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ABSTRACT

The present study aimed to investigate whether environmental enrichment had significant effects on rainbow trout's welfare through nature-, functions- and feeling-based approaches. Fish group behavior was analyzed during the rearing phase, as well as growth performance and health status. We assessed individual's emotional reactivity through a novel-tank test by measuring fear-related behaviors and stress-related physiological responses. Fish boldness and neophobia were then evaluated towards a novel object. We showed that more complex environments decreased aggression levels and improved growth, without impacting fish immune status. Enriched fish were also found less fearful when isolated in a novel tank and bolder when facing a novel object. We concluded that complexifying the environment through the addition of physical structures which stimulate and encourage fish to explore promotes rainbow trout's welfare in farming conditions, according to the three different welfare approaches.

1. Introduction

In recent years, there has been a growing interest in finding strategies for improving the welfare of farmed fish, as evidenced by the increasing number of publications on fish welfare (Ashley, 2007; Kristiansen et al., 2020; Naslund and Johnsson, 2016; Salena et al., 2021). In land-based aquaculture systems, many husbandry parameters can compromise fish welfare if not controlled adequately, such as water quality, high densities, sorting, transportation (Huntingford et al., 2012) and also the lack of environmental stimulations (Franks, 2018). When these situations are prolonged, they may lead to negative affects which can be functional (sickness) or emotional depending on the animal's cognitive perception of its environment (anxiety, fear, boredom, anhedonia) (Mellor, 2016). A growing body of evidence tells us that animals are sentient beings with an ability to feel emotions which can be negative but also positive (Dawkins, 1990; Fraser and Ducan, 1998). Since recently, researchers consider these statements also true for fish (Fife-Cook and Franks, 2019), which are capable of experiencing pain (Sneddon, 2015) and are endowed with various cognitive skills (Brown et al., 2011). The concept of positive welfare can be defined as the physical and mental states that exceed what is strictly necessary for short-term survival (Fife-Cook and Franks, 2019), by allowing the animal to have "a life worth living" (Mellor, 2016). Therefore, it becomes essential not only to prevent negative emotions (fear, anxiety, pain) but also to give captive fish greater opportunities to experience positive affects by generating various forms of comfort, pleasure, stimulation, interest, sense of safety and control in order to induce long-lasting positive affective states, close to the positive welfare concept.

Environmental enrichment is one of the strategies investigated to improve the living conditions of captive animals, including fish. Environmental enrichment is defined as "a deliberate increase in environmental complexity with the aim to reduce maladaptive and aberrant traits in fish reared in otherwise stimuli-deprived environments. Traits could be physiological, behavioral, morphological and psychological

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and considered maladaptive with respect to fitness components (health, survival, reproduction, etc.)" (Naslund and Johnsson, 2016). One of the principal categories of environmental complexity is physical enrichment, made up of physical structures (e.g., stones, plants, kelps, sand, gravels, artificial objects) which are important factors in the natural environment of fish. Fish welfare can be approached from three different angles: nature-, functions- and feelings-based (Huntingford et al., 2012; Huntingford et al., 2006). Physical enrichment is closer to the nature-based approach to consider animal welfare meaning that fish should live in an environment close to the natural habitat of the species, in order to promote more natural behavior (Martins et al., 2012; Wechsler, 2007). In territorial fish, such as salmonids, increased environmental complexity has been shown to limit aggression by reducing visual contacts among dominants individuals (Dolinsek et al., 2007) and by creating shelters where subordinates could hide (Höjesjö et al., 2004). Reducing visual contacts and adding shelters allow the implementation of natural social strategies and promote a sense of safety and security for fish, close to the positive welfare concept. Increased environmental stimulations also reduce stereotypic behaviors (Mason et al., 2007), known as a poor-welfare indicator frequently observed in impoverished environments, even in fish (Martins et al., 2012). According to the functions-based welfare approach, fish must be able to maintain their biological functions and zootechnical performances (Huntingford et al., 2012; Huntingford et al., 2006). Recent studies have tested suspended arrays (rods or colored balls) in rainbow trout (*Oncorhynchus mykiss*) reared in circular tanks and showed that this husbandry practice increased growth and conversion index (Crank et al., 2019; Kientz et al., 2018; Krebs et al., 2018). Enriched rearing environments can also maintain health status by enhancing survival and disease resistance during parasite epidemics in salmonids species (Karvonen et al., 2016). Conversely, a deprived environment increases susceptibility to pathogen infections (Masud et al., 2020). Furthermore, stressors could impact the teleostean innate immune defense, including the bactericidal enzyme lysozyme and microbe-clearing complement components frequently used as innate immune markers (Seibel et al., 2021; Tort, 2011). However, the impact of enrichment on fish immunocompetence has been poorly studied. The third approach to consider fish welfare is "feeling-based" and set in terms of subjective mental states (Huntingford et al., 2012; Huntingford et al., 2006). From this point of view, the requirement for good welfare is that negative emotional experiences must be decreased and that positive experiences should be promoted. Providing fish with environmental enrichment designed to increase complexity may be an effective way to promote positive experiences by stimulating fish, encouraging exploration and facilitating curiosity, thereby meeting the positive welfare concept (Mellor, 2016), from the feeling-based approach.

Therefore, environmental enrichments investigated in some fish species seem to reach many criteria proposed by the different welfare approaches. However, the three different perspectives are often disconnected and rarely evaluated within a whole experiment. Moreover, the effects of environmental complexity were more rarely investigated on farmed fish species. Among these species, rainbow trout is the first continental fish species produced in Europe (FEAP Production Report 2020), and deserves more attention and investigations for finding practical ways to alleviate welfare issues caused by low-stimulating environments often encountered in farming systems.

