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

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Abstract

In aquaculture, fish are exposed to unavoidable stressors that can be detrimental for their health and welfare. However, welfare in farmed fish can be difficult to assess, and, so far, no standardized test has been universally accepted as a welfare indicator. This work contributes to the establishment of behavioural welfare indicators in a marine teleost in response to different water quality acute stressors. Groups of ten fish were exposed to high Total Ammonia Nitrogen concentration (High TAN, 18 mg.L^{-1}), Hyperoxia (200 % O_2 saturation), Hypoxia (20 % O_2 saturation), or control water quality (100% O_2 saturation and TAN < 2.5 mg.L^{-1}) over 1 hour. Fish were then transferred in a novel environment for a group behaviour test under the same water quality conditions over 2 hours. Videos were recorded to assess thigmotaxis, activity and group cohesion. After this challenge, plasma cortisol concentration was measured in a subsample, while individual behavioural response was measured in the other fish using novel tank diving test. Prior to this study, the novel tank diving test was validated as a behavioural challenge indicative of anxiety state, by using nicotine as anxiolytic drug. Overall, all stress conditions induced a decrease in activity, thigmotaxis and group cohesion while only fish exposed to Hypoxia and High TAN conditions displayed elevated plasma cortisol concentrations. In *post*-stress condition, activity was still affected but normal behaviour was recovered within the 25 minutes of the test duration. Our work suggests that the activity, thigmotaxis and group cohesion are good behavioural indicators of exposure to degraded water quality, and could be used as standardized measures to assess fish welfare.

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

1. Introduction

Fish production has expanded importantly during the last decades, both because of the world's diminishing natural wild resources and the increase in demand for fish products (FAO, 2018). Aquaculture represented 53 % of the total fish production (including non-food uses) in 2016 (FAO, 2018) and is now recognized as a major food production industry. Thus, as well as for terrestrial farming industry, concerns about sustainability, environmental issues and animal welfare in aquaculture are increasing (Conte, 2004; Ashley, 2007; Martins et al., 2010; Martins et al., 2012; Hixson, 2014; FAO, 2018; Lembo et al., 2019). It is common that under aquaculture conditions, and in every fish husbandry system, variations of water quality variables such as temperature, pH, oxygen (O_2), carbon dioxide (CO_2) or Total Ammonia Nitrogen concentrations (TAN) occur. Such variations when they reach a certain threshold, depending on the species *preferendum*, could be considered as stress factors (stressor) and therefore deleterious for fish health and welfare. The exposure to stressors, such as degraded water quality may mobilize fish energy for coping with the stressor hereby decreasing the available energy allocated to growth and reproduction, or directly causes death if the magnitude of stress is too high (Barton, 2002; Sneddon et al., 2016). Therefore, it can finally affect fish production and has economic consequences for farmers (Conte, 2004; Lembo et al., 2019).

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

Thus, behavioural measurements have proven to be sensitive indicators of the complex existing biochemical and physiological changes that occur in response to stress (Schreck, 1990; Scherer, 1992; Schreck et al., 1997; Martins et al., 2012). Behaviours, such as changes in food-anticipatory activity, feed intake, ventilation rate, individual and group swimming activity are commonly used as welfare indicators (Huntingford et al., 2006; Martins et al., 2012; Huntingford and Kadri, 2014; Carbonara et al., 2015; Carbonara et al., 2019). Group swimming behaviour is defined as the spatial distribution and swimming activity of the group of fish held within an aquaculture production unit and it covers shoal structure, the horizontal and vertical distribution of the group, their swimming speed and direction (Martins et al., 2012). For instance, exposure to negative *stimuli*, such as poor water quality, is known to lead to rapid escape movements (Stien et al., 2007; Bratland et al., 2010) or to alter group cohesion (Domenici et al., 2002; Espmark and Baeverfjord, 2009; Sadoul et al., 2014; Sadoul et al., 2017). Thus, group swimming behaviour appears to be a sensitive welfare indicator even if it is still lacking calibration efforts to be precisely translated into an operational welfare indicator; nevertheless some examples exist (Papandroulakis et al., 2014; Pettersen et al., 2014). Moreover, the appraisal of negative or positive stimuli and, hence, the psychological dimension of stress as defined for fish by Galhardo and Oliveira (2009) is seldom tackled in welfare research. There exists however a complementary measure which is the individual behavioural responses to novel environment and in particular the novel tank diving test which is worldwide used along with the measure of stereotypies, such as thigmotaxis to assess anxiety in zebrafish (*Danio rerio*) in ecotoxicology and pharmacology research (Levin et al., 2007; Egan et al., 2009; Vignet et al., 2014; Macaulay et al., 2015; Alfonso et al., 2019a). In further details, the novel tank diving test was validated as a tool for evaluating anxiety by using drugs, such as nicotine. Short exposure to nicotine is known to reduce anxiety in fish, through its action on nicotinic acetylcholine receptors as demonstrated by the use of specific inhibitors (Levin et al., 2007; Bencan & Levin, 2008). In the context of novel tank diving test, nicotine-exposure (bathing) has been shown to be anxiolytic by triggering change in fish space utilization, such as higher time spent in the top area of the novel tank which translate a relief from bottom dwelling behaviour that fish would express under predator threat for example. The novel tank diving test could thus be a helpful non-invasive tool to monitor farmed fish anxiety state *post* stress exposure hereby assessing psychological stress and contributing to the assessment of positive or negative emotions and, hence better welfare state determination.

Overall, the objectives of the present study were to further contribute to the establishment of behavioural welfare indicators including the psychological dimension of stress in a model marine teleost in response to different water quality stressors. Firstly, the novel tank diving test outcome was validated as a behavioural indicator of anxiety in European sea bass *Dicentrarchus labrax*, using nicotine as an anxiolytic reference drug. Secondly, behavioural responses of fish group in response to a novel environment under acute and severe water quality deterioration, including Total Ammonia Nitrogen (High TAN) increase (18 $mg.L^{-1}$), Hyperoxia (200 % O_2 saturation) and Hypoxia (20 % O_2 saturation) were evaluated along with cortisol measurement. Finally, individual behaviour expressed following the same water quality exposures were assessed using the novel tank diving test translated from ecotoxicology studies.

2. Material and methods

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

2.1. Fish rearing

Juvenile European sea bass were hatched and reared at Ifremer Palavas-les-flots research station (France, 34250) until 280 days *post* fertilization (dpf) according to sea bass rearing standard (Chatain, 1994). They were then transferred to Ifremer L'Houmeau (France, 17137). No mortality occurred during the transfer between the two facilities. They were then randomly separated in groups of 100 fish into 4 tanks of 400 L (90x90x50 cm). Tanks shared a recirculating system with a flow rate of 4 m^3 per hour and water was renewed at a rate of 20 % per day. Water temperature was maintained at 21.5 ± 1°C, oxygen around 100 % saturation, and salinity and pH were respectively set to 20.5 ± 1 and 8.3. The light regime was 13:11 L/D. Total Ammonia Nitrogen (TAN) concentration was < 2.5 $mg.L^{-1}$ (equivalent to 0.1 $mg.L^{-1}$ [NH_3]). The fish were hand-fed using commercial diet from Le Gouessant (France) once a day each morning at 9:00 at 1 % of biomass. Fish were reared in L'Houmeau for 3 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 $mg.L^{-1}$ 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-1 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 areas, indicative of fish activity level were also measured. Variables were recorded in each frame and they were summed over periods of 5 min or over the whole test duration (*i.e.* 25 min).

2.3. Exposure to stress condition and water quality characterization

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

For the control condition, fish were maintained under the same standard condition than in the rearing room (*i.e.,* at 21.5 ± 1°C, 100 % O_2 saturation and TAN < 2.5 mg.L^{-1}). For high TAN condition, 8.5 g of ammonium chloride NH_4Cl (Fluka 09711, Sigma Aldrich) dissolved in 0.5 L of water was added twice at 30 min interval to reach a targeted TAN concentration of 18 mg.L^{-1} which corresponds to 1.6 mg.L^{-1} of NH_3. Three water samples (50 mL) were taken to quantify *a posteriori* the TAN concentration: (i) before adding ammonium chloride, (ii) at the beginning and (iii) at the end of the novel environment in group. Samples were stored at -22°C before further analysis. Then, TAN concentration was quantified using a spectrophotometer with continuous flow (Alliance Integral Futura, Frépillon, France) and analytical method is described below.

