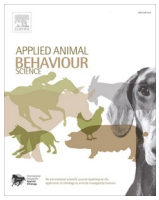
Applied Animal Behaviour Science 239 (2021) 105335

Contents lists available at ScienceDirect

Applied Animal Behaviour Science

journal homepage: www.elsevier.com/locate/applanim



# Loss of light colour preference after chronic embryonic stress in rainbow trout fry: A novel and potential indicator of fish welfare?

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ARTICLE INFO

*Keywords:*
Air exposure
Alarm cue
Colour preference
Fish
Stress
Ontogeny

ABSTRACT

For many fish species, environmental colour may act either as a source of stress or as a stress-buffer, alleviating behavioural and physiological responses after a stressful situation. While much is known on the effects of environmental colour on fish stress parameters, knowledge on the effects of stress on fish colour preferences is still lacking. In order to test the effects of stress on colour preference in fish, in this work, we exposed rainbow trout embryos (*Oncorhynchus mykiss*) to stressful conditions (air exposure, pheromone alarm cue or control, with minimal stress) from 19 to 44 days post fertilization (dpf). They were then raised up to 56 dpf in bright, dark, green or blue environments. After that, fry were individually tested for colour preference in a three-chambered arena where they could choose between green and blue areas. The time spent in the blue and in the green chamber was compared between experimental groups. Rainbow trout fry exposed to minimal stress (control) or to biotic stress (pheromone alarm) showed increased time in the blue environment, with little effect of ambient colour where they were raised. However, fish that experienced air exposure stress showed a lack of colour preference irrespective of the colour they were raised in afterwards. These results imply that early life stress affects colour preference in rainbow trout, suggesting that abiotic stressors, such as air exposure, may affect colour perception or behavioural plasticity in young fish. If the results presented herein are corroborated by future studies in fish at different life stages, beyond the embryonic phase, colour preference tests may be used as an additional and potential welfare indicator to estimate, in a retrospective manner, which stressors were faced by the individuals during early stages. By knowing whether or not their fish were exposed to certain stressful conditions may allow farmers to better adapt fish rearing conditions and to implement strategies that alleviate any long-term impacts that may exist, and, therefore, improve fish welfare.

## 1. Introduction

The concept of animal welfare has been pushed over the recent years to change and adapt to public and legislative demands, prioritizing the so-called positive welfare, instead of only avoiding and alleviating unfavourable and poor rearing conditions (Fife-Cook and Franks, 2019; Lawrence et al., 2019). Fish farmers are much more attentive to how animals perceive and react to their physical and social environments. Considering fish motivations and preferences has become an essential step in assessing their welfare (ANSES, 2018).

An important feature related to farmed fish welfare is the colour of the environment where individuals are reared. Environmental colour may act either as a source of stress (e.g., the red colour for multiple fish species, Ruchin, 2020) or as a stress-buffer, alleviating behavioural and

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https://doi.org/10.1016/j.applanim.2021.105335
Received 4 March 2021; Received in revised form 15 April 2021; Accepted 18 April 2021
Available online 24 April 2021

physiological responses after a stressful situation (e.g., the blue colour for Nile tilapia, *Oreochromis niloticus*, Maia and Volpato, 2013). Studies have shown that some species may have their own colour preferences that need to be considered when housing them. Adults and larvae of Zebrafish (*Danio rerio*), for example, prefer blue zones and avoid yellow-coloured zones (Peeters et al., 2016). On the contrary, Nile tilapia show a preference for yellow zones and avoid red zones (Luchiari et al., 2007). However, these preferences are under the influence of multiple parameters, such as individual experiences (Luchiari et al., 2007; Roy et al., 2019), shoaling (Wang et al., 2020), and season of the year (Luchiari and Pirhonen, 2008). The within-species variation in colour preference has resulted so far in contradictory outcomes, offering partial and inconclusive results (McLean, 2021; Peeters et al., 2016; Ruchin, 2020). While much is known on the effects of environmental colour on fish stress parameters (Barcellos et al., 2009; Heydarnejad et al., 2013; Karakatsouli et al., 2012), knowledge on the effects of stress on fish colour preferences is still lacking.

Rainbow trout (*Oncorhynchus mykiss*), the primary freshwater fish farmed in Europe (Eurostat, 2020), are known to have a strong preference and motivation to access blue backgrounds (Maia et al., 2017). Luchiari and Pirhonen (2008), testing multiple colour preferences in rainbow trout, showed that fish preferences may be altered following different environmental conditions, such as water temperature during the tests: at 1 °C fish preferred equally blue and green backgrounds over yellow and red ones. At 12 °C, however, fish preferred green over all other colours. The immediate environment of fish may, therefore, alter the perceptions they have of their environment. We recently showed that stressful events may alter their perceptions and reactions to the physical environment. Juvenile trout that underwent biotic stress (pheromone alarm cues) during embryogenesis exhibited less fear-related behaviour than control fish. Conversely, fish that were exposed to air, a type of abiotic stress, did not show any disruption in their behaviour (Poisson et al., 2017). Whether embryonic stresses alter rainbow trout spontaneous colour preferences is still unknown.

In this study, our main objective was to investigate whether and how embryonic biotic and abiotic stresses influenced colour preferences. We tested preference for blue versus green, colours that are better perceived by animals (Roy et al., 2019), and are the most preferred colours following previous results by Maia et al. (2017) and Luchiari and Pirhonen (2008) in rainbow trout fry. Based on our previous results (Poisson et al., 2017), we hypothesized that embryonic biotic stress would significantly impact fry colour preferences more than abiotic stress. As the colour of the tanks where fry are reared may influence the individuals' colour preference (Luchiari and Oliveira, 2014), our second objective was to test how a short period of fry incubation under different colour lights would impact fry colour preferences. Since early ontogenetic experience may influence an individual's habitat preferences (Davis and Stamps, 2004), we expected control fish to prefer the colour matching its rearing environment, while this pattern would be disrupted in stressed animals.

