groups of fish (*i.e.* High TAN, Hyperoxia and Hypoxia), were located in the centre area from the  
284 beginning of the test. In addition, fish dispersion increased over the test duration for exposed animals,  
285 while fish from the control condition stayed aggregated during the entire experiment.

286 Under stress conditions, distance travelled by fish differed between conditions ( $F=212.7$ ,  $df=3$ ,  
287  $p<0.001$ ), and periods ( $F=33.2$ ,  $df=3$ ,  $p<0.001$ ) with a significant interaction observed over time  
288 ( $F=10.3$ ,  $df=9$ ,  $p<0.001$ ). Control fish travelled progressively more distance over the test duration;  
289  $3178 \pm 268$  cm during period 0-5 min vs.  $5148 \pm 254$  cm during period 90-95 min ( $p<0.001$ ). Fish  
290 from High TAN, Hyperoxia and Hypoxia conditions showed a lower distance travelled compared to  
291 control fish during the entire test ( $p<0.05$  for all conditions) and it was stable over time (**Figure 5.A**).

292 Interindividual distances, indicative of group cohesion, differed between conditions ( $F=449.3$ ,  $df=3$ ,  
293  $p<0.001$ ), periods ( $F=17.4$ ,  $df=3$ ,  $p<0.001$ ) with a significant interaction between conditions and  
294 periods ( $F=29.1$ ,  $df=9$ ,  $p<0.001$ ). Fish from control condition progressively decreased their

295 interindividual distances during the challenge;  $48.3 \pm 1.4$  cm during period 0-5 min vs.  $41.9 \pm 1.1$  cm  
296 during period 90-95 min ( $p < 0.001$ ). During the 0-5 min period, fish from control and Hypoxia  
297 conditions showed similar interindividual distances ( $p = 0.06$ ), whereas fish from High TAN and  
298 Hyperoxia conditions swam closer to each other ( $p < 0.001$  for both conditions). However, starting from  
299 the 60-65 min to the 90-95 min period, fish from both High TAN and Hypoxia conditions showed  
300 higher dispersion than control fish ( $p < 0.001$ ) while fish from Hyperoxia condition continued to display  
301 lower dispersion than control fish from the beginning to the end of the testing period ( $p < 0.001$ ; **Figure**  
302 **5.B**).

303 Time spent in periphery area also differed between conditions ( $F = 15.3$   $df = 3$ ,  $p < 0.001$ ), not between  
304 periods ( $F = 0.5$ ,  $df = 3$ ,  $p = 0.66$ ) but the interaction between conditions and periods was significant  
305 ( $F = 10$ ,  $df = 9$ ,  $p < 0.001$ ). Fish from control condition spent more time in the centre area from the second  
306 time period (30-35 min) until the end (90-95 min) compared to the first 0-5 min period ( $p = 0.003$ ,  
307  $p = 0.003$  and  $p < 0.001$  for 30-35 min, 60-65 min and 90-95 min respectively). During the first 0-5 min  
308 period of the test, fish from High TAN, Hyperoxia and Hypoxia conditions spent less time in the  
309 peripheric area than control fish ( $p < 0.001$  for both conditions) whereas they spent the same time in the  
310 periphery during the rest of the test, with the exception of High TAN which still spent more time in  
311 Centre than Control during 30-35 period (**Figure 5.C**).

### 312 **3.3. Novel tank diving test *post*-stress condition**

313 Irrespective of the condition, fish progressively explored the top area of the novel tank ( $F = 5.9$ ,  $df = 4$ ,  
314  $p < 0.001$ ). There was an effect of condition ( $F = 11.8$ ,  $df = 3$ ,  $p < 0.001$ ) and an interaction between  
315 conditions and period were also found ( $F = 4.4$ ,  $df = 12$ ,  $p < 0.001$ ; **Figure 6.A**). The total time spent in  
316 top area differed according to conditions ( $F = 11.8$ ,  $df = 3$ ,  $p < 0.001$ ; **Figure 6.B**) with High TAN fish  
317 spending significantly more time in the top area than the three other conditions ( $p < 0.001$ ).

318 Distance travelled by fish varied in relation to the duration of the test ( $F = 13.0$ ,  $df = 4$ ,  $p < 0.001$ ),  
319 conditions ( $F = 15$ ,  $df = 3$ ,  $p < 0.001$ ) and interactions between period and conditions were significant  
320 ( $F = 3$ ,  $df = 12$ ,  $p < 0.001$ ; **Figure 6.C**). Total distance travelled by fish also differed between conditions

321 (F=15, df=3, p<0.001; **Figure 6.D**). High TAN, Hyperoxia and Hypoxia fish travelled less distance  
322 than control fish during the novel tank test (p<0.001 for all conditions). No difference was observed  
323 for the number of transitions between the two areas between conditions (F=2.3, df=3, p=0.8; **Figure**  
324 **6.E**) as well as for the latency to enter into the top area (F=0.5, df=3, p=0.7; **Figure 6.F**).

### 325 **3.4. HPI axis response**

326 Plasma cortisol concentrations for fish from Hyperoxia condition were not different from control fish  
327 values (Z=0.1, p=0.93, **Figure 7**). On the contrary, plasma cortisol concentrations were higher for both  
328 High TAN and Hypoxia conditions compared to control values (Z=-3.5, p<0.001 and Z=-2.1, p=0.04  
329 respectively).