Samples were filtered using GF/F 0.7µm filter (Whatman, Maidstone, United Kingdom). TAN concentration was quantified using a spectrophotometer with continuous flow (Alliance Integral Futura, Frépillon, France) using colorimetric method. The solutions used for the calibration analyses that were performed the same day came from stock solutions from 0.1g.L^{-1} to 1g.L^{-1} of ammoniacal nitrogen (NH_4^+) stocked at a temperature of 8°C. The calibration curve of ammoniacal nitrogen showed a high R^2 validating the procedure (NH_4^+=1.439 x OD - 0.002; R^2 = 0.99, where OD is the optical density measured using the spectrophotometer).

The NH_3 concentration was determined using the following equation as described by Johansson and Wedborg (1980):

$$[NH3] = \frac{[NH_4{}^+]}{K_1 \times [H^+]}$$

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

For Hypoxia condition, oxygen concentration was slowly lowered to reach 20 % of O_2 saturation using nitrogen bubbling. For Hyperoxia condition, oxygen concentration was slowly increased to reach 200 % of O_2 saturation using oxygen bubbling. Oxygen concentration was monitored every 5 min during the entire experiment using an Oxygen probe (Oxi 3310, WTW, Xylem Analytics Germany Sales GmbH & Co. KG). Oxygen concentration, NH_4^+ and NH^3 concentrations recorded over the experimental duration are presented below.

For control condition, water oxygen concentration was maintained at 100% saturation (at 21.5 ± 1°C) and TAN concentration was < 2.5 $mg.L^{-1}$ during both bathing and novel environment in group. For High TAN condition, oxygen concentration was above 100 % and TAN concentrations were 15.3 ± 4.2 and 18.8 ± 7.6 $mg.L^{-1}$ at the beginning and at the end of the test respectively, corresponding to UIA-N concentrations of 1.34 ± 0.37 and 1.64 ± 0.67 $mg.L^{-1}$. For both Hyperoxia and Hypoxia conditions, TAN concentration was maintained at less than 2.5 $mg.L^{-1}$. For the hyperoxia condition, oxygen concentration increased slowly during the bathing period to reach 208 ± 5.6 % of saturation, while for the hypoxia it slowly decreased to reach 21.3 ± 3.8 % of saturation before the start of the novel environment challenge in group. All conditions were then maintained during two hours until the end of the group test (**Figure 2**). In the high TAN condition, the concentration of NH_3 represents half of the LC50 concentration reported for European sea bass after 96 h of exposure under similar hydrological conditions (i.e. temperature=17.5°C, salinity=34, and pH=8.15) than in our experiment (Person-Le Ruyet et al., 1995).

After the initial bathing period, the fish group was challenged in a novel environment with the same water quality (see section 2.4.1). After 2 hours, fish from two replicates were then monitored individually into novel tank diving test in plain system water (see section 2.4.2, n=20 for control, n=20 for High TAN and n=18 for Hyperoxia and Hypoxia). The third replicate was used to quantify plasma cortisol concentration following the challenge in group (n=10 per condition, see section 2.5). Fish were randomly selected for blood sampling or for performing novel tank diving test. After blood sampling (n=9 or 10 fish per condition) or novel tank diving test, all fish were measured for weight (g, to the nearest mg) and standard length (cm, to the nearest mm) under the same anaesthesia conditions (see section 2.5).

2.4. Behavioural procedures

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

2.4.1. Novel environment in group

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

For space occupancy, the visualization of heat maps was produced for each 5-min period using Ethovison XT 13 (Noldus The Netherlands). For further analysis, the arena was separated into two areas: Centre area including one half of the volume and periphery area including the other half; time spent in periphery (s), indicative of thigmotaxis behaviour (Ferrari et al., 2014), was recorded. Distance travelled by each fish (cm), indicative of individual fish activity, and the interindividual distances (cm), indicative of group cohesion (Buske and Gerlai, 2011), were also recorded. Variables were recorded for each frame and they were summed (for Distance travelled) or averaged (for time spent in periphery and interindividual distances) over period of 5 min every 30 minutes (*i.e.,* four sampling periods: 0-5 min, 30-35 min, 60-65 min and 90-95 min).

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

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

2.5. Cortisol measurement

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

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

2.6. Statistical analyses

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

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

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

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

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

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

3. Results

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

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

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

3.2. Novel environment in group

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

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

Interindividual distances, indicative of group cohesion, differed between conditions (F=449.3, df=3, p<0.001), periods (F=17.4, df=3, p<0.001) with a significant interaction between conditions and periods (F=29.1, df=9, p<0.001). Fish from control condition progressively decreased their interindividual distances during the challenge; 48.3 ± 1.4 cm during period 0-5 min *vs.* 41.9 ± 1.1 cm during period 90-95 min (p<0.001). During the 0-5 min period, fish from control and Hypoxia conditions showed similar interindividual distances (p=0.06), whereas fish from High TAN and Hyperoxia conditions swam closer to each other (p<0.001 for both conditions). However, starting from the 60-65 min to the 90-95 min period, fish from both High TAN and Hypoxia conditions showed higher dispersion than control fish (p<0.001) while fish from Hyperoxia condition continued to display lower dispersion than control fish from the beginning to the end of the testing period (p<0.001; **Figure 5.B**).

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

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

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

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

($F=15$, df=3, $p<0.001$; **Figure 6.D**). High TAN, Hyperoxia and Hypoxia fish travelled less distance than control fish during the novel tank test ($p<0.001$ for all conditions). No difference was observed for the number of transitions between the two areas between conditions ($F=2.3$, df=3, $p=0.8$; **Figure 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**).

3.4. HPI axis response

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

4. Discussion

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

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

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

The four experimental conditions had very distinct water qualities. In the control condition, oxygen saturation was at 100 % and TAN concentration was under 2.5 $mg.L^{-1}$ while TAN concentration was approximately 8 fold higher in High TAN condition and oxygen saturation was 2 fold higher and 5 fold lower in Hyperoxia and Hypoxia conditions respectively. TAN concentrations in the High TAN condition corresponded to a concentration of NH_3 of 1.6 $mg.L^{-1}$ (half of the LC50, Person-Le Ruyet et al., 1995). Oxygen saturations chosen during the Hyperoxia and Hypoxia conditions were previously described to affect fish physiology and behaviour during chronic exposure without being lethal (Chapman and Mckenzie, 2009; Espmark and Baeverfjord, 2009; Rimoldi et al., 2016). Both concentrations of oxygen and TAN used in the present study were quite extreme but can occasionally occur (or even co-occur) in aquaculture conditions in case of technical failures.