In the present study, we aimed to assess positive welfare of rainbow trout from the nature-, functions- and feeling-based approaches, when fish were reared in either enriched environments (provided with stones, pipes and artificial plants) or in standard conditions. Positive welfare was evaluated through behavioral, functional (growth, health) and emotional (fearfulness, boldness) indicators. For the nature-based approach, groups of trout were observed in their home tanks (enriched vs. barren) to analyze the impacts of a complex habitat on fish group behavior (dispersion, aggression and stereotypies), expected to be more natural than in barren tanks. Fin erosion was also recorded as a marker of aggression. Growth and metabolic parameters (weight kinetic, condition factor and food conversion ratio) were monitored and immune analyses (lysozyme, complement component ACH50) were carried out to assess whether environmental enrichment also influences fish welfare from the functions-based approach. Two behavioral tests were performed to assess fish welfare from the feeling-based perspective: (i) an emotional reactivity test (also called novel-tank test) to evaluate the fear-related responses (behavior and cortisol release) of the fish when isolated in a novel environment, and (ii) a standard novel object paradigm to characterize fish boldness. These tests seem to be the most robust ways to assess fearfulness and boldness in fish (White et al., 2013). It was shown that positive welfare during rearing conditions was linked to a greater motivation to explore in fish subjected to an emotional reactivity test (Franks, 2018). The novel object test is a well-established paradigm used to assess shyness (or neophobia) in a variety of animals (horses (Leiner and Fendt, 2011), calves (Zhang et al., 2021a), birds (Meehan and Mench, 2002), fish (Frost et al., 2007; Sneddon et al., 2003)), and is defined as the avoidance of an unfamiliar object in a familiar environment (Barnett, 1967). Using these behavioral tests, we hypothesized that rainbow trout reared in a stimulating environment would exhibit more exploratory behaviors, more curiosity and lower neophobia towards novelty (environment or object) than trout reared in barren tanks.

2. Materials and methods

2.1. Ethic declaration

All experimental procedures were performed under the European directive 2010/63/EU on the protection of animals used for scientific purposes. They were approved by the Ethic committee for the animal experimentation of Rennes and received the approval of French minister of national education, research and innovation under the authorization number APAFIS#28962–2,021,011,323,275,224 v2.

2.2. Experimental animals

Female triploids rainbow trout (*O. mykiss*) were used in this study and originated from eggs fertilized at INRAE-PEIMA (Sizun, France). Trout fry were transferred to the Fish Physiology and Genomic Laboratory (LPGP) of INRAE (Rennes, France) at 99 days post-fertilization (dpf). They were split in two experimental treatments: an enriched environment (E) and a barren environment (B). The enriched environment was composed by PVC pipes, plastic plants (grapes of leaves) and white stones (number and size of each structure are given in Table 1), representing a complete panel of the different structures tested in the literature reviewed by Naslund and Johnsson (2016): pipes as shelters to provide hiding places and to limit aggression in salmons (Naslund et al., 2013); artificial vegetation as shelters but also as a manner to complexify the environment, which reduces startling responses in pike (*Esox masquinongy*) (Einfalt et al., 2013); stones as landmarks to complexify the environment and promote behavioral flexibility and better adaptation in case of threatening situations in salmons (Salvanes et al., 2013). Floor covering was estimated by using photographs of the tanks (with a known surface), where each structure was superposed by a corresponding geometrical shape which surface was known. Then surfaces were added and converted as a percentage of the tank surface. Floor covering varied according to fish growth, tank size and tank load, leaving free space enough for allowing fish navigation. From 99 to 189 dpf, tanks were kept uncovered allowing for video observations. Water was clear and light reflections were avoided to guarantee a perfectly visible above-tank view. For each stage, fish mean weight, tank size, number of individuals per tank, number of tanks per treatment, number and types of physical enrichments, as well as estimated floor coverage (%) are presented in Table 1.

Fish load was always lower than 25 kg/m^3. Water temperature was

Table 1
Mean fish weight, tank size, number of individuals, number, type and size of structures, and estimated floor covering at each stage.

Days post-fertilization	Average fish weight at the beginning of each period	Tank size	Number tank replicates and number of individuals	Number (n) and type of structures used in the enriched group			Floor covering
							
99–136	2.83 g	72 L (55 × 45 × 29 cm) Uncovered tanks	2 tanks/treatment 100 individuals each	$n = 1$ (10 × 5 × 5 cm)	$n = 2$ (24 × 15 × 11 cm)	$n = 2$ (9 × 8 × 8 cm)	~70%
137–189	11 g		3 tanks/treatment 30 individuals each			$n = 2$ (9 × 8 × 8 cm)	~70%
189–312	32.83 ± 0.67 g	336 L (102,5 × 102,5 × 32 cm) Covered tanks	1 tank/treatment 42 individuals each		$n = 1$ (35 × 35 × 44 cm)	$n = 1$ (11 × 9 × 6 cm)	~25%
313–364	295.16 ± 4.67 g		1 tank/treatment 18 individuals each	$n = 3$ (10 × 5 × 5 cm)	$n = 1$ (23 × 38 × 36 cm)	$n = 2$ (21 × 18 × 14 cm) $n = 1$ (19 × 14 × 28 cm)	~40%

maintained at 12 ± 0.2 °C, the artificial photoperiod was 12:12 h and the water quality was regularly checked (NH_4^+, NO_2^-, NO_3^-). All breeding and test tanks were supplied by circulating and recycled water. Fish were fed daily with extruded pellets (39% proteins and 24% lipids, Le Gouessant, France), the diameter and the quantity being regularly adapted according to fish mean weight (1.5-5 g: 3.1% of mean weight/Ø = 1.1 cm, 5-15 g: 2.6% of mean weight/Ø = 1.5 cm, 15-80 g: 2% of mean weight/Ø = 1.9 cm, 80-200 g: 1.7% of mean weight/Ø = 4 cm).

2.3. In situ group behavior

During 4 weeks (from 162 to 187 dpf), we measured fish group behavior (n = 3 uncovered tanks per treatment) by video recording twice a week for 30 min with the scan sampling method (one scan every 5 min). Rearing tanks were equipped with digital cameras positioned directly above the tanks (one camera per tank). The group dispersion was measured by manually counting the number of fish that were not in contact with another fish and not superposed, from the above-camera view. This number was converted as a percentage of the total number of fish per tank (30 individuals per tank). A low percentage indicated heightened shoal cohesion. We also determined manually the group's activity level during the 10 s around the scan (5 s before and after), according to the following index: 1 = less than 10% of active individuals, 2 = between 10% and 50% of active individuals, 3 = between 50% and 90% of active individuals, 4 = more than 90% of active individuals. Focal samplings were also performed during the first 5 min and the number of flight behaviors (i.e., accelerating movement at a peak swimming speed compared to the initial speed) were counted. The origins or consequences of this behavior may be aggression (biting, chasing), jumping, or stereotypies (repetitive swimming against the walls) but were not distinguished during the manual sampling. The same experimenter performed all behavioral observations.