## 330 **4. Discussion**

331 Overall, this study demonstrates that commonly used behavioural tests in neurobiology or  
332 ecotoxicology on model species are relevant for evaluating anxiety in a model marine farmed fish and  
333 this opens new opportunities to evaluate psychological stress following environmental perturbation.  
334 Moreover, we have shown that activity, group cohesion and thigmotaxis are relevant behavioural  
335 indicators revealing acute exposures to High Total Ammonia Nitrogen (TAN), Hyperoxia and  
336 Hypoxia in a group situation. Individual bottom dwelling behaviour was also assessed in response to  
337 the same stressors using the novel tank diving test and proved to be sensitive.

338 Fish display typical swimming features in a new environment, e.g. bottom dwelling, which have been  
339 used to define a suitable procedure to measure anxiety or anxiety-like responses. Common protocols  
340 exist for using these behavioural features for anxiety phenotyping in zebrafish (Levin et al., 2007) and  
341 they were successfully transferred to other small model species such as three-spine stickleback and  
342 fathead minnow (Margiotta-Casaluci et al., 2014; Thompson et al., 2016). To our knowledge, our  
343 study represents the first attempt to adapt this test for a larger fish of commercial and ecological  
344 importance such as European Sea bass (Vandeputte et al., 2019). Since this species is known to be a  
345 highly stress responding fish (Levin et al., 2007; Fanouraki et al., 2011; Alfonso et al., 2019b), we  
346 extended the test duration to 25 minutes. Despite this longer test period (usually between 5 and 10

347 minutes for other species) the time spent in the upper part of the tank remained limited to  
348 approximately 10%.

349 Nicotine has been found to reduce anxiety in zebrafish through activation of acetylcholine nicotinic  
350 receptors (Levin et al., 2007; Bencan & Levin, 2008). In the context of novel tank diving test,  
351 nicotine-exposure has been shown to be anxiolytic by triggering change in fish space utilization. In the  
352 present study, acute treatment with nicotine induced the same relief of anxiety as observed in zebrafish  
353 (Levin 2007). It is to note however that the concentration we used, 5 mg.L<sup>-1</sup>, was much lower than the  
354 one efficient in zebrafish (100 mg.L<sup>-1</sup>) and a concentration of 50 mg.L<sup>-1</sup> was not able to induce change  
355 in bottom dwelling behaviour in zebrafish (Levin et al., 2007). Indeed, in zebrafish, exposure to  
356 nicotine results in a release from bottom dwelling behaviour (i.e. swimming in the top area of the  
357 novel tank) over test duration and increases the locomotor activity (Levin et al., 2007; Bencan and  
358 Levin, 2008). In the present study, after exposure to nicotine, sea bass similarly spent more time in the  
359 top area and were more active (i.e., travelled more distance and showed a higher transitions number  
360 between tank areas) compared to control. Altogether, these results advocate that anxiolytic effects of  
361 nicotine are behaviourally observable in European sea bass, as in zebrafish. These results firstly  
362 suggest conservation of the sensitivity to nicotine and the associated behavioural response in  
363 phylogenetically relatively distant fish species. This is supported by the high conservation of nicotinic  
364 acetylcholine receptors in fishes as recently shown (Pedersen et al., 2019) and the fact that we have  
365 found sequence coding for protein sharing high similarity with several nicotinic receptors in the  
366 genome of European sea bass (not shown). Secondly, this study supports the fact that the novel tank  
367 diving test is a promising tool for monitoring anxiety in marine teleost such as European sea bass.

368 The four experimental conditions had very distinct water qualities. In the control condition, oxygen  
369 saturation was at 100 % and TAN concentration was under 2.5 mg.L<sup>-1</sup> while TAN concentration was  
370 approximately 8 fold higher in High TAN condition and oxygen saturation was 2 fold higher and 5  
371 fold lower in Hyperoxia and Hypoxia conditions respectively. TAN concentrations in the High TAN  
372 condition corresponded to a concentration of NH<sub>3</sub> of 1.6 mg.L<sup>-1</sup> ( half of the LC50, Person-Le Ruyet et  
373 al., 1995). Oxygen saturations chosen during the Hyperoxia and Hypoxia conditions were previously



374 described to affect fish physiology and behaviour during chronic exposure without being lethal  
375 (Chapman and Mckenzie, 2009; Espmark and Baeverfjord, 2009; Rimoldi et al., 2016). Both  
376 concentrations of oxygen and TAN used in the present study were quite extreme but can occasionally  
377 occur (or even co-occur) in aquaculture conditions in case of technical failures.