In the novel environment in group, control fish displayed thigmotaxis behaviour and spent most of the time in the periphery area during the first 5 minutes of the test. Indeed, handling fish from bathing tank to a novel environment is known to induce a typical thigmotaxis behavioural response. This thigmotaxis behaviour has been shown to indicate an anxiety state (Prut and Belzung, 2003; Schnorr et al., 2012). Interestingly, fish from High TAN, Hyperoxia and Hypoxia conditions spent less time in the periphery area than control fish, which suggest that they were not displaying anxiety-like behaviour contrary to control fish in response to handling. High TAN, Hyperoxia and Hypoxia are known to directly affect fish survival (Magaud et al., 1997; Person-Le Ruyet and Boeuf, 1998; Shimps et al., 2005; Rimoldi et al., 2016). Indeed, fish survival can be impacted in many ways following their exposure to one of these stressors. For instance, in hypoxic conditions, the oxygen quantity is limited, therefore fish have to adapt their physiology and behaviour to maximize oxygen uptake and avoid death by asphyxia (Chapman & Mckenzie, 2009). Then, Hyperoxia may alter the equilibrium of ions in the gill, causes a decrease in the ventilatory frequency inducing oxidative damages or triggers diseases and thus can cause fish death (Dejours et al. 1977; Liepelt et al.1995; Brauner et al. 2000). Finally, Ammonia exposure can affect osmoregulation, represses the immune system, or causes asphyxiation leading to hyperventilation, convulsions and death (Randall and Tsui, 2002; Eddy, 2005; Camargo and Alonso, 2006). By contrast to these stressors, handling is mostly documented for its effects on the HPI axis, through cortisol release (Barton 2002). To our knowledge, handling does not have direct effects on survival except if fish are injured which was not the case in our experiment. Therefore, we hypothesise that when fish are coping with stressors affecting survival, the thigmotaxis expression in response to a psychological stressor is overruled, possibly through endorphin release. Further mechanistic studies are needed to better understand what changes within the fish are related to these behavioural adaptations under multi-stressors exposure. Furthermore, during the test in group situation, control fish travelled progressively more distance over the test duration whereas High TAN, Hyperoxia and Hypoxia conditions showed lower and stable distance travelled. Such lower swimming activity under high TAN was described before in rainbow trout (Shingles et al., 2001) and under hypoxia conditions in common sole (*Solea solea*; Dalla Via et al. (1998)) and dogfish (*Scyliorhinus canicula*; Metcalfe and Butler (1984)) whereas hyperoxia was shown to lead to higher variability of the activity pattern in Atlantic salmon (Espmark and Baeverfjord, 2009). It is, however, important to note that behavioural responses under hypoxia were found to be clearly dependant on the intensity and duration of the hypoxia challenge and the species (Chapman and Mckenzie, 2009). NH_3 is known to cause asphyxiation by reducing the blood oxygen-carrying capacity and hence alter the swimming performances reported above (Shingles et al., 2001; Camargo and Alonso, 2006). Indeed, decrease in oxygen or increase in TAN concentration both lead to reduced active metabolic rate (Muusze et al., 1998; Shingles et al., 2001) and may explain why exposed fish are being less active than controls. It seems that fish adopted a "wait and see" strategy consisting in minimizing energy expenditure and waiting for an improvement in water quality (van Raaij et al., 1996; Clingerman et al., 2007). Finally, concerning interindividual distances, during the 0-5 min period of novel environment in group, fish from Control and Hypoxia conditions expressed the same group cohesion whereas fish from high TAN and Hyperoxia conditions were closer to each other. Fish from Hyperoxia condition stayed closer during all test duration while both fish from High TAN and Hypoxia conditions displayed lower group cohesion than control fish from 60-65 to 90-95 min periods. Interestingly, Domenici et al. (2002) reported the same larger dispersion in Atlantic herring under O_2 saturation of 20 % and same results were observed in Atlantic salmon under O_2 saturation of 150 % (Espmark and Baeverfjord, 2009). Lower group cohesion was also observed in rainbow trout under other stress factor such as hypercapnia by Sadoul et al. (2017). When given the possibility, it is well known that fish are able to avoid stressful environment such as hypoxic waters, high ammonia or high CO_2 concentrations (Richardson et al., 2001; Clingerman et al., 2007; Skjæraasen et al., 2008; Herbert et al., 2010). Therefore we suggest that fish interindividual distance increases to maximize oxygen uptake (Domenici et al., 2017) and thus could be used as a relevant welfare indicator (Juell, 1995; Espmark and Baeverfjord, 2009). This behavioural strategy can also be perceived as a decrease in attention and/or used for saving energy for a better coping with stress favouring a return to a homeostatic state. Additional experiments are however required to fully understand the observed disruptions of the shoal cohesion but it overall fits well with the on-growing research effort about the effects of abiotic factors on fish group behaviour (Weetman et al., 1999; Domenici et al., 2002; Espmark and Baeverfjord, 2009; Colchen et al., 2017; Domenici et al., 2017; Sadoul et al., 2017; Colson et al., 2019).

Concerning the HPI axis responsiveness, plasma cortisol concentrations are always high for European sea bass upon stress exposure since this species is known to be high cortisol responder (Rotllant et al., 2003; Samaras et al., 2016; Alfonso et al., 2019b). In undisturbed conditions, plasma cortisol values are expected to be around 100 $ng.L^{-1}$ but it can reach a value 10 fold higher upon stress (Samaras et al., 2018; Alfonso et al., 2019b). In addition to the confinement stress in the bathing tank, fish also experienced two handling stresses, one transfer between the rearing and the bathing tanks and then between the bathing and the experimental tanks explaining the high cortisol values measured in all conditions. Measured plasma cortisol concentrations were similar between fish exposed to Hyperoxia and control fish but were higher in fish exposed to Hypoxia and High TAN conditions than control. A similar increase in cortisol release was previously reported in Atlantic salmon following chronic exposure to ammonia (0.15 $mg.L^{-1}$, (Knoph and Olsen, 1994) and hyperoxia (150 % O_2 saturation, (Espmark and Baeverfjord, 2009) or acute exposure to hypoxia (25 % of O_2 saturation) in rainbow trout (van Raaij et al., 1996). Behavioural disruptions observed in Hyperoxia treated fish did not translate in terms of cortisol values compared to control fish, suggesting the possible implication of other physiological mechanisms, such as plasma catecholamines as previously shown in rainbow trout (van Raaij et al., 1996; Gesto et al., 2013; 2015). Interestingly, within a species, individuals are known to differ from each other in terms of amplitude of primary stress response (e.g. cortisol and catecholamines) and, along with divergent behavioural responses, that defines individual coping styles (Castanheira et al., 2017). Therefore, it would be interesting in future studies to include complementary coping style characterizations to investigate the link between coping style, behavioural responses within a group and primary responses upon stress exposure. This would help in assessing whether different strategies co-exist to cope with these kinds of stressors within a fish group.

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

Conclusion

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

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References

Alfonso, S., Blanc, M., Joassard, L., Keiter, S.H., Munschy, C., Loizeau, V., Begout, M.L., Cousin, X., 2019a. Examining multi- and transgenerational behavioral and molecular alterations resulting from parental exposure to an environmental PCB and PBDE mixture. Aquat Toxicol 208, 29-38.

Alfonso, S., Sadoul, B., Gesto, M., Joassard, L., Chatain, B., Geffroy, B., Begout, M.L., 2019b. Coping styles in European sea bass: The link between boldness, stress response and neurogenesis. Physiol Behav 207, 76-85.

Ashley, P.J., 2007. Fish welfare: Current issues in aquaculture. Applied Animal Behaviour Science 104, 199–235.

Barton, B., 2002. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integ. and Comp. Biol 42, 517-525.

Bencan, Z., Levin, E.D., 2008. The role of alpha 7 and alpha 4 beta 2 nicotinic receptors in the nicotine-induced anxiolytic effect in zebrafish. Physiology & Behavior 95, 408-412.

Bratland, S., Stien, L.H., Braithwaite, V.A., Juel, J.E., Folkedal, O., Nilsson, J., Oppedal, F., Fosseidenge, J.E., Kristiansen, T.S., 2010. From fright to anticipation: using aversive light stimuli to investigate reward conditioning in large group sof Atlantic salmon (*Salmo salar*). Aquaculture International 18, 991-1001.

Buske, C., Gerlai, R., 2011. Early embryonic ethanol exposure impairs shoaling and the dopaminergic and serotoninergic systems in adult zebrafish. Neurotoxicol Teratol 33, 698-707.

Brauner CJ, Seidelin M, Madsen SS, Jensen F.B. 2000. Effects of freshwater hyperoxia and hypercapnia and their influences on subsequent seawater transfer in Atlantic salmon Salmo salar smolts. Can J Fish Aquat Sci 57:2054–2064.

Camargo, J.A., Alonso, A., 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. Environ Int 32, 831-849.

Carbonara, P., Scolamacchia, M., Spedicato, M.T., Zupa, W., McKinley, R.S., Lembo, G., 2015. Muscle activity as a key indicator of welfare in farmed European sea bass (Dicentrarchus labrax L. 1758). Aquaculture research 46(9), 2133-2146.