2.4. Fin erosion

At 188 dpf, we analyzed fin erosion (dorsal, caudal, anal and pectoral fins) by photographs taken on 15 anesthetized trout per treatment, during one of the weight samplings points (see paragraph 2.9 for anesthesia procedure during weighing). The identification key for fin erosion was created based on those set up by (Hoyle et al., 2007) and (Noble et al., 2020), using an erosion index ranging from 1 to 3. Score 1: no lesion; score 2: between 0 and 50% of the fin surface was damaged; score 3: more than 50% of the fin surface was damaged.

2.5. Emotional reactivity test

At 208 dpf, we assessed fish emotional reactivity by using our established protocol (Poisson et al., 2017; Valotaire et al., 2020). We isolated 12 fish (~ 45 g) per treatment (E and B) into a novel tank (72 L: 55 × 45 × 29 cm) for 40 min. Six fish (3/treatment) could be simultaneously tested since we have 6 dedicated test-tanks of this size, thus 4 sessions were needed to test 24 fish. The first 18 min of the test were video recorded and the following variables were then analyzed by EthoVision® XT software (v. 14.0.1234) by a 1-min time step: maximum speed (cm/s), distance traveled (cm), time spent not moving (%), time spent in the periphery (s), angular velocity (°/s) and number of rotations.

2.6. Novel object test

We relied on three studies that used the novel object test to assess boldness and neophobia in rainbow trout (Basic et al., 2012; Frost et al., 2013; Sneddon et al., 2003). The present test was performed between 250 and 260 dpf, and 12 fish (~80 g) per treatment were individually tested in 72 L tanks, during 4 sessions of 6 fish tested simultaneously on two consecutive days. The novel-object test design is given in Fig. 1. For each fish, after an 18-h period of acclimation (Day 1, 10 am), a first object (object 1) was gently introduced three times during 3 h on two consecutive days (t1: day 1 (10 am), t2: day 1 (2 pm), t3: day 2 (10 am)). Then, a novel object (object 2) with a different shape and colors from object 1 was introduced into the test-tank during 15 min (t4: day 2 (2 pm)). The 15 min before and after the introduction of objects were video recorded ("before" and "after" periods). Objects were made of LEGO® DUPLO® bricks. The object 1 was made of a blue brick between two yellow bricks (3 × 3 × 6 cm) and the object 2 was made of three red bricks (6 × 3 × 4 cm). Objects were weighted with dermatologically tested UHU® adhesive paste and a 60 cm iron wire was hook up to each object to introduce and remove them easily from the test-tanks. Each object was placed in the center of the width of the tank at 2/3 of its length (on the water inlet side). Tested trout were not fed during 27 to 30 h before the period of acclimation, and were fed after each removal of object 1 (1/3 of the daily ration).

The following variables were analyzed using EthoVision® XT software (v. 14.0. 1234) during 15 min (before/after each object introduction): latencies to enter (s) and number of entries in the objects area (bounded at a 7-cm perimeter around the object), time spent in the objects area (s), minimum distance to objects (cm), distance traveled (cm), maximum velocity (cm/s), time spent not moving (%) and angular velocity (°/s). The software tracked the fish from its center of gravity. The number of mouth contacts and mouth contact latency (s) were manually observed during the 15 min of each video.

2.7. Plasma cortisol responses

To measure fish basal cortisol levels, eight fish per treatment were not subjected to the emotional reactivity test but were directly netted

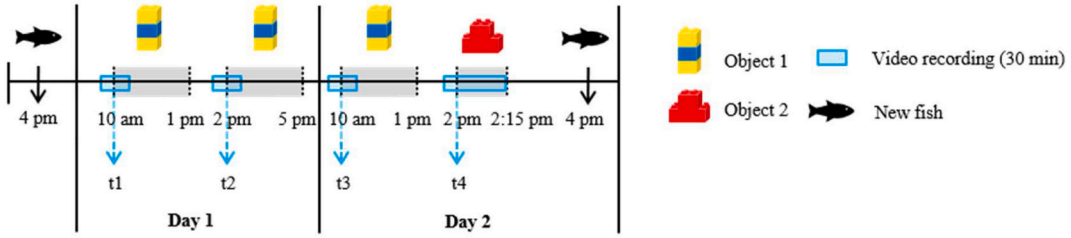


Fig. 1. Overview of the novel-object test design, see text for details.

from their rearing tank and euthanized using a lethal dose of tricaine methane sulphonate (200 mg/l; PHARMAQ, Hampshire, UK). Blood (~ 0.15 ml) was sampled from caudal sinuses into heparinized syringes and samples were stored on ice. After sampling, blood cells and plasma were separated by centrifugation (10 min at 3000 rpm). Plasma was collected and frozen at −20 °C until basal cortisol and immune analyses. To measure plasma cortisol elevation after an acute stressor, the 12 fish per treatment subjected to the emotional reactivity test were left until 40 min in social isolation in their test-tank. Forty minutes is an average delay needed to observe a peak of plasma cortisol following an acute stressor in rainbow trout (30 min: (Sadoul et al., 2016), 45 min: (Gesto et al., 2015) or 60 min: (Auperin and Geslin, 2008)). They were then euthanized and blood was collected, centrifugated and plasma was stored at −20 °C until cortisol analyses. Plasma cortisol assay was carried out by ELISA following manufacturer instructions (BioSource, Nivelles, Belgium). The used plasma samples were not diluted. Twenty microliters of each cortisol calibrator (standard curve in the range of 5–600 ng/ml), control and plasma samples were dispensed in each well of a 96 well plate. 100 µl of cortisol horseradish peroxidase conjugate were added into each well. After thoroughly mix for 10 s, 45 min incubation using a horizontal shaker, and 3 times washing (wash solution provided in the kit), 150 µl of enzyme substrate solution containing tetramethylbenzidine and hydrogen peroxide were added into each well. Then, the plate was incubated for 20 min at room temperature and the reaction was stopped by adding 100 ml of H_2SO_4 1 M. The optical density was immediately read at 450 nm with a microtiter plate reader. The detection limit was 2.5 ng/ml, the inter- and intra-assay CV were 6.9% and 5.6% respectively. The recovery range (specificity validation) was evaluated at 85–111%, depending on doses.