## 2. Material and methods

A full description of fertilization, incubation, and conspecific pheromone exposure is described in Poisson et al. (2017). This study was conducted at the INRAE-LPGP experimental facilities (Rennes, France) from April 13$^{th}$ to June 27$^{th}$ in 2016. These facilities have authorization for animal experimentation (C35-238-6). This present study was approved by the local ethic committee provided by the French legislation under the official licence no. 07. The project's agreement number is: APAFIS#1749- 201509071335794 v2.

### 2.1. Fertilization and incubation

Oocytes and milt from four females and four males of Spring rainbow trout strain, domesticated over 30 years by INRAE, were stripped. After fertilization, fertilized eggs were separated into three experimental groups: a control group with minimal pre-hatching stress (Control), a stressed group in which eggs were air exposed for 1 min (air stressed - AS), and a stressed group which eggs were exposed to conspecific alarm pheromone (pheromone stressed - PS) for 1 min (detailed below). The abiotic (air) and biotic (pheromone) stressing procedures were performed three times per week (at 12 pm, midday) from the eyed stage (19 days postfertilization [dpf]) to 44 dpf, totalling 12 times of exposure to the stressing procedures. The succession of acute stressors may become a chronic stressor, as seen for different fish species (Lankford et al., 2005; Piato et al., 2011).

Approximately 1650 fertilized eggs (550 per experimental group) were distributed within 12 trays (20 × 50 cm), each one containing 2 incubators (10 × 10 cm box with bottom holes for water circulation) with 70 eggs/incubator, and supplied with 10°C flow-through recycled water. Each experimental group was composed of 4 trays, i.e., 8 incubators/experimental group. Each tray was covered with a plain lid, to create a darkness ambient, which is the classical incubation condition used in rainbow trout farming. Dead embryos and fry were counted and removed from the incubators at 20 dpf, 28 dpf, and 35 dpf. The initial number of fertilized eggs per incubator was obtained by taking pictures of each incubator and pointing eggs one by one using ImageJ software. At 35 dpf, when hatching has already occurred, living fry were counted to obtain the final mortality percentages.

### 2.2. Stressor exposure

To generate a whole-body pheromone extract, as described by Brown et al. (2011), eight juvenile trout (~10 g) were killed with a blow to the head and decapitated, this killing method being accepted by the European legislation. Fifty cm$^2$ of skin/trout were collected and immediately placed in 80 mL of distilled water. The tissue was crushed and filtered through a colander, and we added distilled water to bring it to a final volume of 800 mL (1 cm$^2$/10 mL). Half of the blend was used to prepare dilutions. The chosen concentration was 10x, corresponding to 3.125 mL/litre. Preliminary pilots showed this concentration induced the most potent behavioural response (e.g., trout were observed immobile for the longest duration) (Poisson et al., 2017). Dilutions were separated into 1.5 mL aliquots and frozen at -20 °C until needed. Each day, frozen tubes were removed from the freezer before diffusions and kept at ambient temperature from 8 a.m. until 12 a.m. hours when diffusions started. Embryos from PS groups were simultaneously exposed to the alarm cue by injecting a constant flow with a peristaltic pump carrying the 1.5 mL of liquid through a pipe. Each PS incubator was equipped with a pipe passing through the lid via a hole of the same diameter, maintaining the eggs under dark conditions. The diffusion lasted approximately 1 min.

The air exposure was performed in the AS group by removing the incubators from the tray for 1 min. At the same time, the room's light was manually turned off, to maintain dark conditions during the two stress procedures.

From 44–47 dpf, stress procedures ceased until the beginning of incubation under different light conditions.

### 2.3. Fry incubation under different light colours

From 47–56 dpf, fry from the experimental groups were reared under four different incubation conditions. These incubation conditions were designed to be really distinctive from each other and were as follows: (1) Dark incubation (i.e. Control condition with solid lids kept over the tray; illuminance measured under the lid at the water surface was 0.2 lx), (2) Bright incubation (solid lids replaced by clear Plexiglas lids, fry receiving the room's light under 12 L:12D (Light: Dark) photoperiod, with lights on at 8 a.m.; illuminance ~ 60 lx), (3) Green incubation: a translucent green plastic cover surmounted by a three-LED light band (DC 12 V, LED $\lambda_{max}$ 578 nm) illuminated each incubator under 24 L:0D photoperiod (illuminance ~ 60 lx, green wavelengths ranged from 500

to 600 nm, $\lambda_{max}$534 nm), and (4) Blue incubation: a translucent blue plastic cover surmounted by a three-LED light band illuminated each incubator under 24 L:0D photoperiod (illuminance ~ 60 lx, blue plastic cover exhibited 3 spectral contributions (blue line with $\lambda_{max}$ centred at 450 nm (40 % of the light perceived), green line with $\lambda_{max}$ centred at 520 nm (40 %) and red line with $\lambda_{max}$ centred around 700 nm (20 %)). There were, in the same circuit, two incubators (i.e., approximately 140 fry) per experimental condition. Illuminance was measured at the water surface by a Digital Lux Meter (ISO-TECH 1332A). Visible spectra measurements have been performed on a HR-Evolution (HORIBA Scientific) micro-Raman spectrometer in the "SIR Platform" (UMS Scan-MAT, Rennes 1 University: https://scanmat.univ-rennes1.fr). Light was collected through a x5 lens on the 400–800 nm spectral range.