378 In the novel environment in group, control fish displayed thigmotaxis behaviour and spent most of the  
379 time in the periphery area during the first 5 minutes of the test. Indeed, handling fish from bathing tank  
380 to a novel environment is known to induce a typical thigmotaxis behavioural response. This  
381 thigmotaxis behaviour has been shown to indicate an anxiety state (Prut and Belzung, 2003; Schnorr et  
382 al., 2012). Interestingly, fish from High TAN, Hyperoxia and Hypoxia conditions spent less time in  
383 the periphery area than control fish, which suggest that they were not displaying anxiety-like  
384 behaviour contrary to control fish in response to handling. High TAN, Hyperoxia and Hypoxia are  
385 known to directly affect fish survival (Magaud et al., 1997; Person-Le Ruyet and Boeuf, 1998; Shimps  
386 et al., 2005; Rimoldi et al., 2016). Indeed, fish survival can be impacted in many ways following their  
387 exposure to one of these stressors. For instance, in hypoxic conditions, the oxygen quantity is limited,  
388 therefore fish have to adapt their physiology and behaviour to maximize oxygen uptake and avoid  
389 death by asphyxia (Chapman & Mckenzie, 2009). Then, Hyperoxia may alter the equilibrium of ions  
390 in the gill, causes a decrease in the ventilatory frequency inducing oxidative damages or triggers  
391 diseases and thus can cause fish death (Dejours et al. 1977; Liepelt et al.1995; Brauner et al. 2000).  
392 Finally, Ammonia exposure can affect osmoregulation, represses the immune system, or causes  
393 asphyxiation leading to hyperventilation, convulsions and death (Randall and Tsui, 2002; Eddy, 2005;  
394 Camargo and Alonso, 2006). By contrast to these stressors, handling is mostly documented for its  
395 effects on the HPI axis, through cortisol release (Barton 2002). To our knowledge, handling does not  
396 have direct effects on survival except if fish are injured which was not the case in our experiment.  
397 Therefore, we hypothesise that when fish are coping with stressors affecting survival, the thigmotaxis  
398 expression in response to a psychological stressor is overruled, possibly through endorphin release.  
399 Further mechanistic studies are needed to better understand what changes within the fish are related to  
400 these behavioural adaptations under multi-stressors exposure. Furthermore, during the test in group

401 situation, control fish travelled progressively more distance over the test duration whereas High TAN,  
402 Hyperoxia and Hypoxia conditions showed lower and stable distance travelled. Such lower swimming  
403 activity under high TAN was described before in rainbow trout (Shingles et al., 2001) and under  
404 hypoxia conditions in common sole (*Solea solea*; Dalla Via et al. (1998)) and dogfish (*Scyliorhinus*  
405 *canicula*; Metcalfe and Butler (1984)) whereas hyperoxia was shown to lead to higher variability of  
406 the activity pattern in Atlantic salmon (Espmark and Baeverfjord, 2009). It is, however, important to  
407 note that behavioural responses under hypoxia were found to be clearly dependant on the intensity and  
408 duration of the hypoxia challenge and the species (Chapman and Mckenzie, 2009). NH<sub>3</sub> is known to  
409 cause asphyxiation by reducing the blood oxygen-carrying capacity and hence alter the swimming  
410 performances reported above (Shingles et al., 2001; Camargo and Alonso, 2006). Indeed, decrease in  
411 oxygen or increase in TAN concentration both lead to reduced active metabolic rate (Muusze et al.,  
412 1998; Shingles et al., 2001) and may explain why exposed fish are being less active than controls. It  
413 seems that fish adopted a “wait and see” strategy consisting in minimizing energy expenditure and  
414 waiting for an improvement in water quality (van Raaij et al., 1996; Clingerman et al., 2007). Finally,  
415 concerning interindividual distances, during the 0-5 min period of novel environment in group, fish  
416 from Control and Hypoxia conditions expressed the same group cohesion whereas fish from high TAN  
417 and Hyperoxia conditions were closer to each other. Fish from Hyperoxia condition stayed closer  
418 during all test duration while both fish from High TAN and Hypoxia conditions displayed lower group  
419 cohesion than control fish from 60-65 to 90-95 min periods. Interestingly, Domenici et al. (2002)  
420 reported the same larger dispersion in Atlantic herring under O<sub>2</sub> saturation of 20 % and same results  
421 were observed in Atlantic salmon under O<sub>2</sub> saturation of 150 % (Espmark and Baeverfjord, 2009).  
422 Lower group cohesion was also observed in rainbow trout under other stress factor such as  
423 hypercapnia by Sadoul et al. (2017). When given the possibility, it is well known that fish are able to  
424 avoid stressful environment such as hypoxic waters, high ammonia or high CO<sub>2</sub> concentrations  
425 (Richardson et al., 2001; Clingerman et al., 2007; Skjæraasen et al., 2008; Herbert et al., 2010).  
426 Therefore we suggest that fish interindividual distance increases to maximize oxygen uptake  
427 (Domenici et al., 2017) and thus could be used as a relevant welfare indicator (Juell, 1995; Espmark  
428 and Baeverfjord, 2009). This behavioural strategy can also be perceived as a decrease in attention

429 and/or used for saving energy for a better coping with stress favouring a return to a homeostatic state.  
430 Additional experiments are however required to fully understand the observed disruptions of the shoal  
431 cohesion but it overall fits well with the on-growing research effort about the effects of abiotic factors  
432 on fish group behaviour (Weetman et al., 1999; Domenici et al., 2002; Espmark and Baeverfjord,  
433 2009; Colchen et al., 2017; Domenici et al., 2017; Sadoul et al., 2017; Colson et al., 2019).