2.8. Immune parameters

Lysozyme and complement system (ACH50) activities were analyzed from the same plasma samples collected before the emotional reactivity test ($n = 8$ individuals/treatment), to assess fish immunity according to the different rearing conditions.

2.8.1. Plasma lysozyme activity

The lysozyme activity of plasma samples was measured using a method based on the ability of lysozyme to lyse the bacterium *Micrococcus lysodeikticus* (Ellis, 1990; Milla et al., 2010). In a 96-well microplate, 10 µl of fish plasma were mixed with 10 µl of phosphate buffered saline (PBS) (0.05 M, PH 6,2) and then with 130 µl of 0.6 mg/ml suspension of *M. lysodeikticus* (Sigma-Aldrich M3770-5G, USA). Optical density (OD) at 450 nm (Thermo Scientific™ Multiskan™ Spectrophotometer) was monitored every five minutes for 20 min. Lysozyme concentrations for samples were converted to U/ml using the reference curve from 6.25 to 150 U/ml established with hen egg white lysozyme (Sigma).

2.8.2. Alternative hemolytic complement activity (ACH50)

The plasma alternative hemolytic complement activity (ACH50) was determined by the hemolytic assay with the rabbit red blood cells (RRBC, Clinisciences) (Danion et al., 2011; Yano, 1992). Plasma samples diluted to 1/32 in Veronal buffer (IDvet, France) were added in increasing amounts, from 10 to 100 µl in each well on the microplate previously filled with Veronal buffer to obtain a final volume of 100 µl. Then, the wells were filled with 50 µl of 2% RRBC suspension in veronal buffer. Control values of 0% and 100% hemolysis were obtained using respectively 100 µl veronal buffer and 100 µl distilled water. Each mixture was incubated at 20 °C for 60 min. The microplates were centrifuged (400 *g*, 15 min, 4 °C) and 75 µl of supernatant from each well were transferred into a 96-well flat-bottom microplate. The absorbance was read in a Thermo Scientific™ Multiskan™ Spectrophotometer at 405 nm. The ACH50 value was defined as the reciprocal of the plasma dilution inducing 50% of RRBC haemolysis.

2.9. Growth

Every 3 weeks, from September 28th 2020 (102 dpf) to May 17th 2021 (333 dpf), all individuals were netted and transferred to buckets containing tank water with tricaine methane sulphonate (anesthetic dose for a trout <40 g: 50 mg/l, >40 g: 80 mg/l), and sodium bicarbonate (dose for a trout <40 g: 100 mg/l, >40 g: 160 mg/l). Then, fish were weighed individually before returning to their respective home tanks. After the emotional reactivity test (208 dpf), the body weight (W) and length (L) were both measured post-mortem following each blood sample. For each fish, the condition-factor was calculated as followed: K-factor = 100 (W/L^3). The feed conversion ratio (FCR) was calculated at the end of the experiment, between two weights as followed: FCR = food intake/(333-dpf weight/309-dpf weight).

2.10. Statistical analysis

All analyses were done with the RStudio© software version 1.4.1106. The packages used in addition to those natively installed are the following: “car” to calculate the ANOVA tables, “lme4” for the mixed models and “emmeans” for the post-hoc tests. The diagrams and the regression curves were made with the “ggplot2” package.

For in situ observations of the group, the percentage of isolated individuals and group activity were analyzed with repeated measures analysis of variance (ANOVA type III). The number of flight behaviors was analyzed with a Generalized Linear Mixed Model (GLMM) since the dataset followed a Poisson distribution. Treatment (E and B) was the fixed explanatory factor and rearing tanks were considered as random factors.

Regarding the emotional reactivity test, the fixed factors were treatment (E and B) and time (1-min steps over 18 min), and the random factor was the individual. A GLMM was performed for the variable “number of rotations” following a Poisson distribution, and GLMMs with Gamma distribution for the others variables. Post-hoc tests of multiple comparisons (Tukey’s HSD) were then performed with the “emmeans” package.

For the novel object test, the fixed factors were treatment (E, B), trial (t1, t2, t3, t4) and period (before/after the deposition of an object). For variables only related to the period when the object was present (after), the fixed factors were treatment and trial. The random factor was the individual. GLMMs (Poisson distribution) were performed to evaluate

the effects of factors and their interaction on the variables "number of entries into the object area" and "number of mouth contacts" and GLMMs (Gamma distribution) for the others variables. Post-hoc tests of multiple comparisons (Tukey's HSD) were then performed with the "emmeans" package.

Cortisol data were analyzed using ANOVA (type III) since the dataset followed a normal distribution. For weight data, we ran an ANOVA for repeated measures using treatment and time as fixed factors, followed by Tukey'HSD tests if the interaction treatment x time was significant ($P < 0.05$). For complement (ACH50) activity, condition-factor and feed-conversion ratio, Student's *t*-tests were performed (independent explanatory variables, normal distribution). We analyzed fin erosions and lysozyme activity according to treatments with Wilcoxon's tests since data did not meet normal distribution.

3. Results

3.1. In situ group behavior

The percentage of isolated fish was significantly higher (ANOVA, $F_{1,4} = 25$, $P < 0.01$) for the trout raised in a barren environment (B: mean ± SEM: 0.23 ± 0.01%) compared to those raised in an enriched environment (E: 0.11 ± 0.01%), indicating more shoal cohesion in this group. The global activity index (ANOVA, $F_{1,4} = 27.16$, $P < 0.01$, E: 1.84 ± 0.12, B: 3 ± 0.09) and the number of flight behaviors (GLMM, $\chi^2 = 82.69$, df = 1, $P < 0.001$, E: 12.38 ± 2.03, B: 73.04 ± 5.02) were also lower in the enriched groups.