From 56 dpf (at vitellus resorption) to 62 dpf, all fry were reared under bright conditions (similar to the bright incubation, with 12 L:12D photoperiod and lights on at 8 a.m.) and were manually fed (3 meals/day) with a commercial diet (Biomar: 48 % protein and 22 % lipid, 0.5 mm diameter pellets) directly into their incubators.

### 2.4. Colour preference test

Between 62 and 65 dpf, rainbow trout fry from each experimental group (12 experimental groups, with 12 fish each) were tested individually and only once to measure their colour preferences according to incubation colour (dark, bright, green or blue) and stress conditions (control, AS or PS).

Adapted from previous experiments on fish preferences (Delicio et al., 2006; Millot et al., 2014), the arena (30 × 10 cm) used for the colour preference test was a 3-chamber tank (each chamber measuring 10 × 10 cm) with the middle chamber used as a starting point (where fish was introduced). Each lateral chamber was illuminated in the exact same conditions as during green or blue incubation: the three-LED light band was placed above a green (green chamber) or a blue (blue chamber) translucent plastic cover (Fig. 1).

For the test, 12 fry were netted at the same time from the incubator and transported in a bowl with water to an adjacent video-equipped room where the arena was placed. The transport of fry between these two rooms lasted less than 30 s. Furthermore, water conditions in the testing were kept the same as in the incubation room. The testing order was chosen at random among the 12 experimental groups (six random fish from each of the two incubators per experimental group). Half of the fry were tested with the blue chamber being on their right and the green chamber being on their left, while for the remaining fry, the position of the chambers was inversed (by rotating the testing arena).

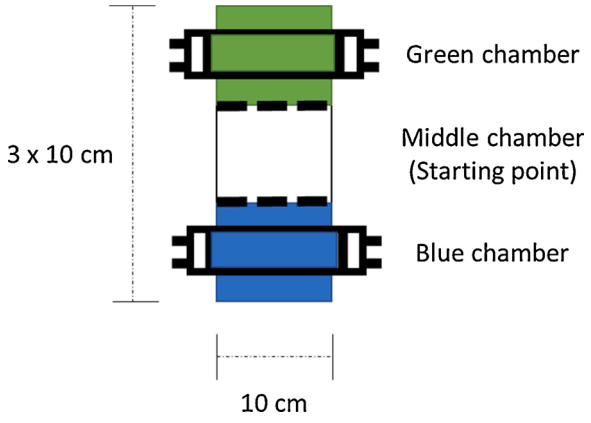


**Fig. 1.** Schematic figure of the arena used for the colour preference test in rainbow trout fry. Each chamber measured 10 × 10 cm and the middle chamber was used as a starting point (where fry were individually introduced). The lateral chambers were illuminated by a three-LED light band placed above a green (green chamber) or a blue (blue chamber) translucent plastic cover. After a 30-min period of fry acclimatation to the middle chamber, two remote-controlled guillotine doors were pulled up (dashed lines), and the cover was removed by a motorized rolling-cylinder, allowing fry to actively access the whole arena.

Upon their arrival in the test room, fry were immediately and individually introduced into the middle chamber (which was covered) of the testing arena for 30 min for acclimation. At the end of the 30-min period, two remote-controlled guillotine doors were pulled up, and the cover was removed by a motorized rolling-cylinder, allowing fry to actively access the whole arena, and enter the blue or the green chamber at opposite sides. Fish behaviour was recorded for 40 min. All fish actively explored the arena and switched from one chamber to another. At the end of the colour preference tests, fry were netted and euthanized by lethal dose of tricaine methane sulphonate (200 mg/litre) in accordance with the European legislation. They were then measured and weighed.

### 2.5. Behavioural analysis

First, five frames per second were extracted from the videos with the VirtualDub software. These images were then analysed with a home-made macro written with the free software Fiji (Schindelin et al., 2012). Briefly, a background image was calculated from a median image of 14 images (7 images preceding and 7 following the image to analyse on the video). The background was subtracted to each image allowing automatic thresholding of the pixel corresponding to the fish body. To avoid any artifact (non-fish body pixels), only the area greater than 100 pixels were kept. The barycentre of the binarized fish body was measured. The value corresponds to the location of the fish in the middle chamber of the tank. Then, the time spent in each chamber was determined with the assumption that if the fish is visible, a barycentre value exists, therefore the fish is in the middle chamber (non-coloured). If the fish is not visible (no barycentre value), its location is either in the blue or green chamber and can be determined according to the proximity of its last visible location (barycentre in the middle chamber) with the coloured area. The data were then grouped together to have, for each fish, the time spent in each chamber.

### 2.6. Statistics

Mortality percentage (summed over 20 dpf, 28 dpf, and 35 dpf) were compared between stress treatments using chi-square tests, followed by post-hoc with Bonferroni adjustments.

Our first objective was to compare the time spent in each chamber between stress treatments and incubation colours. For this, the time spent in each chamber was transformed into percentages. That is, for each individual the time spent in each chamber (blue, middle, or green) was divided by the total time of the test and multiplied by 100. Then, this variable was analysed using a linear mixed model (LMM, 'lmerTest' R package) and degrees of freedom were estimated through the Satterthwaite approximation. Normality of residuals was verified through graphical evaluations (histograms and QQ plots). To meet normality assumptions, our dependent variable was square-root transformed. The fixed factors included in the first model were: the stress treatments (control, air stress, and pheromone stress), the incubation conditions (dark, bright, green, and blue), the chambers (blue, middle, and green), as well as their two-way and three-way interactions. Covariates included in the model were: the position of the blue chamber during test (right or left), fry weight and size. Finally, the random variables were individual ID and incubator. The complete model used was, therefore: Model <- lmer(timesqrt ~ treatment * incubation * chamber + position + weight + size + (1 |id) + (1 | incubator), data = data). The three-way interaction "treatment*incubation*chamber", as well as the two-way interactions between treatment*incubation and incubation*chamber were not significant and were excluded from the final model. Significant interactions or main effects were analysed through a post hoc ANOVA of estimated marginal means ('emmeans' R package), carried out with Tukey adjustment.