Carbonara, P., Alfonso, S., Zupa, W., Manfrin, A., Fiocchi, E., Pretto, T., Spedicato, M.T. & Lembo, G. (2019). Behavioral and physiological responses to stocking density in sea bream (Sparus aurata): Do coping styles matter?. Physiol Behav 212, 112698.

Castanheira, M. F., Conceição, L. E. C., Millot, S., Rey, S., Bégout, M. L., Damsård, B., Kristiansen, T., E, H., Øverli, Ø. & Martins, C. I. M. (2017). Coping styles in farmed fish: consequences for aquaculture. Reviews in Aquaculture 9, 23-41.

Chapman, L.J., Mckenzie, D.J., 2009. Chapter 2 Behavioral Responses and Ecological Consequences. Fish physiology Hypoxia (Ed Richard J.G, Farrell A.P and Brauner C.J), 27, 25-77.

Chatain, B., 1994. Estimation et amélioration des performances zootechniques de l'élevage larvaire de *Dicentrarchus labrax* et de *Sparus auratus*.

Clingerman, J., Bebak, J., Mazik, P.M., Summerfelt, S.T., 2007. Use of avoidance response by rainbow trout to carbon dioxide for fish self-transfer between tanks. Aquacultural Engineering 37, 234-251.

Colchen, T., Teletchea, F., Fontaine, P., Pasquet, A., 2017. Temperature modifies activity, inter-individual relationships and group structure in a fish. Current Zoology 63, 175-183.

Conte, F.S., 2004. Stress and the welfare of cultured fish. Applied Animal Behaviour Science 86, 205–223.

Colson, V., Mure A., Valotaire, C., Le Calvez, J.M., Goardon, Labbe, L., Leguen I., Prunet P., 2019. A novel emotional and cognitive approach to welfare phenotyping in rainbow trout exposed to poor water quality. Applied animal behaviour science 210, 103-112.

Dalla Via, J., Van Den Thillart, G., Cattani, O., Cortesi, P., 1998. Behavioural responses and biochemical correlates in Solea solea to gradual hypoxic exposure. Canadian Journal of Zoology 76, 2108-2113.

Dejours, P., Toulmond, A., Truchot, J.P., 1977. The effect of hyperoxia on the breathing of marine fishes. Comp. Biochem. Physiol A 58, 409-411.

Domenici, P., Ferrari, R.S., Steffensen, J.F., Batty, R.S., 2002. The effect of progressive hypoxia on school structure and dynamics in Atlantic herring Clupea harengus. Proc Biol Sci 269, 2103-2111.

Domenici, P., Steffensen, J.F., Marras, S., 2017. The effect of hypoxia on fish schooling. Philos Trans R Soc Lond B Biol Sci 372.

Eddy, F.B., 2005. Ammonia in estuaries and effects on fish. J. Fish Biol 67(6), 1495-1513.

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A.V., 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behavioural Brain Research 205, 38-44.

Espmark, A.M., Baeverfjord, G., 2009. Effects of hyperoxia on behavioural and physiological variables in farmed Atlantic salmon (*Salmo salar*) parr. Aquaculture International 17, 341-353.

Fanouraki, E., Mylonas, C.C., Papandroulakis, N., Pavlidis, M., 2011. Species specificity in the magnitude and duration of the acute stress response in Mediterranean marine fish in culture. General and Comparative Endocrinology 173, 313-322.

FAO, 2018. The state of world fisheries and aquaculture., Food and Organisation of the United Nations, p. 227 pp.

Ferrari, S., Benhaim, D., Colchen, T., Chatain, B., Begout, M.L., 2014. First links between self-feeding behaviour and personality traits in European seabass, Dicentrarchus labrax. Applied Animal Behaviour Science 161, 131-141.

Galhardo, L., Oliveira, R.F., 2009. Psychological stress and welfare in fish. Annual review of Biomedical Sciences, 1-20.

Gesto, M., Lopez-Patino, M.A., Hernandez, J., Soengas, J.L., Miguez, J.M., 2013. The response of brain serotonergic and dopaminergic systems to an acute stressor in rainbow trout: a time course study. Journal of Experimental Biology 216, 4435-4442.

Gesto, M., Lopez-Patino, M.A., Hernandez, J., Soengas, J.L., Miguez, J.M., 2015. Gradation of the stress response in rainbow trout exposed to stressors of different severity: the role of brain serotonergic and dopaminergic systems. J Neuroendocrinol 27, 131-141.

Herbert, N.A., Skjæraasen, J.E., Nilsen, T., Salvanes, A.G.V., Steffensen, J.F., 2010. The hypoxia avoidance behaviour of juvenile Atlantic cod (*Gadus morhua L.*) depends on the provision and pressure level of an O2 refuge. Marine Biology 158.

Hixson, S.M., 2014. Fish Nutrition and Current Issues in Aquaculture: The Balance in Providing Safe and Nutritious Seafood, in an Environmentally Sustainable Manner. Journal of Aquaculture Research Development 5, 234.

Huntingford, F.A., Adams, C., Braithwaite, V.A., Kadri, S., Pottinger, T.G., Sandøe, P., Turnbull, J.F., 2006. Current issues in fish welfare. Journal of Fish Biology 68, 332-372.

Huntingford, F.A., Kadri, S., 2014. Defining, assessing and promoting the welfare of farmed fish. Revue Scientifique Et Technique-Office International Des Epizooties 33, 233-244.

Johansson, O., Wedborg, M., 1980. Ammonia-Ammonium Equilibrium in Seawater at Temperatures between 5 and 25-Degrees-C. Journal of Solution Chemistry 9, 37-44.

Juell, J.E., 1995. The behaviour of Atlantic salmon in relation to efficient cage-rearing. Reviews in Fish Biology and Fisheries 5, 320-335.

Knoph, M.B., Olsen, Y.A., 1994. Subacute Toxicity of Ammonia to Atlantic Salmon (Salmo-Salar L) in Seawater - Effects on Water and Salt Balance, Plasma-Cortisol and Plasma Ammonia Levels. Aquatic Toxicology 30, 295-310.

Lembo, G., Carbonara, P., Fabris, A., Manfrin, A., Zupa, W., 2019. Welfare issues and veterinary treatments.

Levin, E.D., Bencan, Z., Cerutti, D.T., 2007. Anxiolytic effects of nicotine in zebrafish. Physiol Behav 90, 54-58.

Liepelt, A., Karbe, L., Westendorf, J. 1995. Induction of DNA strand breaks in rainbow trout Oncorhynchus mykiss under hypoxic and hyperoxic conditions. Aquat. Toxicol 33, 177-181.

Macaulay, L.J., Bailey, J.M., Levin, E.D., Stapleton, H.M., 2015. Persisting effects of a PBDE metabolite, 6-OH-BDE-47, on larval and juvenile zebrafish swimming behavior. Neurotoxicol Teratol 52, 119-126.

Magaud, H., Migeon, B., Morfin, P., Garric, J., Vindimian, E., 1997. Modelling fish mortality due to urban storm run off: interacting effects of hypoxia and un-ionized ammonia. Water research 31, 211-218.

Margiotta-Casaluci, L., Owen, S.F., Cumming, R.I., de Polo, A., Winter, M.J., Panter, G.H., Rand-Weaver, M., Sumpter, J.P., 2014. Quantitative Cross-Species Extrapolation between Humans and Fish: The Case of the Anti-Depressant Fluoxetine. Plos One 9.

Martins, C.I., Eding, E.H., Verdegem, M.C.J., Heinsbroek, L.T.N., Schneider, O., Blancheton, J.P., Roque d'Orbcastel, E., Verreth, J.A.J., 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. Aquacultural Engineering 43, 83-93.

Martins, C.I., Galhardo, L., Noble, C., Damsgard, B., Spedicato, M.T., Zupa, W., Beauchaud, M., Kulczykowska, E., Massabuau, J.C., Carter, T., Planellas, S.R., Kristiansen, T., 2012. Behavioural indicators of welfare in farmed fish. Fish Physiol Biochem 38, 17-41.