3.2. Fin erosion

Only the dorsal fins were eroded, with an erosion index significantly higher in barren fish (W = 24.5, $P < 0.001$, E: 1.08 ± 0.08, B: 2.07 ± 0.20).

3.3. Emotional reactivity test

Only two variables were significantly different between treatments: time spent not moving and angular velocity which are represented in Fig. 2. All statistical results are given in Table 2.

Post-hoc tests showed that barren fish spent significantly more time motionless at the 2-min step (Tukey's HSD, $P < 0.05$, Fig. 2A) and displayed a higher angular velocity at different time-points ($P < 0.05$, Fig. 2C). Maximum velocity also tended to be higher in barren fish at the 9-min step ($P = 0.0807$, Fig. 2F).

3.4. Novel object test

We found significant interactions between treatments, trials and periods for the time spent not moving, angular velocity, distance traveled and maximum velocity. All statistical results are given in Table 3.

In both treatments, time spent not moving (Fig. 3A) and angular velocity (Fig. 3B) increased between periods "before" and "after" ($P < 0.001$, at each trial), whereas distance traveled and maximum velocity decreased ($P < 0.001$). At period "before", post-hoc tests showed that the variable "time spent not moving" significantly increased between trial 1 and the three following trials in both treatments ($P < 0.001$, Fig. 3). At period "after", time spent not moving decreased from trial 2 to trial 3 both in enriched ($P < 0.001$) and barren fish ($P < 0.05$, Fig. 3A). When the object 2 was introduced at t4 (period "after"), barren fish spent significantly more time not moving than enriched fish ($P < 0.05$, Fig. 3A), and angular velocity was significantly higher for barren fish ($P < 0.05$, Fig. 4B). Distance traveled (E: 214.59 ± 6.77 cm, B: 114.81 ± 6.67 cm) and maximum velocity (E: 130.49 ± 13.15 cm/s, B: 60.4 ± 6.18 cm/s) were significantly higher for enriched fish compared to barren fish ($P < 0.05$).

When the objects were present, we found significant interactions between treatments and trials for all variables ($P < 0.001$), except for the latency to enter in the object area ($P = 0.97$) and the mouth contact latency ($P = 0.16$) (see Table 3 for statistical differences). Post-hoc tests

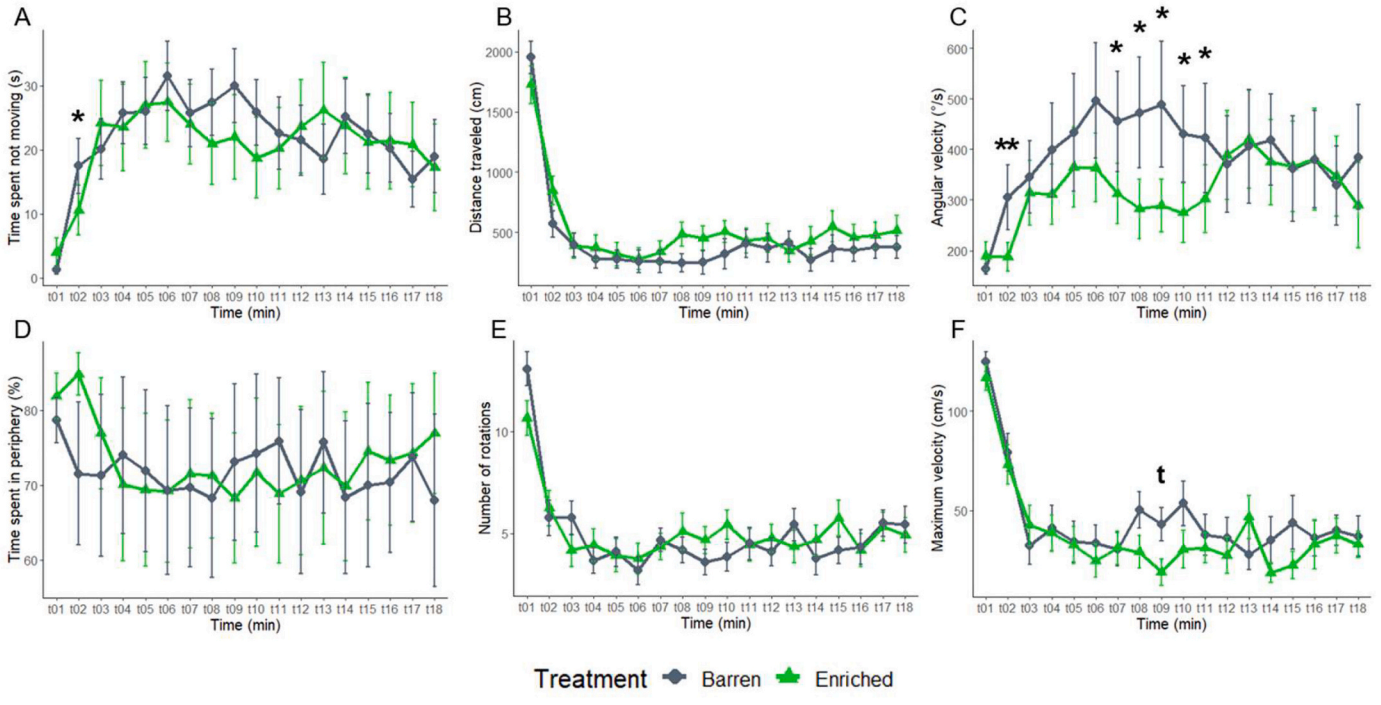


Fig. 2. Time spent not moving (s) (A), distance traveled (cm) (B), angular velocity (°/s) (C), time spent in periphery (s) (D), number of rotations (E) and maximum velocity (cm/s) (F) of trout raised in enriched environment (E: green line) or barren environment (B: grey line) during the first 18 minutes of the emotional reactivity test. Differences between treatments are represented by **($P < 0.01$), * ($P < 0.05$), and t ($0.05 < P < 0.1$). Values are means and the mean standard error is represented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Statistical effects of the fixed factors treatment, time and their interaction on the behavioral variables obtained from the novel-tank test.