Weight, body size and condition factor (100 x [Weight/Body size$^3$]) of each fry were analysed following the same previous procedures. Fixed factors included in the first model were: the stress treatments and the incubation conditions, while the random variable was the incubator. Interactions were not significant and were excluded from the final model. All three variables were square-root transformed to fit the residual normality assumptions. One extreme outlier in the condition factor variable was excluded.

Analyses were conducted using IBM SPSS 22 and R version 3.6.1., with statistical significance being assigned at $p \leq 0.05$.

## 3. Results

There was a significant association between mortality percentage and stress treatments ($X^2(2) = 7.52$, $p = 0.02$). Post-hoc analyses revealed that both stress treatments, PS and AS, had similar mortality percentages, however they were both higher compared to individuals in the Control situation (PS: 20.5 %, AS: 20.5 %, and Control: 14.7 %).

The percentage of time spent in each chamber (blue, middle, and green), for each experimental group is shown in Table 1. Chamber was the only main effect significantly influencing the time spent by the fry in different chambers during the test ($F_{2, 417} = 309.66$, $p < 0.001$). Post-hoc analyses on stress treatments revealed that blue was the chamber where fry spent most of their time, followed by the green chamber, and finally, the least time was spent in the middle chamber (all $p < 0.001$). Stress treatments ($F_{2, 417} = 0.01$, $p = 0.98$), incubation conditions ($F_{3, 417} = 0.189$, $p = 0.9$), fry weight ($F_{1, 417} = 0.26$, $p = 0.6$), fry size ($F_{1, 417} = 0.26$, $p = 0.6$), or the position of the blue chamber ($F_{1, 417} = 0.12$, $p = 0.72$) did not influence the time fry spent in the different chambers. We found a significant interaction between chamber and treatment ($F_{4, 417} = 4.71$, $p < 0.001$, Fig. 2). Post-hoc analyses revealed that control fish and PS fish spent more time in the blue chamber, followed by the green chamber, and then the middle chamber (all $p < 0.001$). The time spent in the blue chamber and green chamber for AS fish was not different ($p = 0.08$). However, the time spent in these chambers were significantly higher compared to the middle chamber (all $p < 0.001$). Additionally, AS fish spent more time in the green chamber compared to control ones ($p = 0.04$, Fig. 2).

At the end of the colour preference tests, fry were weighed and measured. Fry weight and body size were not influenced by stress treatments (weight: $F_{2, 18} = 0.61$, $p = 0.55$, body size; ($F_{2, 18} = 0.78$, $p = 0.48$), nor by incubation colours (weight: $F_{3, 18} = 2.03$, $p = 0.14$, body size; ($F_{3, 18} = 0.91$, $p = 0.45$). While stress did not influence condition factor ($F_{2, 17.36} = 0.18$, $p = 0.82$), there was a significant effect of incubation ($F_{3, 18} = 3.23$, $p = 0.047$). Post-hoc analyses revealed that fish incubated in the dark showed reduced condition factor compared to bright conditions ($p = 0.04$, Table 2), but did not differ from the other conditions (all $p > 0.05$).

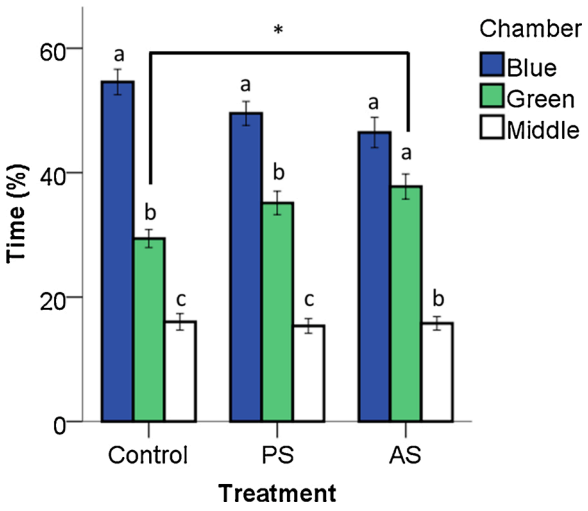


**Fig. 2.** Rainbow trout fry percentage of time spent in each chamber (blue, green, and middle chambers) during a colour preference test. Experimental groups (n = 48) faced different types of embryonic stress treatments (control, PS – pheromone alarm cue stress, and AS – air exposure stress). Different letters indicate significant differences in the time spent in the different chambers within each treatment. Asterisk indicates differences in the time spent in the different chambers between treatments. * ≤ 0.05. Mean ± SD are given.

## 4. Discussion

To our knowledge, this is the first report on how biotic (alarm pheromone from conspecifics) and abiotic stressors (air exposure), incubation colour (dark, bright, green, and blue), and their interaction influence light colour preference (blue or green) in rainbow trout fry. Contrary to our initial expectations, control fish and fish exposed to the

**Table 1**
Rainbow trout fry percentage of time spent in each chamber (blue, green, and middle chambers) during a colour preference test. Each line indicates one combination between stress treatments (control, PS and AS) and incubation colour (dark, bright, blue and green). Mean ± SD are given.