434 Concerning the HPI axis responsiveness, plasma cortisol concentrations are always high for European  
435 sea bass upon stress exposure since this species is known to be high cortisol responder (Rotllant et al.,  
436 2003; Samaras et al., 2016; Alfonso et al., 2019b). In undisturbed conditions, plasma cortisol values  
437 are expected to be around 100 ng.L<sup>-1</sup> but it can reach a value 10 fold higher upon stress (Samaras et al.,  
438 2018; Alfonso et al., 2019b). In addition to the confinement stress in the bathing tank, fish also  
439 experienced two handling stresses, one transfer between the rearing and the bathing tanks and then  
440 between the bathing and the experimental tanks explaining the high cortisol values measured in all  
441 conditions. Measured plasma cortisol concentrations were similar between fish exposed to Hyperoxia  
442 and control fish but were higher in fish exposed to Hypoxia and High TAN conditions than control. A  
443 similar increase in cortisol release was previously reported in Atlantic salmon following chronic  
444 exposure to ammonia (0.15 mg.L<sup>-1</sup>, (Knoph and Olsen, 1994) and hyperoxia (150 % O<sub>2</sub> saturation,  
445 (Espmark and Baeverfjord, 2009) or acute exposure to hypoxia (25 % of O<sub>2</sub> saturation) in rainbow  
446 trout (van Raaij et al., 1996). Behavioural disruptions observed in Hyperoxia treated fish did not  
447 translate in terms of cortisol values compared to control fish, suggesting the possible implication of  
448 other physiological mechanisms, such as plasma catecholamines as previously shown in rainbow trout  
449 (van Raaij et al., 1996; Gesto et al., 2013; 2015). Interestingly, within a species, individuals are known  
450 to differ from each other in terms of amplitude of primary stress response (e.g. cortisol and  
451 catecholamines) and, along with divergent behavioural responses, that defines individual coping styles  
452 (Castanheira et al., 2017). Therefore, it would be interesting in future studies to include  
453 complementary coping style characterizations to investigate the link between coping style, behavioural  
454 responses within a group and primary responses upon stress exposure. This would help in assessing  
455 whether different strategies co-exist to cope with these kinds of stressors within a fish group.

456 After 2 h-exposure to any of the tested conditions (High TAN, Hyperoxia and Hypoxia), fish travelled  
457 less distance in novel tank diving test than control fish during the first 5 minutes in system water.  
458 Thereafter all fish reached the same activity level. Such behavioural recovery from hypoxia in  
459 European sea bass is rapid and similar to that reported in Nile Tilapia (*Oreochromis niloticus*, Xu et al.  
460 (2006)). To our knowledge, it is the first time that behavioural recovery from Hyperoxia and High  
461 ammonia concentration has been monitored. In addition to distance travelled, fish previously exposed  
462 to High TAN explored the top area more than control fish and decreased progressively time spent in  
463 top to reach control's level after 20 min in system water. On the contrary, the two other conditions  
464 (*i.e.*, Hyperoxia and Hypoxia) explored progressively the top area in the same way than control fish.  
465 The fast release from bottom dwelling (or the search for access to water surface) observed in High  
466 TAN exposed fish resembled the surface swimming observed for nicotine-exposed fish. As explained  
467 before for the novel environment test in group situation, the lower anxiety-like behaviour observed in  
468 High TAN fish could also be the result of fish setting priority to cope with TAN instead of expressing  
469 psychological stress effects *i.e.* a relief of anxiety.

## 470 **Conclusion**

471 In conclusions, our results showed that thigmotaxis, swimming activity, group cohesion and bottom  
472 dwelling behaviour are reliable behavioural indicators of health and welfare status in European sea  
473 bass. Moreover, this study reports the fact that it is important to distinguish between stressors that  
474 could affect survival and psychological stressors such as handling or confinement alone. Besides  
475 cortisol, other molecular factors should be sought for to fully understand individual stress responses  
476 depending on the type and/or duration of stressors. Finally, the novel tank diving test seems to be  
477 sensitive for screening recovery state and thus better evaluate welfare status in farmed fish.

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691

692 **Captions**

693 **Figure 1.** Experimental protocol followed for fish exposure to different water qualities (stress  
694 condition) and behavioural and physiological measurements.

695 **Figure 2.** Mean  $\pm$  SEM of TAN and UIA-N ( $\text{mg}\cdot\text{L}^{-1}$ ) and oxygen saturation ( $\% \text{O}_2$ ) for High TAN,  
696 Hyperoxia and Hypoxia conditions during bathing period (at 0, 30 and 60 min) and at the start and end  
697 of novel environment challenge in group.

698 **Figure 3.** Swimming characteristics in the novel tank diving test. Mean  $\pm$  SEM of **(A)** time spent in  
699 top area in relation to the observation period (s); **(B)** total time spent in top area (s); **(C)** distance  
700 travelled in relation to the observation period (cm); **(D)** total distance travelled (cm); **(E)** number of  
701 transitions between top and bottom areas and **(F)** latency to enter in top area (s) in nicotine-bathed and  
702 control-conditions (n=24 per condition). **(A, C)** Tukey HSD *post-hoc*: \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*,  
703  $p < 0.001$ . **(B,D,E,F)** Mann-Whitney U-test: \*\*\*:  $p < 0.001$ .