Variables	Treatment			Time			Treatment x Time		
	χ^2	df	*P*-value	χ^2	df	*P*-value	χ^2	df	*P*-value
Time spent not moving (s)	0.04	1	0.85	37.58	17	*0.002*	11.32	17	0.84
Distance traveled (cm)	0.04	1	0.84	40.75	17	*0.001*	8.63	17	0.95
Angular velocity (°/s)	0.03	1	0.87	39.15	17	*0.002*	23.8	17	0.12
Time spent in periphery (%)	0.09	1	0.76	0.71	17	1	0.4	17	1
Number of rotations	1.63	1	0.2	91.58	17	*<0.001*	20.23	17	0.26
Maximum velocity (cm/s)	0.01	1	0.92	36.51	17	*0.004*	12.29	17	0.78

revealed that when presenting object 2 at t4, the number of entries in the object area was significantly higher in enriched fish compared to barren fish (34.91 ± 7.39 vs 19.92 ± 4.8, respectively; Tukey's HSD, $P < 0.05$). Moreover, enriched fish significantly increased the number of mouth contacts at t4 with the novel object compared to t1, t2 and t3 ($P < 0.05$, Fig. 4A). At t4, the number of contacts was also higher in enriched fish than in barren fish ($P < 0.05$). The minimum distance to object 2 was significantly lower in the enriched group as compared to the barren group (10.91 ± 0.35 cm vs 12.83 ± 0.4 cm, respectively; Tukey's HSD: $P < 0.05$) as well as mouth contact latency (E: 380.58 ± 112.65 s, B: 701.5 ± 96.82 s; GLM: $P < 0.05$, Fig. 4B).

3.5. Plasma cortisol responses

We found significant differences between basal and final cortisol levels (ANOVA, $F_3 = 24.8$), within enriched group (Tukey's HSD: $P < 0.001$, basal: 18.67 ± 3.42 ng/ml, final: 132.59 ± 17.64 ng/ml) and barren group ($P < 0.001$, basal: 27.05 ± 3.55 ng/ml, final: 147.82 ± 27.26 ng/ml), but no difference between treatments within the basal ($P = 0.52$) or final cortisol levels ($P = 0.99$).

3.6. Immune parameters

No differences were found between treatments for complement (ACH50) activity ($t = 0.96$, df = 18.4, $P > 0.05$, E: 96.252 ± 3.95 U, B: 88.15 ± 7.44 U) and lysozyme activity ($W = 93$, $P > 0.05$, E: 137.25 ± 10.22 U/ml, B: 166,15 ± 19.36 U/ml).

3.7. Growth

We found a significant interaction between treatment and time for weights data (ANOVA, $F_{7,147} = 7.07$, $P < 0.001$). Tukey's HSD showed that enriched fish were significantly heavier than fish reared in barren tanks at 288 dpf ($P < 0.05$), 309 dpf ($P < 0.01$) and 333 dpf ($P < 0.001$) (Fig. 5). The condition-factor K was significantly higher in barren fish ($t = -3.14$, df = 23.39, $P < 0.01$, E: 1.15 ± 0.02, B: 1.24 ± 0.02), and the FCR was not significantly different between treatments ($t = -0.98$, df = 12.86, $P = 0.35$, E: 1.41 ± 0.03, B: 1.49 ± 0.08).

4. Discussion

This study highlights important behavioral differences between rainbow trout reared in enriched tanks and those held in barren tanks. When observed in their rearing tanks, enriched fish displayed more shoal cohesion and fewer agonistic and/or stereotypic behaviors, which explained the lower activity level observed in this group. Environmental enrichment also improved growth, without any effect on immune parameters. When subjected to the emotional reactivity test, they displayed fewer fear-related behavior but plasma cortisol levels remained similar between groups. When exposed to a novel object, enriched fish were bolder and less neophobic, spending more time close to the object, without exhibiting anxiety-like behaviors.

When observing fish behavior in their home tank, the presence of complex structures decreased aggression levels, as measured by significantly fewer flight behaviors and almost no fin erosion in the enriched groups. Habitat complexity has already been shown to decrease aggressiveness in several fish species (Atlantic salmon (*Salmo salar*): (Naslund et al., 2013), gilthead seabream (*Sparus aurata*): (Batzina and Karakatsouli, 2012), redbreast tilapia (*Tilapia rendalli*): (Torrezani et al., 2013), black rockfish (*Sebastes schlegelii*) and fat greenling (*Hexagrammos otakii*): (Zhang et al., 2021b)), but not always (Nile tilapia (*Oreochromis niloticus*): (Barreto et al., 2011), zebrafish (*Danio rerio*): (Woodward et al., 2019)). This discrepancy can be linked to the different fish species used in these studies, but it may also depend on the number of the structural enrichments used and the surface covered by the structures, which is an important factor especially for territorial species. A high number of physical enrichments might restrict territorial range and visual contact, thereby decreasing the probability of encountering and consequently reduced aggressive behavior towards conspecifics (Zhang et al., 2021b). During the behavioral observation period, the average covered area by the structures composed of 4 plants, 1 stone and 2 pipes was around 70% which seems to be a correct ratio to provide enough visual obstruction, without lowering fish maneuverability. In contrast, introducing too few objects may induce more conspecifics aggression since the physical structure becomes a competing resource (Barley and Coleman, 2010). Another obvious function of physical enrichment is to provide shelter where subordinate fish can escape from aggressive attempts providing a sense of safety, as encountered in the natural habitat of rainbow trout. From the nature-based approach, the possibility for those fish to hide and to express these nature-like phenotypes is likely to promote positive welfare. Furthermore, the lower aggression levels led to a decrease in damaged fins, which can become a point of entry for many pathogens that can affect fish health (Goede and Barton, 1990; Noble et al., 2020). This finding is consistent with several studies reporting less fin erosion in salmonids held with submerged structures, overhead covers or vertical structures made of spheres (Berejikian and Tezak, 2005; Kientz et al., 2018; Rosengren et al., 2017). Enriched rearing has also a positive effect on survival and disease resistance of Atlantic salmon during different parasitic infections (Karvonen et al., 2016) or during bacterial (*Flavobacterium columnare*) exposure (Räihä et al., 2019). Structural enrichments have been suspected to represent a bacterial reservoir and vectors for pathogens (Tuckey and Smith, 2001). In contrast, a recent study revealed that a beneficial microbial community introduced by the structures may prevent the development of pathogens through competitive phenomenon (Karvonen et al., 2021). Therefore, fish reared in enriched environments could have an increased resistance through an improved immunocompetence. However, in the present study, the innate immune markers investigated (lysozyme and complement activities) were not different between treatments, which might be explained by the treatment, maybe not strong enough for generating an impact on such humoral molecules involved in the teleostean innate immune defense (Tort, 2011).