| Stress treatment | Incubation colour | Time (%) Blue | Middle | Green |
|---|---|---|---|---|
| Control | Dark | 54.71 ± 12.63 | 14.61 ± 4.31 | 30.65 ± 9.72 |
| | Bright | 54.45 ± 19.26 | 17.86 ± 14.58 | 27.69 ± 6.96 |
| | Blue | 58.25 ± 8.96 | 15.99 ± 9.10 | 25.74 ± 9.53 |
| | Green | 50.86 ± 15.07 | 15.60 ± 6.21 | 33.5 ± 12.53 |
| PS | Dark | 52.51 ± 12.27 | 14.60 ± 4.54 | 32.91 ± 8.92 |
| | Bright | 44.83 ± 10.86 | 13.07 ± 8.51 | 42.04 ± 15.23 |
| | Blue | 50.88 ± 17.14 | 17.43 ± 12.51 | 31.69 ± 14.50 |
| | Green | 49.83 ± 12.98 | 16.30 ± 5.38 | 33.85 ± 11.42 |
| AS | Dark | 45.14 ± 14.13 | 17.47 ± 8.13 | 37.39 ± 12.33 |
| | Bright | 48 ± 18.01 | 15.48 ± 9.27 | 36.50 ± 13.73 |
| | Blue | 43.25 ± 21.91 | 16.48 ± 8.24 | 40.26 ± 18.32 |
| | Green | 49.41 ± 13.58 | 13.66 ± 3.88 | 36.91 ± 11.88 |

**Table 2**
Rainbow trout fry body weight, size, and condition factor. Experimental groups (n = 12, except for Control * Bright groups where n = 11, due to one outlier being excluded) were a combination of stress condition (control, PS – pheromone alarm cue stress, and AS – air exposure stress) and incubation colour (dark, bright, blue, and green). Mean ± SD are given.

| Incubation colour | Stress treatment | Weight (g) | Size (cm) | Condition factor |
|---|---|---|---|---|
| Dark[a] | Control | 0.12 ± 0.02 | 2.63 ± 0.15 | 0.68 ± 0.07 |
| | PS | 0.10 ± 0.02 | 2.50 ± 0.11 | 0.65 ± 0.07 |
| | AS | 0.11 ± 0.02 | 2.55 ± 0.17 | 0.68 ± 0.08 |
| Bright[b] | Control | 0.12 ± 0.03 | 2.53 ± 0.17 | 0.75 ± 0.13 |
| | PS | 0.13 ± 0.03 | 2.6 ± 0.14 | 0.74 ± 0.11 |
| | AS | 0.14 ± 0.02 | 2.65 ± 0.12 | 0.77 ± 0.08 |
| Blue[ab] | Control | 0.13 ± 0.03 | 2.63 ± 0.14 | 0.73 ± 0.09 |
| | PS | 0.13 ± 0.02 | 2.61 ± 0.14 | 0.75 ± 0.08 |
| | AS | 0.13 ± 0.02 | 2.62 ± 0.15 | 0.74 ± 0.07 |
| Green[ab] | Control | 0.12 ± 0.01 | 2.61 ± 0.1 | 0.68 ± 0.06 |
| | PS | 0.13 ± 0.01 | 2.6 ± 0.11 | 0.76 ± 0.04 |
| | AS | 0.14 ± 0.05 | 2.7 ± 0.19 | 0.72 ± 0.11 |

Different lower case letter ([a, b, ab]) above the incubation colours indicate statistical significance (LMM post-hoc) between groups (dark ≠ bright) for the condition factor ($p < 0.05$).

alarm pheromone tended to have equivalent reactions during tests: they showed a stronger preference for the blue chamber than air-exposed fish, which exhibited no preferences between the blue or the green chambers.

These results confirm previous studies and add new knowledge to the research on fish colour preferences. Like juvenile rainbow trout (Maia et al., 2017), non-stressed control fry preferred blue environments over green ones. Maia et al. (2017) suggested that the species' environment shapes preference in natural conditions: rainbow trout are commonly found in dark-blue waters, where the contrast between their body and the background is less apparent. Blue environmental colour is also known to positively affect multiple domains, such as fish reproduction, growth, and stress responses (Maia and Volpato, 2013; Ruchin, 2020; Volpato et al., 2004), which further explains the individuals' preference for this colour. Interestingly, even though it is known that, from 44 dpf, rainbow trout fry present optomotor responses, and have an increasing vision acuity and sensibility to light variation (Carvalho et al., 2004), our results demonstrate that the ten-day incubation of fish under different light/colour conditions was insufficient to change their overall innate preferences. Similar outcomes were reported for juvenile turbot (*Scophthalmus maximus*) and barramundi (*Lates calcarifer*): irrespectively of being reared in coloured tanks, these fish species still displayed preferences for white and blue backgrounds, respectively (Li et al., 2016; Ullmann et al., 2011). Optomotor tests using the same wavelengths of our preferences tests in order to measure fry visual abilities after different stress situations would be highly valuable to better understand fish behavioural responses.