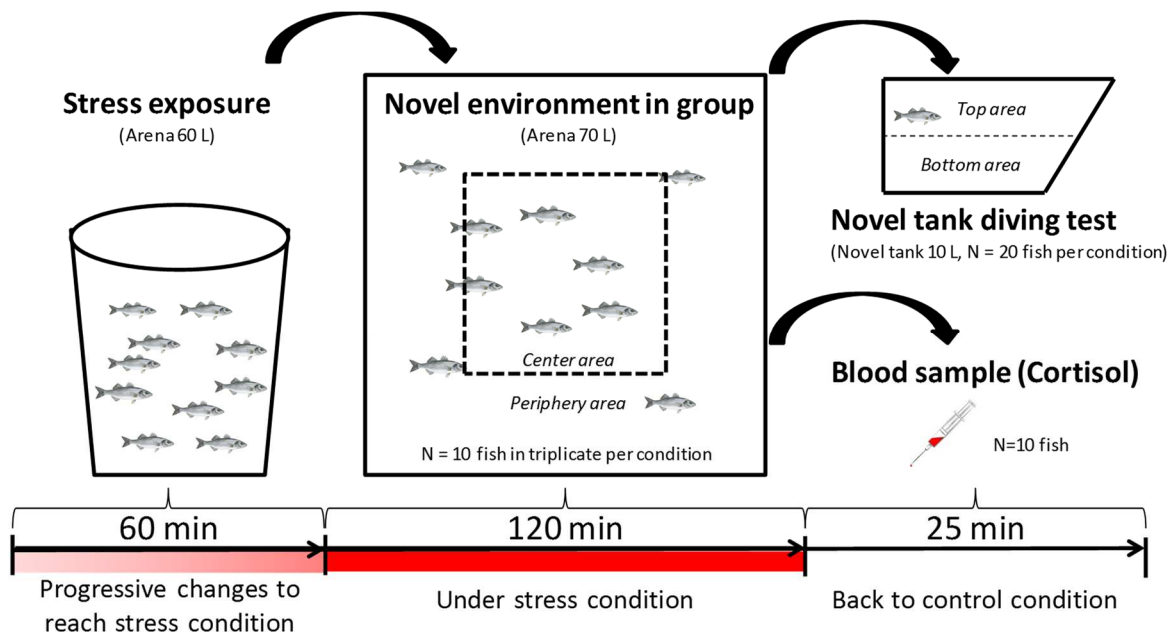
704 **Figure 4.** Spatial location of fish during the novel environment in group. Averaged heatmaps of fish  
705 spatial location (n=30 per condition) over the different time periods. From low (blue colour) to high  
706 location frequency (red colour).

707 **Figure 5.** Swimming characteristics in the novel environment in group challenge. Mean  $\pm$  SEM of **(A)**  
708 distance travelled (cm); **(B)** interindividual distance (cm); **(C)** proportion of time spent in different  
709 areas (%) for each condition (n=30 control, High TAN, Hyperoxia and Hypoxia). Tukey HSD *post-*  
710 *hoc*: different letters indicate significant differences between conditions within observation periods.

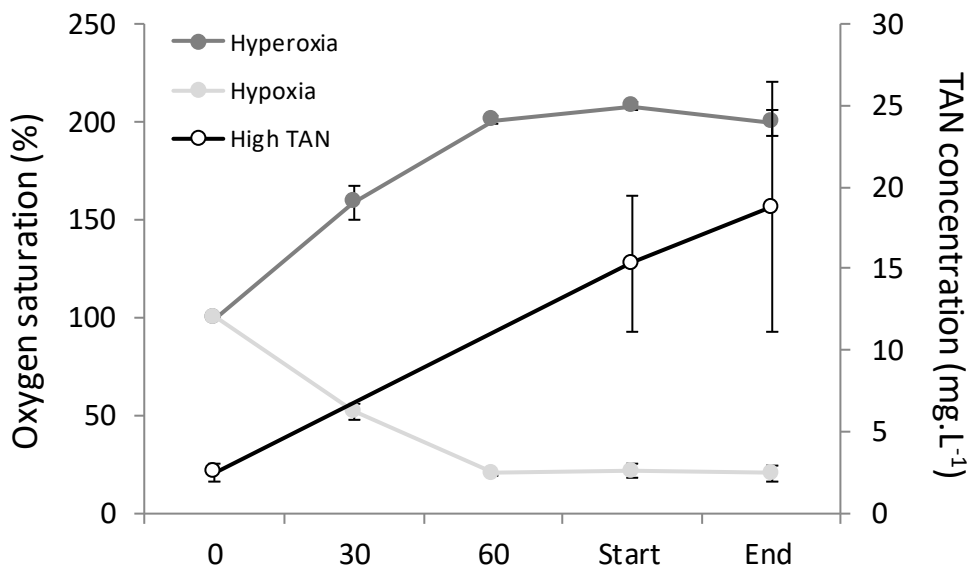
711 **Figure 6.** Swimming characteristics in the novel tank diving test. Mean  $\pm$  SEM of **(A)** time spent in  
712 the top area (s); **(B)** total time spent in the top area (s); **(C)** distance travelled (cm); **(D)** total distance  
713 travelled (cm); **(E)** number of transitions between the two areas and **(F)** latency to enter in the top area  
714 (s) *post-stress* for all conditions (n=20 control, n=20 High TAN, n=18 Hyperoxia; n=18 Hypoxia).  
715 Tukey HSD *post-hoc*: different letters indicate significant differences between conditions.