Metcalfe, J.D., Butler, P.J., 1984. Changes in activity and ventilation in response to hypoxia in unrestrained, unoperated dogfish (Scyliorhinus canicula L.). J Exp Biol 108, 411-418.

Muusze, B., Marcon, J., van den Thillart, G., Almeida-Val, 1998. Hypoxia tolerance of Amazon fish Respirometry and energy metabolism of the cichlid *Astronotus Ocellatus*. Comparative Biochemistry and Physiology Part A 120, 151-156.

Papandroulakis, N., Lika, K., Kristiansen, T.S., Oppedal, F., Divanach, P., Pavlidis, M., 2014. Behaviour of European sea bass, Dicentrarchus labrax L., in cages - impact of early life rearing conditions and management. Aquaculture Research 45, 1545-1558.

Pedersen, J.E., Bergqvist, C.A., Larhammar, D., 2019. Evolution of vertebrate nicotinic acetylcholine receptors. BMC Evol Biol 19(1):38.

Person-Le Ruyet, J., Boeuf, G., 1998. L'Azote ammoniacal, un toxique potentiel en élevage de poissons : le cas du turbot. Bulletin Francais de la Peche et de la Pisciculture 350-351, 393-412.

Person-Le Ruyet, J., Chartois, H., Quemener, L., 1995. Comparative acute ammonia toxicity in marine fish and plasma ammonia response. Aquaculture 136, 181-194.

Pettersen, J.M., Bracke, M.B.M., Midtlyng, P.J., Folkedal, O., Stien, L.H., Steffenak, H., Kristiansen, T.S., 2014. Salmon welfare index model 2.0: an extended model for overall welfare assessment of caged Atlantic salmon, based on a review of selected welfare indicators and intended for fish health professionals. Reviews in Aquaculture 6, 162-179.

Prut, L., Belzung, C., 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 463, 3-33.

Randall, D.J., Tsui, T.K.N., 2002. Ammonia toxicity in fish. Marine Pollution Bulletin 45(1-12), 17-23.

Richardson, J., Williams, E.E., Hickey, W., 2001. Avoidance behaviour of freshwater fish and shrimp exposed to ammonia and low dissolved oxygen separately and in combination. New Zealand Journal of Marine and Freshwater Research 35, 625-633.

Rimoldi, S., Terova, G., Zaccone, G., Parker, T., Kuciel, M., Dabrowski, K., 2016. The Effect of Hypoxia and Hyperoxia on Growth and Expression of Hypoxia-Related Genes and Proteins in Spotted Gar Lepisosteus oculatus Larvae and Juveniles. J Exp Zool B Mol Dev Evol 326, 250-267.

Rotllant, J., Ruane, N.M., Caballero, M.J., Montero, D., Tort, L., 2003. Response to confinement in sea bass (Dicentrarchus labrax) is characterised by an increased biosynthetic capacity of interrenal tissue with no effect on ACTH sensitivity. Comp Biochem Physiol A Mol Integr Physiol 136, 613-620.

Sadoul, B., Evouana Mengues, P., Friggens, N.C., Prunet, P., Colson, V., 2014. A new method for measuring group behaviours of fish shoals from recorded videos taken in near aquaculture conditions. Aquaculture 430, 179-187.

Sadoul, B., Friggens, N.C., Valotaire, C., Labbe, L., Colson, V., Prunet, P., Leguen, I., 2017. Physiological and behavioral flexibility to an acute CO2 challenge, within and between genotypes in rainbow trout. Comp Biochem Physiol A Mol Integr Physiol 209, 25-33.

Sadoul, B., Vijayan, M.M., 2016. Stress and growth. Fish physiology Biology of stress in fish, academic Press, 167-205.

Samaras, A., Dimitroglou, A., Sarropoulou, E., Papaharisis, L., Kottaras, L., Pavlidis, M., 2016. Repeatability of cortisol stress response in the European sea bass (Dicentrarchus labrax) and transcription differences between individuals with divergent responses. Sci Rep 6, 34858.

Samaras, A., Esperito Santo, C., Papandroulakis, N., Mitrizakis, N., Pavlidis, M., Höglund E., Pelgrim, T.N.M., Zetholf, J., Spanings F.A.T., Vindas, M.A., Ebbesson, L.O.E., Flik, G., Gorissen, M., 2018. Allostatic Load and Stress Physiology in European Seabass (Dicentrarchus labrax L.) and Gilthead Seabream (Sparus aurata L.). Front Endocrinol 9, 451.

Scherer, E., 1992. Behavioural responses as indicators of environmental alterations: approaches, results, developments. J. Appl. Ichthyol. 8, 122-131.

Schnorr, S.J., Steenbergen, P.J., Richardson, M.K., Champagne, D.L., 2012. Measuring thigmotaxis in larval zebrafish. Behaviour Brain Research 228, 367-374.

Schreck, C.B., 1990. Physiological, behavioral, and performance indicators of stress. Am. Fish. Soc. Symp. 8, 29-37.

Schreck, C.B., Olla B., L., Davis, M.W., 1997. Behavioral responses to stress, in: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish stress and Health in Aquaculture, Cambridge University Press, Cambridge, U.K., pp. 145-170.

Schreck, C.B., Tort, L., 2016. The concept of stress in fish. Fish physiology Biology of stress in fish, academic Press, 1-34.

Shimps, E.L., Rice, J.A., Osborne, J.A., 2005. Hypoxia tolerance in two juvenile estuary-dependent fishes. Journal Experimental of Marine Biology and Ecology 325, 146-162.

Shingles, A., McKenzie, D.J., Taylor, E.W., Moretti, A., Butler, P.J., Ceradini, S., 2001. Effects of sublethal ammonia exposure on swimming performance in rainbow trout (Oncorhynchus mykiss). J Exp Biol 204, 2691-2698.

Skjæraasen, J.E., Nilsen, T., Meager, J.J., Herbert, N.A., Moberg, G.P., Tronci, V., Johansen, T., Salvanes, A.G.V., 2008. Hypoxic avoidance behaviour in cod (Gadus morhua L.): The effect of temperature and haemoglobin genotype. Journal Experimental of Marine Biology and Ecology 358, 70-77.

Sneddon, L.U., Wolfenden, D.C.C., J.S., T., Tort, L., 2016. Stress management and welfare. Fish physiology Biology of stress in fish, academic Press, 463-539.

Stien, L.H., Bratland, S., Austevoll, I., Oppedal, F., Kristiansen, T.S., 2007. A video analysis procedure for assessing vertical fish distribution in aquaculture tanks. Aquacultural Engineering 37, 115-124.

Sumpter, J.P., 1997. The endocrinology of stress. Fish Stress and Health in Aquaculture, Cambridge University Press, Cambridge Society for Experimental Biology, Seminar series:62, 95-118.

Thompson, R.R.J., Paul, E.S., Radford, A.N., Purser, J., Mendl, M., 2016. Routine handling methods affect behaviour of three-spined sticklebacks in a novel test of anxiety. Behavioural Brain Research 306, 26-35.

van Raaij, M.T., Pit, D.S., Balm, P.H., Steffens, A.B., van den Thillart, G.E., 1996. Behavioral strategy and the physiological stress response in rainbow trout exposed to severe hypoxia. Horm Behav 30, 85-92.

Vandeputte, M., Gagnaire, P.A., Allal, F., 2019. The European sea bass: a key marine fish model in the wild and in aquaculture. Anim Genet 50, 195-206.

Vignet, C., Devier, M.H., Le Menach, K., Lyphout, L., Potier, J., Cachot, J., Budzinski, H., Begout, M.L., Cousin, X., 2014. Long-term disruption of growth, reproduction, and behavior after embryonic exposure of zebrafish to PAH-spiked sediment. Environ Sci Pollut Res Int 21, 13877-13887.

Weetman, D., Atkinson, D., Chubb, J.S., 1999. Water temperature influences the shoaling decisions of guppies, *Poecilia reticulata*, under predation threat. Animal behavior 58, 735-741.