The innate blue colour preference was disrupted when individuals faced chronic abiotic stress conditions during the embryonic stages. While for control and PS fish there were significant preferences for the blue chamber, AS fish did not show any sign of colour preferences, neither towards the blue, nor the green chambers. This altered preference pattern of AS fish can be further seen through their increased time spent in the green chamber compared to control individuals, which suggests that some stressors may also enforce a preference for a particular colour. Adult and embryo fish exposure to air is known to have deleterious impacts, such as acute hypoxia, physical damage to the gill lamellae, and to activate multiple physiological stress responses, such as cortisol increase (Cook et al., 2015). Embryos of Atlantic cod, *Gadus morhua*, exposed to air have a higher expression of oxidative stress-related genes (Caipang et al., 2015). Maternal air exposure during late pregnancy in southern fiddler rays (*Trygonorrhina dumerilii*) caused embryos to have lower body mass and yolk sac volume at birth, a granulocyte-to-lymphocyte ratio indicative of a stressed condition, and altered behaviour patterns, such as reduced boldness, compared to individuals from non-air exposed mothers (Finotto et al., 2021). Furthermore, in humans, chronic stress may affect cognitive and emotional responses that lead to impaired contrast perception (Bubl et al., 2010). In the present study, fish were challenged to distinguish between green ($\lambda_{max}$ 534 nm) and blue ($\lambda_{max}$ 450 and 520 nm) in order to demonstrate a preference. Our results add to the fish literature and show that, beyond the known action of chemical pollutants (Qiu et al., 2020) and colour regimes on fish colour preferences (Luchiari and Oliveira, 2014), typical abiotic stressors fish face in farms or laboratories, such as air exposure, may also alter the perception or motivation towards their environment and their natural preferences. In line with our results, Wood et al. (2011), studying African cichlid fish (*Astatotilapia burtoni*), showed that fish may fall within three different phenotypes when learning a task: learners, non-learners, and non-attempters. While learners and non-learners participated in the task, non-attempters never participated in the task, presenting little to no motivation to reach the available social reward upon completion of the task. Interestingly, non-attempters had higher levels of plasma cortisol compared to learners and non-learners, further confirming the inverse relationship between stress and fish' natural motivations.

Stress has been discussed as a major concern in aquaculture, particularly in hatcheries, since fish may develop a functional glucocorticoid system at early stages (Wilson et al., 2014). When stressful conditions are present during the embryonic phase, undesirable consequences may occur and predispose fish to increased mortality, diseases and compromised growth (Rehman et al., 2017). In the present work, besides natural mortality (observed in the control group), stressed groups (both PS and AS) presented increased values of mortality rate. This differential mortality between groups did not occur, however, in a previous work, which used the same experimental stressful conditions (Poisson et al., 2017). Further studies are, therefore, needed to better understand the differences between these two studies and better elucidate the impacts of stressful conditions during the embryonic phase of rainbow trout. Furthermore, according to Huntingford et al. (2006), stress in relation to welfare should be also considered at the psychological level, rather than only the physiological level. Stressors are usually measured in terms of cortisol response (besides other hormones, cytokines and some behavioural responses), but these measures are restrictive and may not show stress effects after the animals are homeostatically recovered. Therefore, it would be important to understand how the cortisol release due to acute or chronic stress affects the brain, i.e., which areas of the brain present differential activation due to the presence of mineralocorticoid and glucocorticoid receptors, and what are the long term consequences of such stimulation (Ellis et al., 2012). For example, the loss of environmental colour preference observed herein, and other behavioural changes described in Poisson et al. (2017), can be attributed to the lasting effects of the stress encountered during the embryonic phase. The exposure to stressful conditions at different periods across ontogeny (prenatal, postnatal, youth, and adulthood) may have different effects on animal behaviour, and, more importantly, since these effects may attenuate over time (Eyck et al., 2019), it is important to replicate this experiment with fish at different life periods in further studies. One may wonder, for example, whether behavioral impairment found here in fry may disappear in adults, and that

preference for blue could be retrieved.

In a previous experiment, we showed that PS fish, but not AS fish, were significantly more impacted by the stressor and became less fearful and more active than control fish (Poisson et al., 2017). These results were unexpected, since in a similar experiment the stressed fish (1 min in 0 °C water, 1 min out of water) did present higher whole body cortisol concentrations compared to unstressed fish at 44 dpf, evidencing that the fish hypothalamic–pituitary–interrenal (HPI) axis is fully functional at this stage (Auperin and Geslin, 2008). We had suggested that one-minute air exposure did not seem to be perceived as a severe enough stress agent by AS fish (Poisson et al., 2017). However, our new results here show the inverse, AS fish being more impacted than PS and control fish, which highlights the importance of testing individuals in multiple situations to fully picture the impacts of the different stress treatments, so some differences do not go unperceived.

Finally, it is essential to mention that neither stress, nor incubation influenced weight or body size at the end of the trials. Further analyses showed no influences of weight or body size on the time spent in the different chambers during colour preference tests. Therefore, different colour preferences cannot be explained by differences in body growth (Poisson et al., 2017). However, fry incubated in the dark showed a lower condition factor than those incubated under bright conditions, but not when compared to the other conditions. Rearing fry under dark conditions, a standard procedure used in trout's production, may be disadvantageous in fry performances. More studies are needed to investigate why these differences were restricted to dark and bright conditions, and to know whether this condition factor difference is no longer present in the later life stages of individuals incubated in dark conditions.

To conclude, our results imply that early life stress may affect the colour preference of rainbow trout. Stressors, such as air exposure, may affect colour perception or behavioural plasticity in young fish. Fish welfare is a multifactorial concept based on individuals' physical, physiological, behavioural and psychological measures (Conte, 2004; Ellis et al., 2012). If the results presented herein are corroborated by future studies in fish at different life stages, beyond the embryonic phase, colour preference tests may be used as an additional and potential welfare indicator to estimate, in a retrospective manner, which stressors were faced by the individuals during early stages. By knowing whether or not their fish were exposed to certain stressful conditions may allow farmers to better adapt fish rearing conditions and to implement strategies that alleviate any long-term impacts that may exist, and, therefore, improve fish welfare.

## Declaration of Competing Interest

The authors report no declarations of interest.

## Acknowledgments

This work is part of the PReSTO'Cog Project, which received funding from the French National Research Agency (ANR) under grants agreement No. ANR-13-BSV7-0002-05. We are really grateful to Alain Moréac from the Physical Institute of Rennes (France) for the LED, green and blue filters visible spectra measurements performed in the "SIR Platform" (UMS ScanMAT, Rennes 1 University: https://scanmat.univ-rennes1.fr).

## References

ANSES, 2018. Avis de l'ANSES relatif au « Bien-être animal: contexte, définition et évaluation » [WWW Document]. https://www.anses.fr/fr/system/files/SABA2016SA0288.pdf.