716 **Figure 7.** Plasma cortisol concentration *post*-stress in the novel environment group challenge (Mean ±  
717 SD) for each condition (n=9 control, n=9 High TAN, n=9 Hyperoxia; n=10 Hypoxia). Mann &  
718 Whitney U-test: \*: p<0.05; \*\*\*: p<0.001

**Figure 1**



**Figure 2**





**Figure 3**

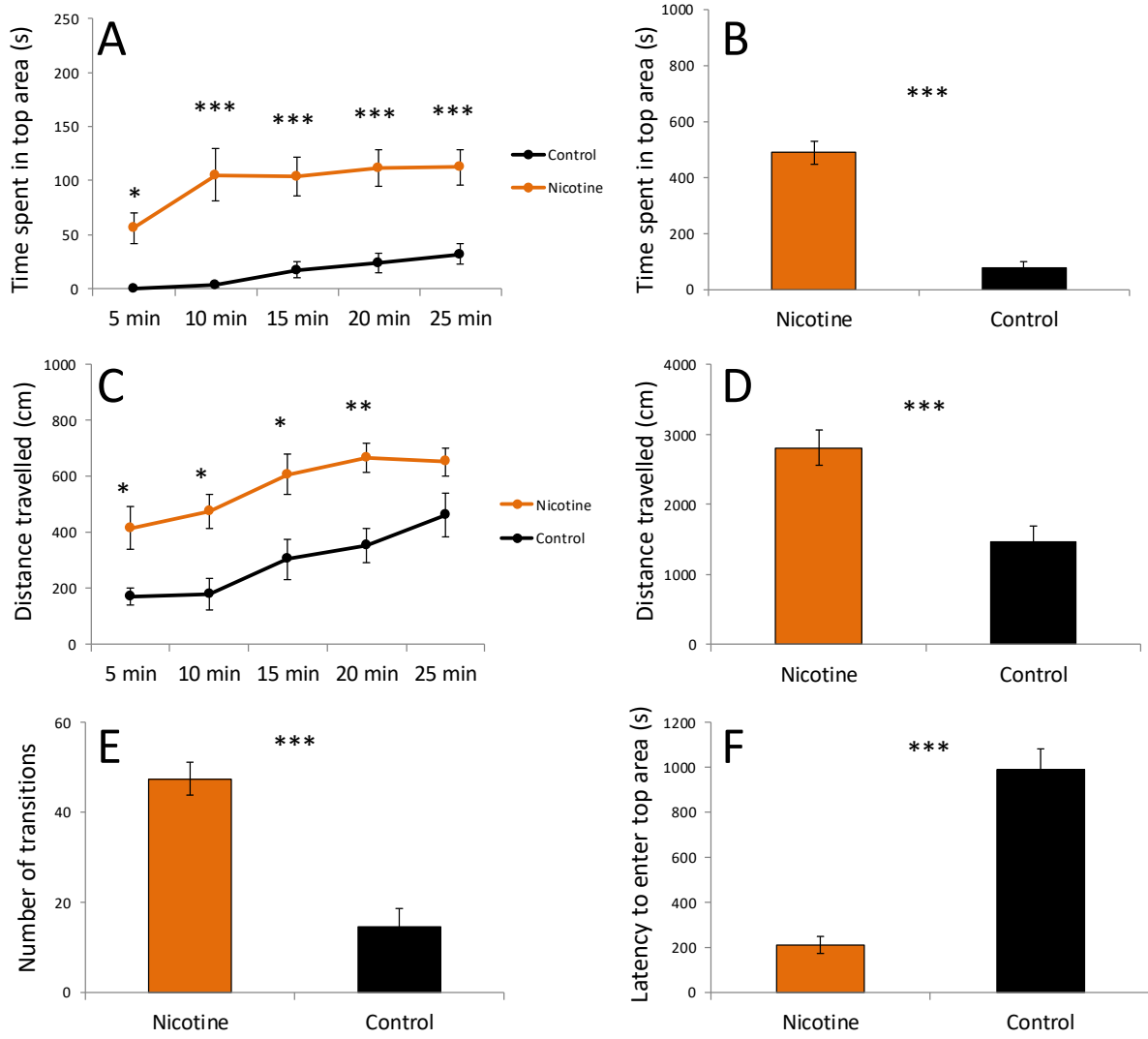
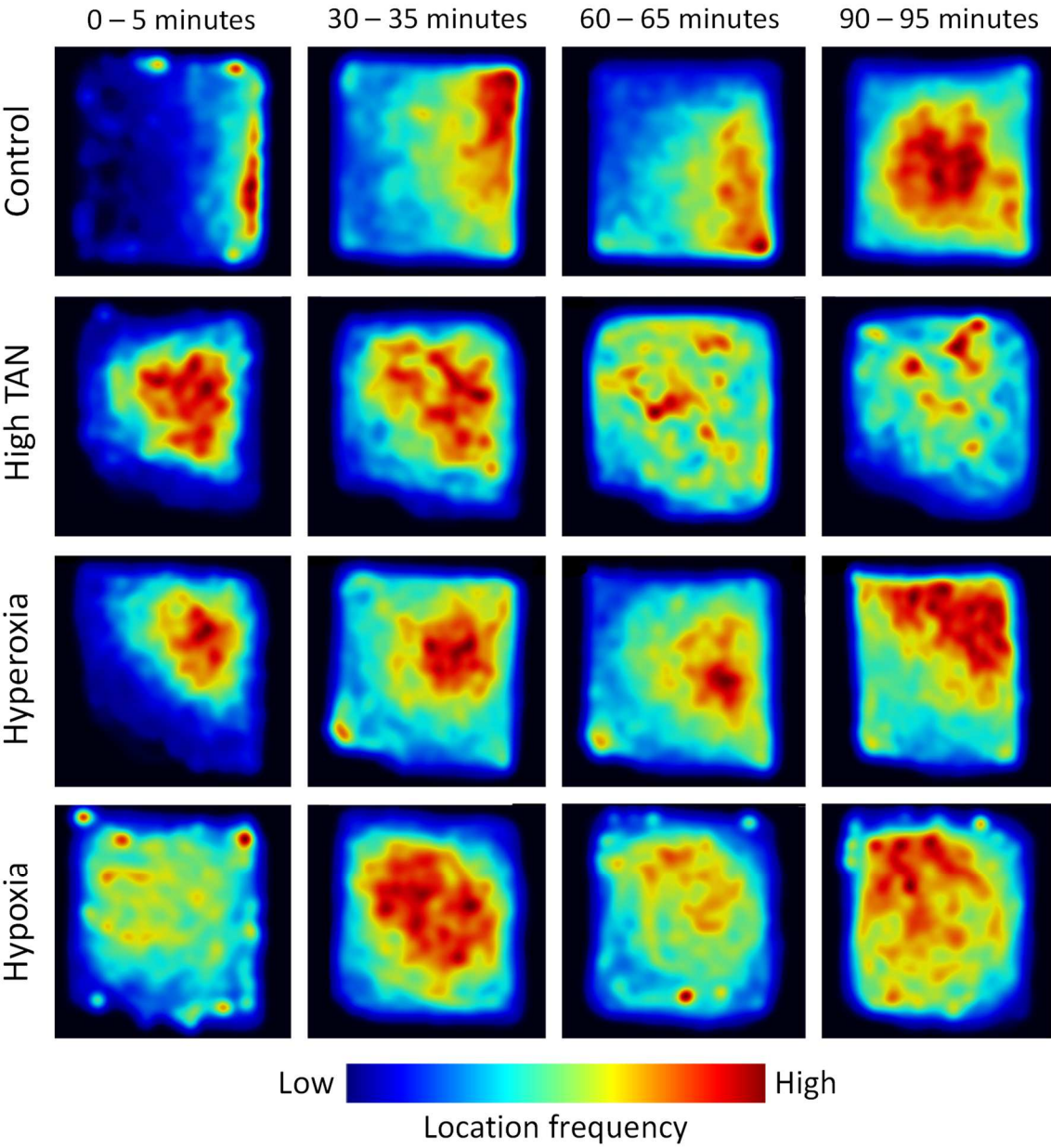
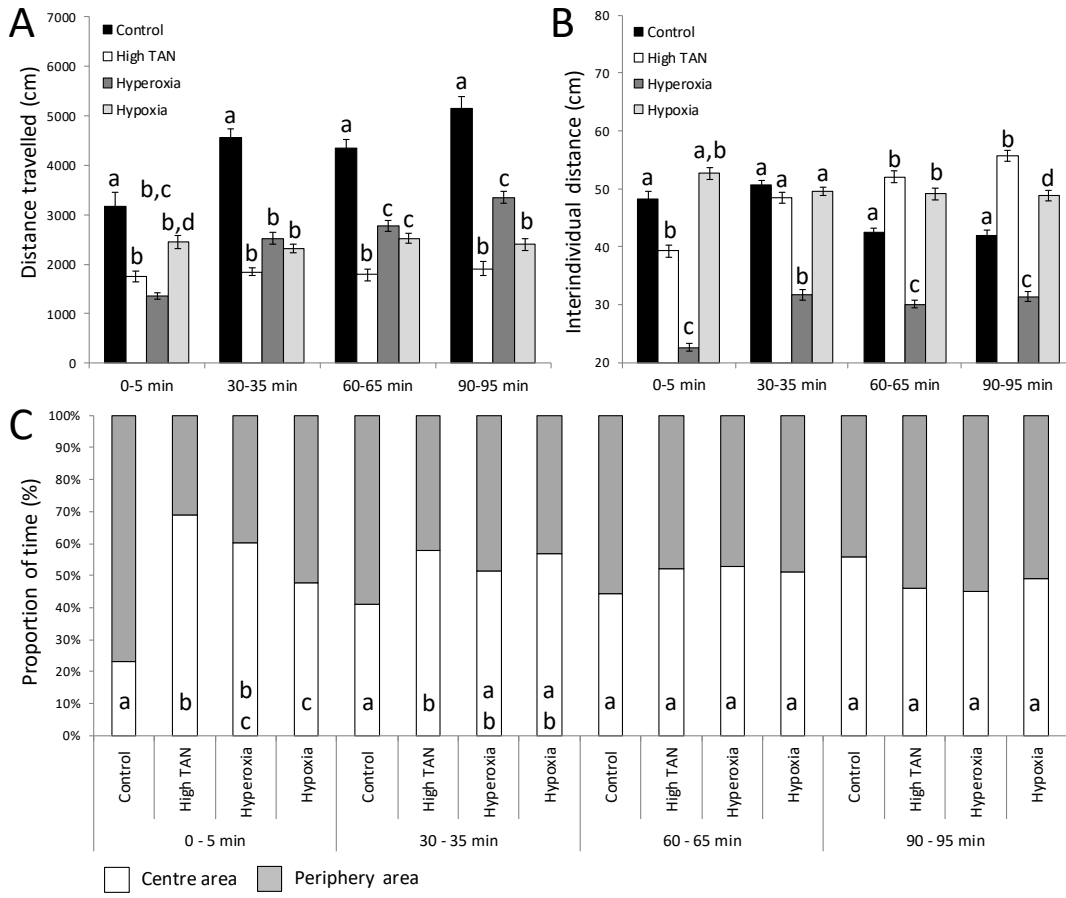