Xu, J., Liu, Y., Cui, S., Miao, X., 2006. Behavioral responses of tilapia (*Oreochromis niloticus*) to acute fluctuations in dissolved oxygen levels as monitored by computer vision. Aquacultural Engineering 35, 207-217.

Captions

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

Figure 2. Mean ± SEM of TAN and UIA-N ($mg.L^{-1}$) and oxygen saturation (% O_2) for High TAN, Hyperoxia and Hypoxia conditions during bathing period (at 0, 30 and 60 min) and at the start and end of novel environment challenge in group.

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

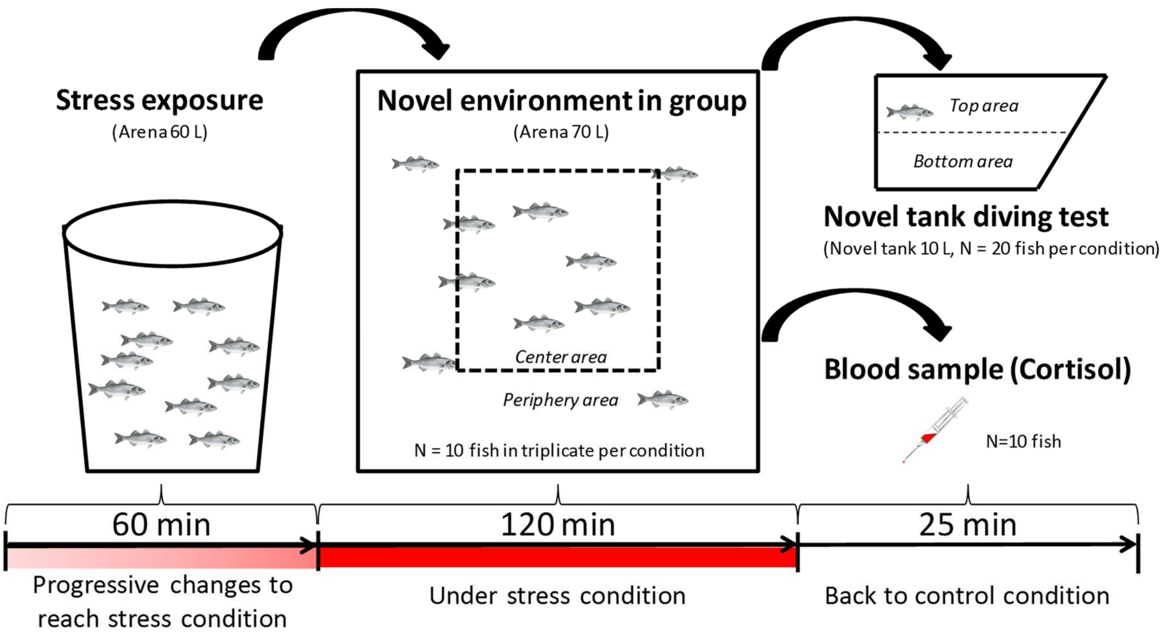
Figure 4. Spatial location of fish during the novel environment in group. Averaged heatmaps of fish spatial location (n=30 per condition) over the different time periods. From low (blue colour) to high location frequency (red colour).

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

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

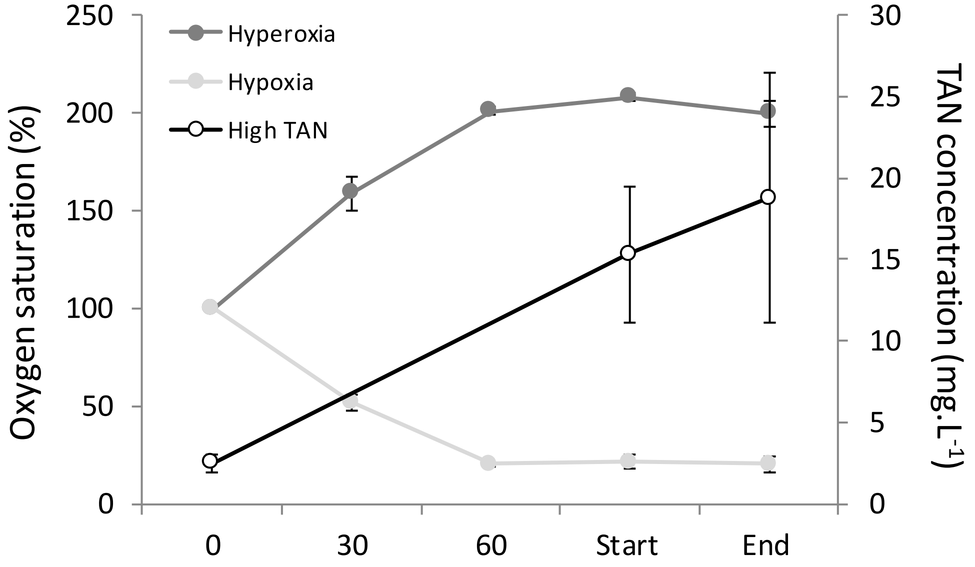
Figure 7. Plasma cortisol concentration *post*-stress in the novel environment group challenge (Mean ± SD) for each condition (n=9 control, n=9 High TAN, n=9 Hyperoxia; n=10 Hypoxia). Mann & Whitney U-test: *: p<0.05; ***: p<0.001

Figure 1



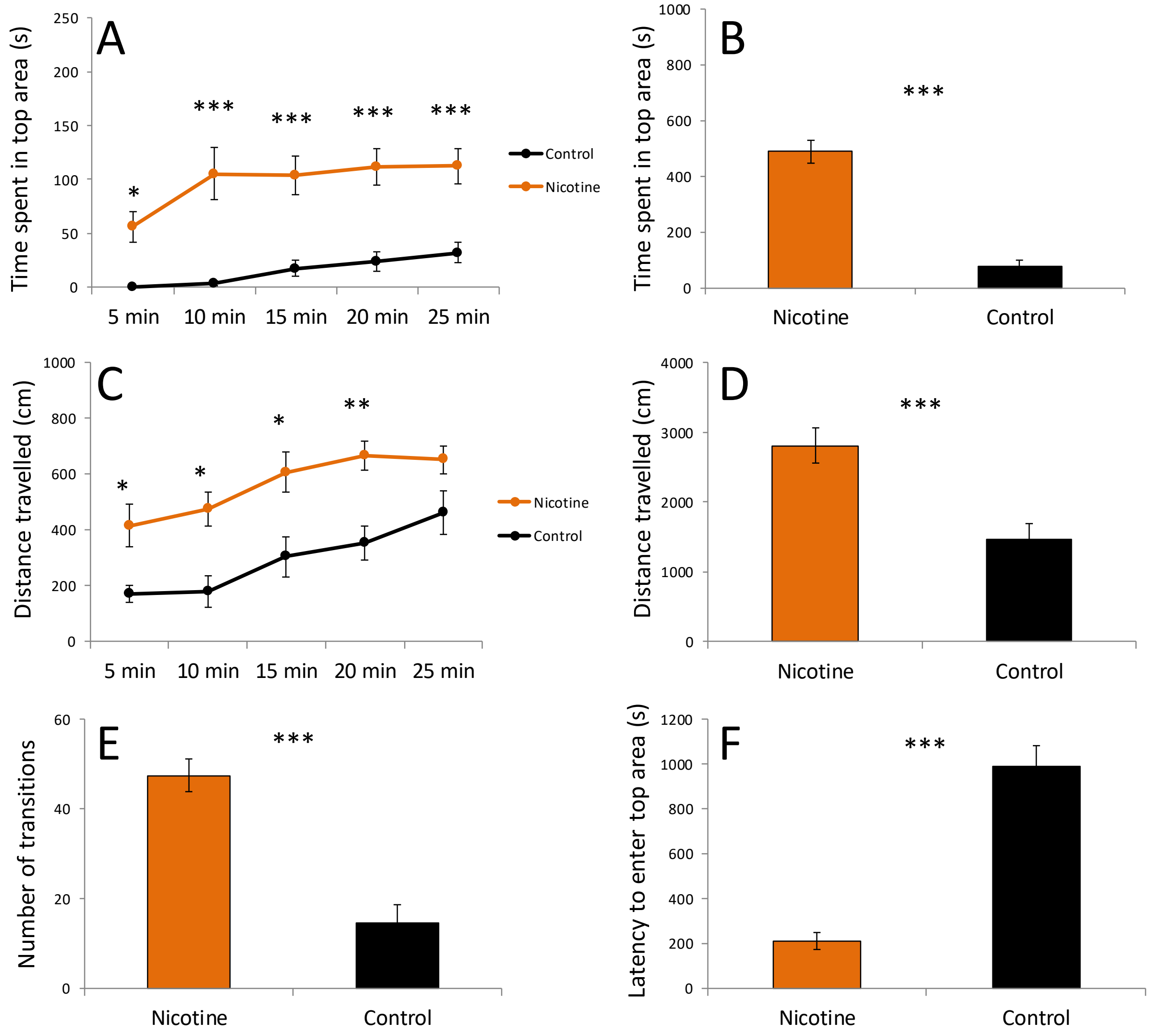
Stress exposure
(Arena 60 L)
Novel environment in group
(Arena 70 L)
Center area
Periphery area
N = 10 fish in triplicate per condition
Top area
Bottom area
Novel tank diving test
(Novel tank 10 L, N = 20 fish per condition)
Blood sample (Cortisol)
N=10 fish
60 min
120 min
25 min
Progressive changes to reach stress condition
Under stress condition
Back to control condition