We found that global fish activity observed in rearing tanks was decreased in enriched groups as compared to barren groups, which is consistent with several studies reporting that environmental enrichment leads to reduced swimming activity in coastal cod (*Gadus morhua*) (Salvanes and Braithwaite, 2005) and zebrafish (von Krogh et al., 2010), and reduced startling response in pike (Einfalt et al., 2013). Obviously,

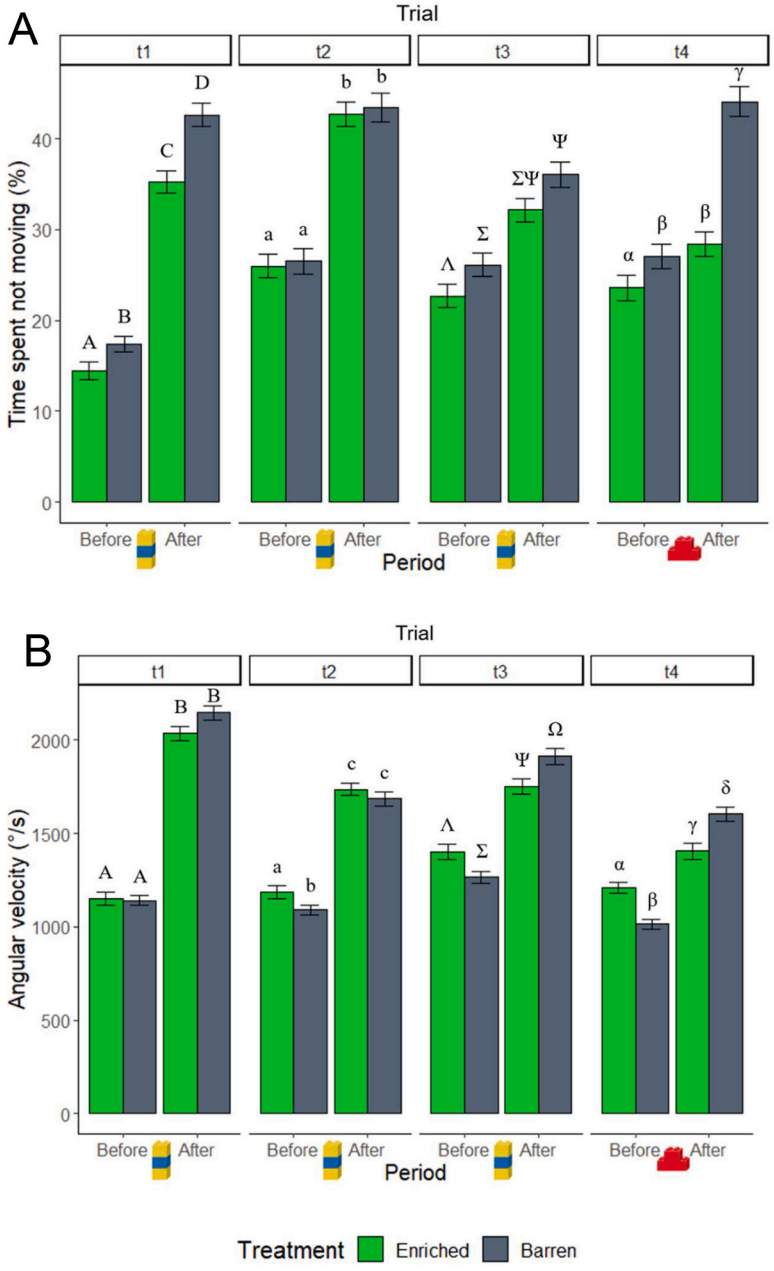
Table 3
Statistical effects of the fixed factors Treatment, Trial, Period and their interactions on the behavioral variables obtained from the novel-object test.

Variables	Treatment			Trial			Period			Treatment x Trial			Treatment x Period			Trial x Period			Treatment x Trial x Period		
	χ^2	df	P-value	χ^2	df	P-value	χ^2	df	P-value	χ^2	df	P-value	χ^2	df	P-value	χ^2	df	P-value	χ^2	df	P-value
Distance traveled (cm)	3.84	1	0.05	85.66	3	*<0.001*	134.57	1	*<0.001*	50.16	3	*<0.001*	19.38	1	*<0.001*	65.13	3	*<0.001*	13.41	3	*0.004*
Maximum velocity (cm/s)	3.86	1	*0.049*	27.23	3	*<0.001*	93.66	1	*<0.001*	155.15	3	*<0.001*	118.29	1	*<0.001*	29.68	3	*<0.001*	118.13	3	*<0.001*
Time spent not moving (%)	0.74	1	0.39	33.78	3	*<0.001*	121.27	1	*<0.001*	19.62	3	*<0.001*	1.71	1	0.19	59.49	3	*<0.001*	6.06	3	0.11
Angular velocity (°/s)	0.005	1	0.95	112.98	3	*<0.001*	277.51	1	*<0.001*	12.5	3	*0.006*	0.99	1	0.32	78.75	3	*<0.001*	19.92	3	*<0.001*
Latency to enter in the object area (s)	0.3	1	0.58	1.48	3	0.69				0.24	3	0.97									
Number of entries in the object area	0.77	1	0.38	257.17	3	*<0.001*				47.64	3	*<0.001*									
Time spent in the object area (s)	0.35	1	0.55	162.3	3	*<0.001*				53.6	3	*<0.001*									
Minimum distance to object (cm)	0.06	1	0.79	28.78	3	*<0.001*				8.82	3	*0.03*									
Number of mouth contacts	0.02	1	0.9	36.62	3	*<0.001*				13.06	3	*0.005*									
Mouth contact latency (s)	0.05	1	0.84	9.48	3	*0.02*				5.16	3	0.16									