Auperin, B., Geslin, M., 2008. Plasma cortisol response to stress in juvenile rainbow trout is influenced by their life history during early development and by egg cortisol content. Gen. Comp. Endocrinol. 158, 234–239. https://doi.org/10.1016/j.ygcen.2008.07.002.

Barcellos, L.J.G., Kreutz, L.C., Quevedo, R.M., da Rosa, J.G.S., Koakoski, G., Centenaro, L., Pottker, E., 2009. Influence of color background and shelter availability on jundiá (Rhamdia quelen) stress response. Aquaculture 288, 51–56. https://doi.org/10.1016/j.aquaculture.2008.11.002.

Brown, G.E., Ferrari, M.C.O., Malka, P.H., Russo, S., Tressider, M., Chivers, D.P., 2011. Generalization of predators and nonpredators by juvenile rainbow trout: Learning what is and is not a threat. Anim. Behav. 81, 1249–1256. https://doi.org/10.1016/j.anbehav.2011.03.013.

Bubl, E., Kern, E., Ebert, D., Bach, M., Tebartz Van Elst, L., 2010. Seeing gray when feeling blue? Depression can be measured in the eye of the diseased. Biol. Psychiatry 68, 205–208. https://doi.org/10.1016/j.biopsych.2010.02.009.

Caipang, C.M.A., Fagutao, F.F., Fatira, E., Lazado, C.C., Pavlidis, M., 2015. Cortisol levels and expression of selected stress- and apoptosis-related genes in the embryos of Atlantic cod, Gadus morhua following short-term exposure to air. Int. Aquat. Res. 7, 75–84. https://doi.org/10.1007/s40071-015-0094-x.

Carvalho, P.S.M., Noltie, D.B., Tillitt, D.E., 2004. Biochemical, histological and behavioural aspects of visual function during early development of rainbow trout. J. Fish Biol. 64, 833–850. https://doi.org/10.1111/j.1095-8649.2004.0350.x.

Conte, F.S., 2004. Stress and the welfare of cultured fish. Appl. Anim. Behav. Sci. 86, 205–223. https://doi.org/10.1016/j.applanim.2004.02.003.

Cook, K.V., Lennox, R.J., Hinch, S.G., Cooke, S.J., 2015. Fish out of water: how much air is too much? Fisheries 40, 452–461. https://doi.org/10.1080/03632415.2015.1074570.

Davis, J.M., Stamps, J.A., 2004. The effect of natal experience on habitat preferences. Trends Ecol. Evol. 19, 411–416. https://doi.org/10.1016/j.tree.2004.04.006.

Delicio, H.C., Barreto, R.E., Normandes, E.B., Luchiari, A.C., Marcondes, A.L., 2006. A place preference test in the fish Nile tilapia. J. Exp. Anim. Sci. 43, 141–148. https://doi.org/10.1016/j.jeas.2006.01.001.

Ellis, T., Yildiz, H.Y., López-Olmeda, J., Spedicato, M.T., Tort, L., Øverli, Ø., Martins, C.I.M., 2012. Cortisol and finfish welfare. Fish Physiol. Biochem. 38, 163–188. https://doi.org/10.1007/s10695-011-9568-y.

Eurostat, 2020. Fishery Statistics - Statistics Explained [WWW Document]. URL https://ec.europa.eu/eurostat/statistics-explained/index.php/Aquaculture_statistics#Aquaculture_statistics (Accessed 4.5.21).

Eyck, H.J.F., Buchanan, K.L., Crino, O.L., Jessop, T.S., 2019. Effects of developmental stress on animal phenotype and performance: a quantitative review. Biol. Rev. 94, 1143–1160. https://doi.org/10.1111/brv.12496.

Fife-Cook, I., Franks, B., 2019. Positive welfare for fishes: rationale and areas for future study. Fishes 4. https://doi.org/10.3390/fishes4020031.

Finotto, L., Walker, T.I., Reina, R.D., 2021. Prolonged alteration of neonate traits following maternal exposure to fishing-capture stress during late pregnancy in a Chondrichthyan Species. Front. Mar. Sci. 8. https://doi.org/10.3389/fmars.2021.631798.

Heydarnejad, S.S., Parto, M., Pilevarian, A.A., 2013. Influence of light colours on growth and stress response of rainbow trout (Oncorhynchus mykiss) under laboratory conditions. J. Anim. Physiol. Anim. Nutr. (Berl.) 97, 67–71. https://doi.org/10.1111/j.1439-0396.2011.01243.x.

Huntingford, F.A., Adams, C., Braithwaite, V.A., Kadri, S., Pottinger, T.G., Sandøe, P., Turnbull, J.F., 2006. Current issues in fish welfare. J. Fish Biol. 68, 332–372. https://doi.org/10.1111/j.0022-1112.2006.001046.x.

Karakatsouli, N., Katsakoulis, P., Leondaritis, G., Kalogiannis, D., Papoutsoglou, S.E., Chadio, S., Sakellaridis, N., 2012. Acute stress response of European sea bass Dicentrarchus labrax under blue and white light. Aquaculture 364–365, 48–52. https://doi.org/10.1016/j.aquaculture.2012.07.033.

Lankford, S.E., Adams, T.E., Miller, R.A., Cech, J.J., 2005. The cost of chronic stress: Impacts of a nonhabituating stress response on metabolic variables and swimming performance in sturgeon. Physiol. Biochem. Zool. 78, 599–609. https://doi.org/10.1086/430687.

Lawrence, A.B., Vigors, B., Sandøe, P., 2019. What is so positive about positive animal welfare?—a critical review of the literature. Animals 9, 1–19. https://doi.org/10.3390/ani9100783.