Figure 2



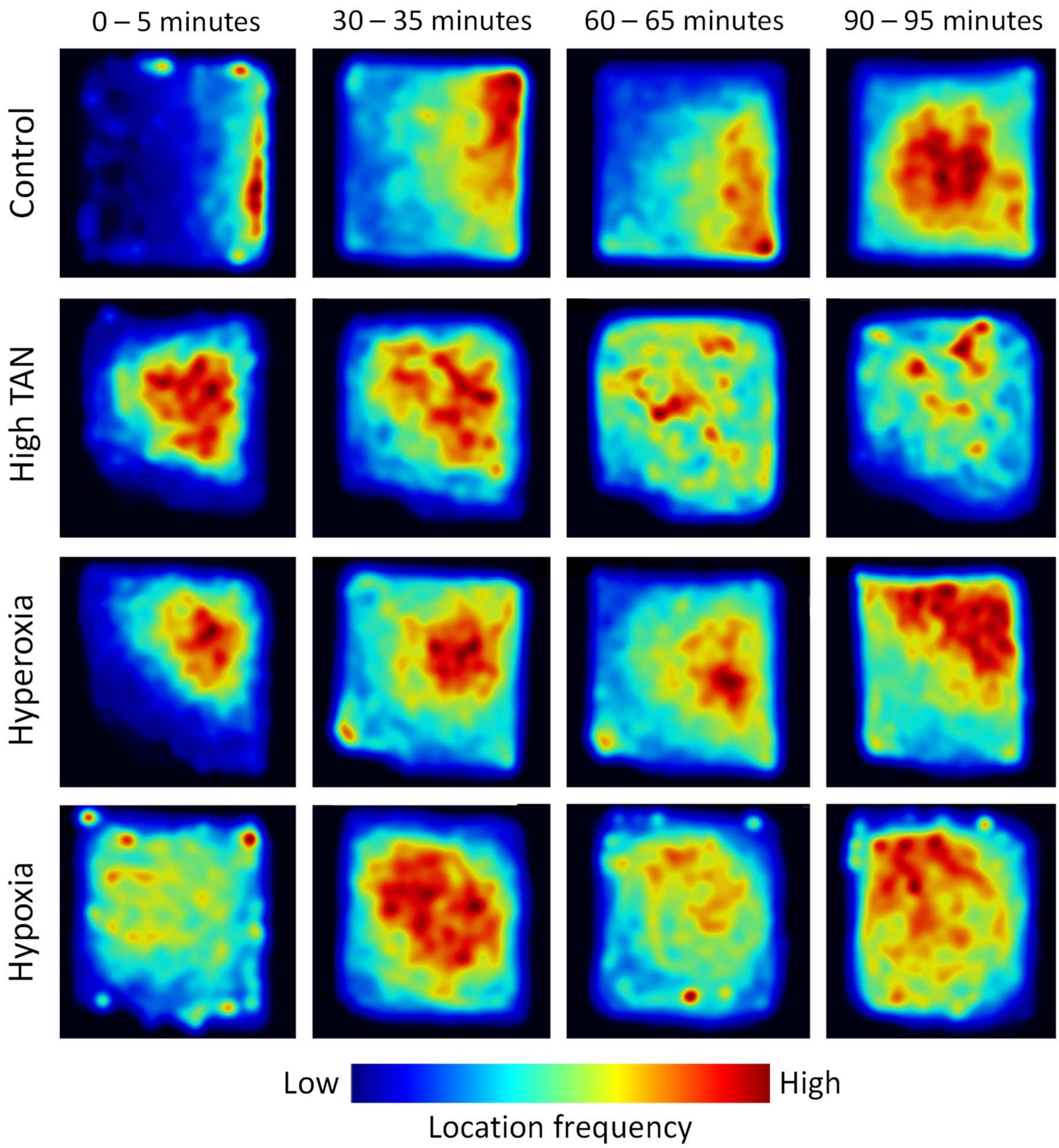
Hyperoxia
Hypoxia
High TAN
Oxygen saturation (%)
TAN concentration (mg.L-1)
250
200
150
100
50
0
30
25
20
15
10
5
0
0
30
60
Start
End

Figure 3



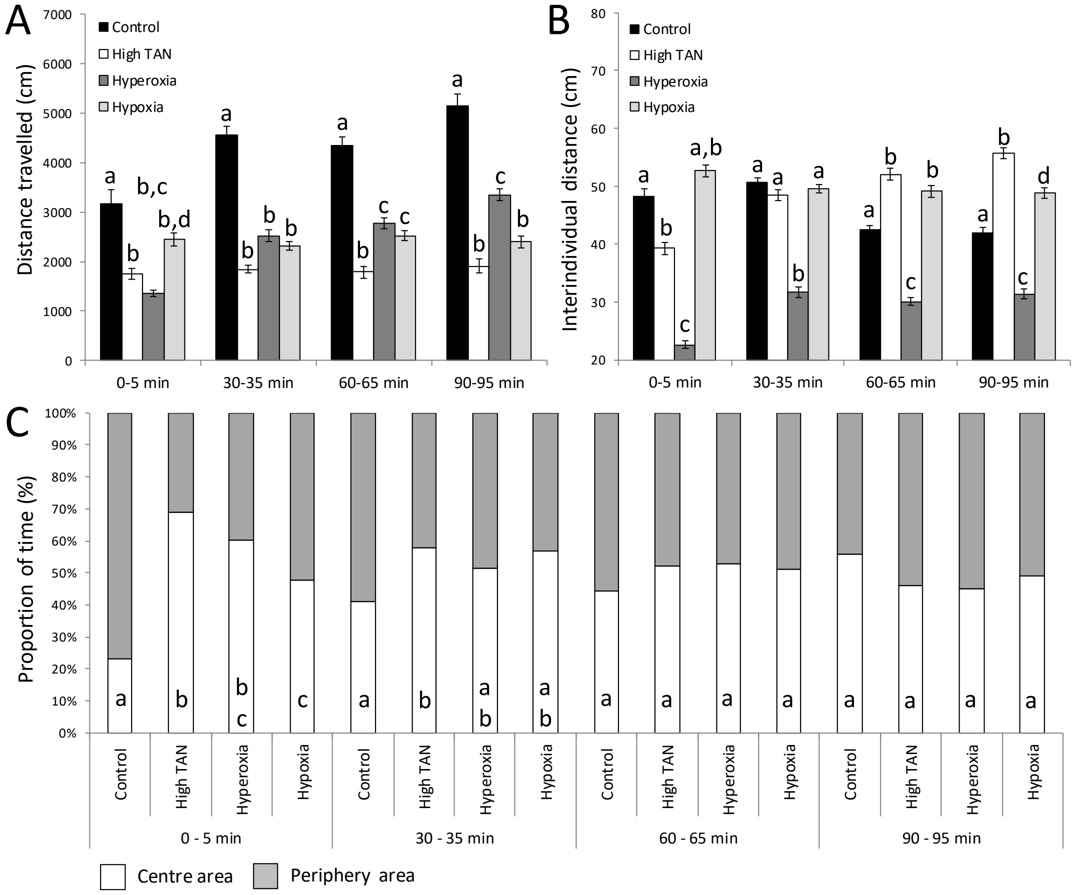
A
Time spent in top area (s)
250
200
150
100
50
0
5 min
10 min
15 min
20 min
25 min
Control
Nicotine
*
***
***
***
***
B
Time spent in top area (s)
1000
800
600
400
200
0
Nicotine
Control
***
C
Distance travelled (cm)
1000
800
600
400
200
0
5 min
10 min
15 min
20 min
25 min
Nicotine
Control
*
*
*
**
D
Distance travelled (cm)
4000
3000
2000
1000
0
Nicotine
Control
***
E
Number of transitions
60
40
20
0
Nicotine
Control
***
F
Latency to enter top area (s)
1200
1000
800
600
400
200
0
Nicotine
Control
***