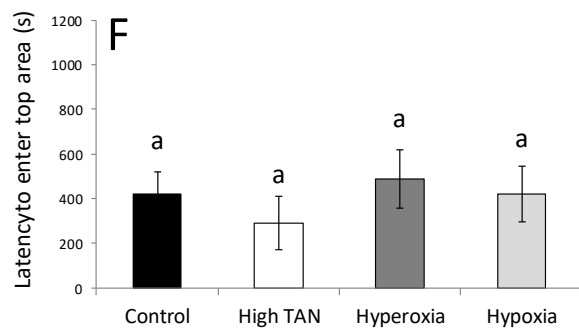
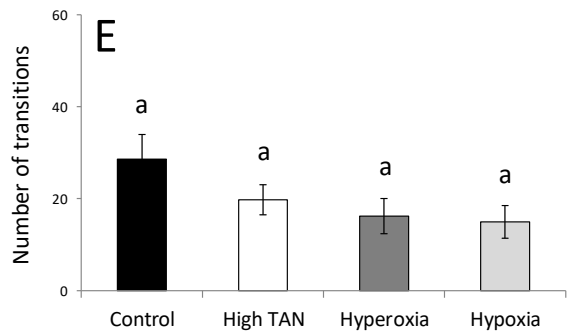
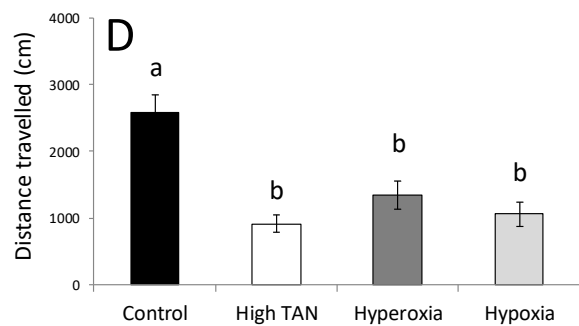
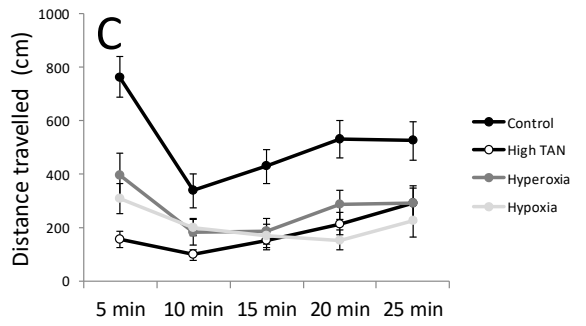
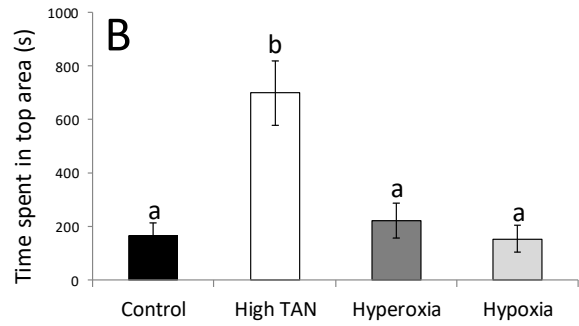
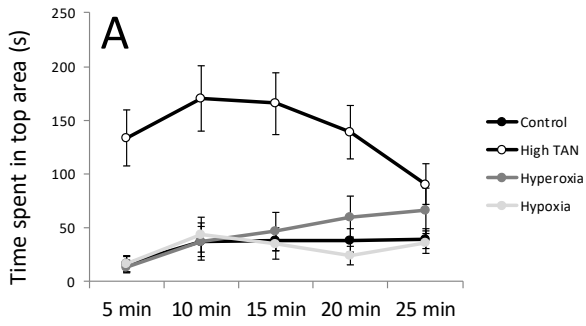
Figure 4



**Figure 5**



**Figure 6**



**Figure 7**