Li, X., Chi, L., Tian, H., Meng, L., Zheng, J., Gao, X., Liu, Y., 2016. Colour preferences of juvenile turbot (Scophthalmus maximus). Physiol. Behav. 156, 64–70. https://doi.org/10.1016/j.physbeh.2016.01.007.

Luchiari, A.C., Oliveira, J.J., 2014. The interaction of innate and imposed colour perception: a behavioural approach. J. Ethol. 32, 179–183. https://doi.org/10.1007/s10164-014-0407-3.

Luchiari, A.C., Pirhonen, J., 2008. Effects of ambient colour on colour preference and growth of juvenile rainbow trout Oncorhynchus mykiss (Walbaum). J. Fish Biol. 72, 1504–1514. https://doi.org/10.1111/j.1095-8649.2008.01824.x.

Luchiari, A.C., Do Amaral Duarte, C.R., De Morais Freire, F.A., Nissinen, K., 2007. Hierarchical status and colour preference in Nile tilapia (Oreochromis niloticus). J. Ethol. 25, 169–175. https://doi.org/10.1007/s10164-006-0013-0.

Maia, C.M., Volpato, G.L., 2013. Environmental light color affects the stress response of Nile tilapia. Zoology 116, 64–66. https://doi.org/10.1016/j.zool.2012.08.001.

Maia, C.M., Ferguson, B., Volpato, G.L., Braithwaite, V.A., 2017. Physical and psychological motivation tests of individual preferences in rainbow trout. J. Zool. 302, 108–118. https://doi.org/10.1111/jzo.12438.

McLean, E., 2021. Fish tank color: an overview. Aquaculture 530, 735750. https://doi.org/10.1016/j.aquaculture.2020.735750.

Millot, S., Cerqueira, M., Castanheira, M.F., Øverli, Ø., Martins, C.I.M., Oliveira, R.F., 2014. Use of conditioned place preference/avoidance tests to assess affective states in fish. Appl. Anim. Behav. Sci. 154, 104–111. https://doi.org/10.1016/j.applanim.2014.02.004.

Peeters, B.W.M.M., Moeskops, M., Veenvliet, A.R.J., 2016. Color preference in Danio rerio: effects of age and anxiolytic treatments. Zebrafish 13, 330–334. https://doi.org/10.1089/zeb.2015.1150.

Piato, A.L., Capiotti, K.M., Tamborski, A.R., Oses, J.P., Barcellos, L.J.G., Bogo, M.R., Lara, D.R., Vianna, M.R., Bonan, C.D., 2011. Unpredictable chronic stress model in zebrafish (Danio rerio): behavioral and physiological responses. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 35, 561–567. https://doi.org/10.1016/j.pnpbp.2010.12.018.

Poisson, A., Valotaire, C., Borel, F., Bertin, A., Darmaillacq, A.S., Dickel, L., Colson, V., 2017. Embryonic exposure to a conspecific alarm cue triggers behavioural plasticity in juvenile rainbow trout. Anim. Behav. 133, 35–45. https://doi.org/10.1016/j.anbehav.2017.09.013.

Qiu, N., Su, L., Wu, B., Hou, M., Xu, C., Wang, J., 2020. Chemicals affect color preference in rare minnow (Gobiocypris rarus). Environ. Sci. Pollut. Res. 27, 23206–23214. https://doi.org/10.1007/s11356-020-08924-9.

Rehman, S., Gora, A.H., Ahmad, I., Rasool, S.I., 2017. Stress in aquaculture hatcheries: source, impact and mitigation. Int. J. Curr. Microbiol. Appl. Sci. 6, 3030–3045. https://doi.org/10.20546/ijcmas.2017.610.357.

Roy, T., Suriyampola, P.S., Flores, J., López, M., Hickey, C., Bhat, A., Martins, E.P., 2019. Color preferences affect learning in zebrafish, Danio rerio. Sci. Rep. 9, 1–10. https://doi.org/10.1038/s41598-019-51145-5.

Ruchin, A.B., 2020. Environmental colour impact on the life of lower aquatic vertebrates: development, growth, physiological and biochemical processes. Rev. Aquac. 12, 310–327. https://doi.org/10.1111/raq.12319.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.

Ullmann, J.F.P., Gallagher, T., Hart, N.S., Barnes, A.C., Smullen, R.P., Collin, S.P., Temple, S.E., 2011. Tank color increases growth, and alters color preference and spectral sensitivity, in barramundi (Lates calcarifer). Aquaculture 322–323, 235–240. https://doi.org/10.1016/j.aquaculture.2011.10.005.

Volpato, G.L., Duarte, C.R.A., Luchiari, A.C., 2004. Environmental color affects Nile tilapia reproduction. Braz. J. Med. Biol. Res. 37, 479–483. https://doi.org/10.1590/S0100-879X2004000400004.

Wang, J., Yin, L., Hu, B., Zheng, L., 2020. Social interaction influences innate color preference of zebrafish shoals. bioRxiv. https://doi.org/10.1101/2020.03.23.003186, 2020.03.23.003186.

Wilson, K.S., Matrone, G., Livingstone, D.E.W., Mullins, J.J., Tucker, C.S., Hadoke, P.W.F., Kenyon, C.J., Denvir, M.A., 2014. Physiological roles of glucocorticoids during early embryonic development of the zebrafish (Danio rerio). J. Physiol. 24, 6209–6220. https://doi.org/10.1113/jphysiol.2013.256826.

Wood, L.S., Desjardins, J.K., Fernald, R.D., 2011. Effects of stress and motivation on performing a spatial task. Neurobiol. Learn. Mem. 95, 277–285. https://doi.org/10.1016/j.nlm.2010.12.002.