Figure 4



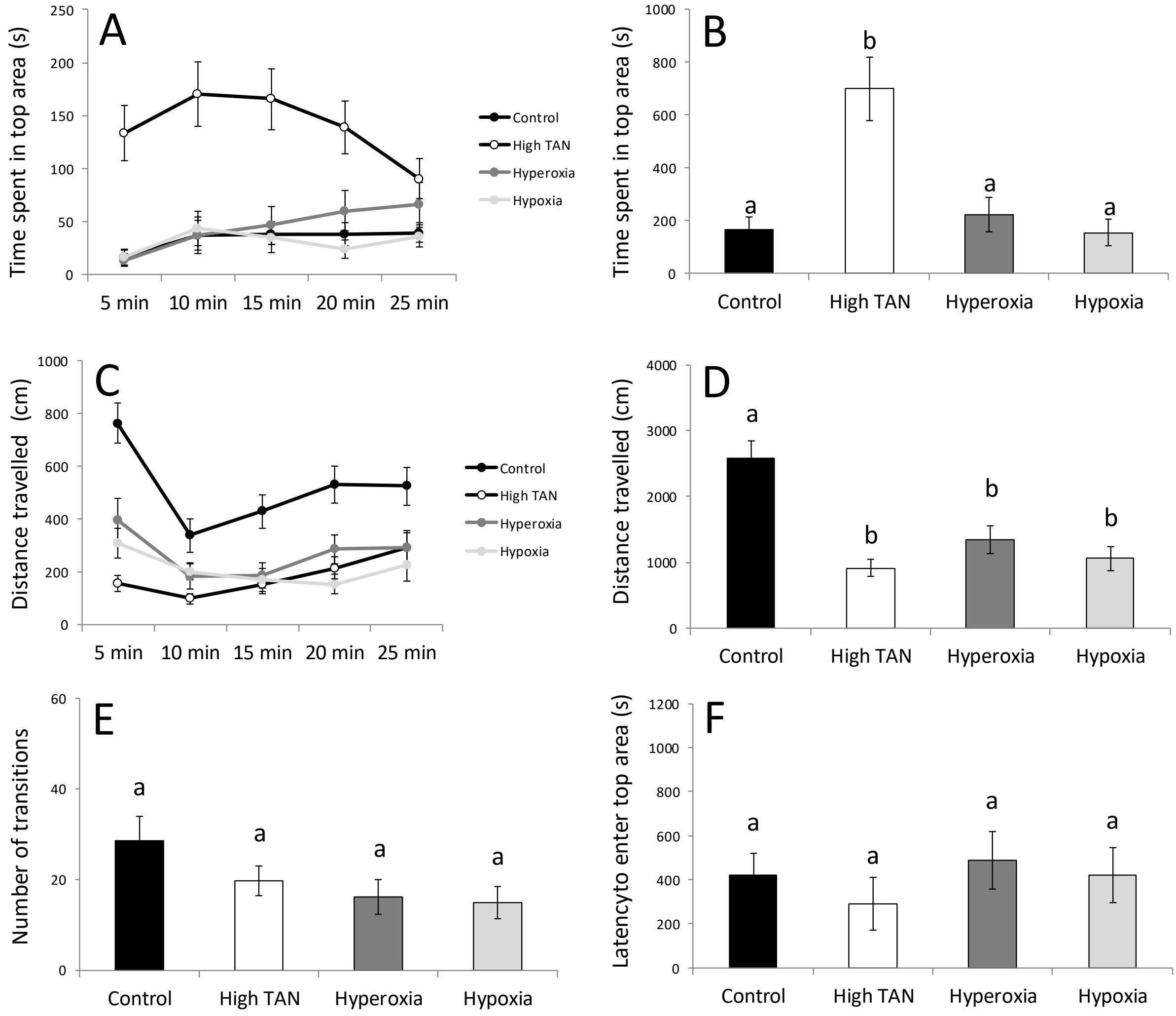
0 – 5 minutes
30 – 35 minutes
60 – 65 minutes
90 – 95 minutes
Control
High TAN
Hyperoxia
Hypoxia
Low
High
Location frequency

Figure 5



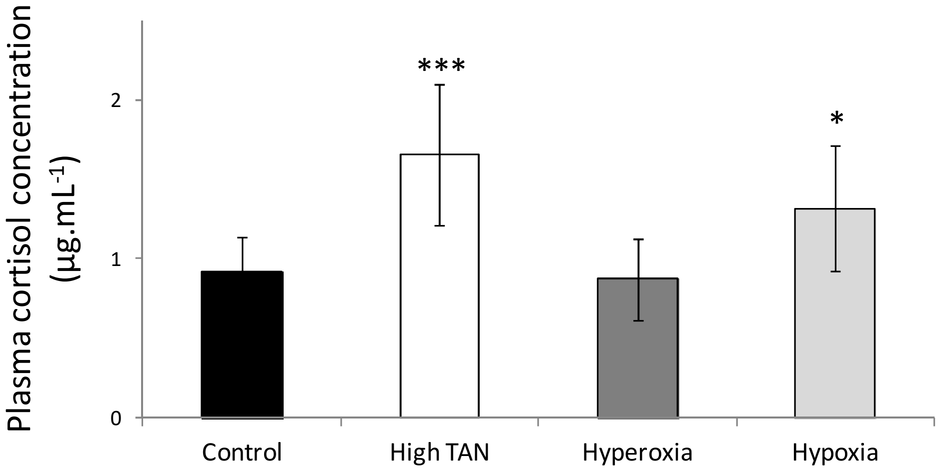
A
B
C
Control
High TAN
Hyperoxia
Hypoxia
Distance travelled (cm)
Interindividual distance (cm)
Proportion of time (%)
0-5 min
30-35 min
60-65 min
90-95 min
Centre area
Periphery area

Figure 6



A
Time spent in top area (s)
250
200
150
100
50
0
5 min
10 min
15 min
20 min
25 min
Control
High TAN
Hyperoxia
Hypoxia
B
Time spent in top area (s)
1000
800
600
400
200
0
a
b
a
a
Control
High TAN
Hyperoxia
Hypoxia
C
Distance travelled (cm)
1000
800
600
400
200
0
5 min
10 min
15 min
20 min
25 min
Control
High TAN
Hyperoxia
Hypoxia
D
Distance travelled (cm)
4000
3000
2000
1000
0
a
b
b
b
Control
High TAN
Hyperoxia
Hypoxia
E
Number of transitions
60
40
20
0
a
a
a
a
Control
High TAN
Hyperoxia
Hypoxia
F
Latencyto enter top area (s)
1200
1000
800
600
400
200
0
a
a
a
a
Control
High TAN
Hyperoxia
Hypoxia

Figure 7



Plasma cortisol concentration (µg.mL-1)
2
1
0
***
*
Control
High TAN
Hyperoxia
Hypoxia