this can be explained by the low level of aggression observed in the enriched groups, but an additional explanation is that frustration of motivations to perform specific activities (deprivation: (Dawkins, 1990) in impoverished environments tends to increase locomotor activity and to induce stereotypic behavior, escape attempts and other 'restless' behavior (Fureix and Meagher, 2015). Indeed, it is well known that a lack of stimulation provokes the apparition of stereotypies in captive animals, including fish (Martins et al., 2012). We did not distinguish stereotypies from aggressions in our manual observations thus it seems that these behavioral phenotypes were both decreased in the complex habitat where fish could avoid aggression and find specific activities to perform (foraging, exploring, resting) at the same time. Our study revealed that enriched fish distributed in a more homogeneous pattern than barren fish, as also reported in black rockfish (Zhang et al., 2021c). Here, we noticed that shoal cohesion was pronounced close to the different structures provided in the enriched tanks, which is not surprising considering the high floor covering of the structures (~70%). Heightened shoal cohesion is considered as a marker of positive emotions in zebrafish (Franks et al., 2018). Therefore, the shoal cohesion observed in our study suggests the existence of long resting periods shared by a majority of conspecifics in peaceful relationships, attracted by a variety of shaded areas provided by the structures mimicking the natural habitat of rainbow trout, and can be considered as a marker of positive welfare from the nature-based approach.

We also found evidence of better growth rates in trout reared in enriched compared to barren environments for the same amount of food distributed (in percentage of total weight). This result is consistent with the literature since it is frequently observed that environmental enrichment increases growth in rainbow trout (Kientz and Barnes, 2016; Kientz et al., 2018; Krebs et al., 2018; Voorhees et al., 2019). While some neutral (Barnes et al., 2019; Imre et al., 2002; Zhang et al., 2020) or even negative effects in case of high densities (Rosengren et al., 2017) of enriched rearing have been reported, the current evidence strongly highlights positive effects of environmental enrichment on salmonids growth. It seems that weight differences may be due to the decreased level of aggression in enriched fish, leading to lower chronic stress and forward less energy used. Some studies have shown that adding physical structures into the tanks decreases the metabolic rate and stress levels in fish (Millidine et al., 2006; Naslund et al., 2013). Conversely, the increased energy expenditure led by a restless state observed in barren tanks might have negatively impacted the metabolic rate, which in turn led to decreased fish growth in our experiment. The immune parameters analyzed in this experiment were not negatively impacted by physical enrichments as discussed earlier, and fish growth was even better than in barren groups suggesting positive effects of enrichments from the functions-based welfare approach.

Experiences encountered during life significantly affect the individual's perception of the environment (threatening, neutral, stimulating) and the responses to it throughout life. These behavioral and physiological responses represent the individual's emotional reactivity, here expected to differ between enriched and barren fish. It may be either adaptive or maladaptive depending on the environments in which the animal will live, likely impacting its welfare (Doyle et al., 2011). The emotional reactivity test (or novel-tank test), as evaluated by isolating a fish in a novel environment, is a forced-choice exploration paradigm known to be highly stressful (Doyle et al., 2011). Using this test, we found that barren fish spent more time not moving at the beginning of the test, and also higher angular velocities compared to enriched fish. Greater time spent not moving (freezing) in open-field tests is indicative of greater anxiety in many species (Forkman et al., 2007). When tested out of the living environment, inactivity has been observed in a variety of situations where welfare was supposed to be poor (Fureix and Meagher, 2015). For example, male rats exposed to social defeat become more inactive and less exploratory in novel environments than non-defeated controls (Meerlo et al., 1996). Here, barren fish also displayed a greater angular velocity (i.e., erratic swimming), which is a



Trial effect:

Treatment	Period	Trial
E	Before	t2 - t3 - t4 > t1
	After	t1 > t4 t2 > t3 – t4
B	Before	t2 - t3 - t4 > t1
	After	t2 - t4 > t3

Trial effect:

Treatment	Period	Trial
E	Before	t3 > t1 - t2 - t4
	After	t1 > t2 - t3 > t4
B	Before	t3 > t1 > t4 t3 > t2
	After	t1 > t3 > t2 - t4

Fig. 3. Percentage of time spent not moving (A) and angular velocity (deg/s) before and after the object presentation at each trial (t1, t2, t3 and t4) of trout raised in an enriched environment (E: green) or barren environment (B: grey) during the novel object test. Values are mean and the standard error of mean is represented. Post-hoc tests (Tukey's HSD) have been run for each trial and differences ($P < 0.05$) within a trial are represented by different letters (t1: Latin capitals, t2: Latin lowercase, t3: Greek capitals, t4: Greek lowercase). The differences between trials for each time period and treatment can be seen in the "Trial Effect" table (Tukey's HSD, $P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

well-known anxiety-related phenotype observed in various fish species, mainly described in zebrafish (Kalueff et al., 2013). Conversely, in enriched fish, the rapid recovery from the stressor (i.e., netting followed by isolation in a novel tank) as illustrated by lower time spent not moving at the beginning of the test without exhibiting erratic swimming, suggests a low anxiety state and a greater motivation to explore the new environment. These results in rainbow trout are consistent with zebrafish literature showing that physical enrichment of the environment decreased anxiety-related behaviors and increased exploration (Collymore et al., 2015; Manuel et al., 2015). As also supposed by Franks (2018), we suggest that exploratory behaviors are indicative of positive emotions and higher level of welfare from the feeling-based approach. These behaviors displayed by enriched fish translate a sense of safety despite the forced-choice exploration paradigm of the novel-tank test, and a willingness to acquire novel information from the unknown environment. The structures provided in their rearing tanks have created hidden areas and enhanced fish propensity to navigate the space to discover what is going on behind each visual obstruction. This propensity to explore seems to continue in the novel-tank test without erratic swimming, and provides evidence for a higher positive affect in fish reared in a stimulating environment.

We did not find any effect of the complex habitat on plasma cortisol levels measured after the novel-tank test (~130 ng/ml in enriched fish and ~ 150 ng/ml in barren fish), although these levels were higher than basal levels in both groups, confirming the relevance of the novel-tank test as an induced acute stressor in rainbow trout (Colson et al., 2019;

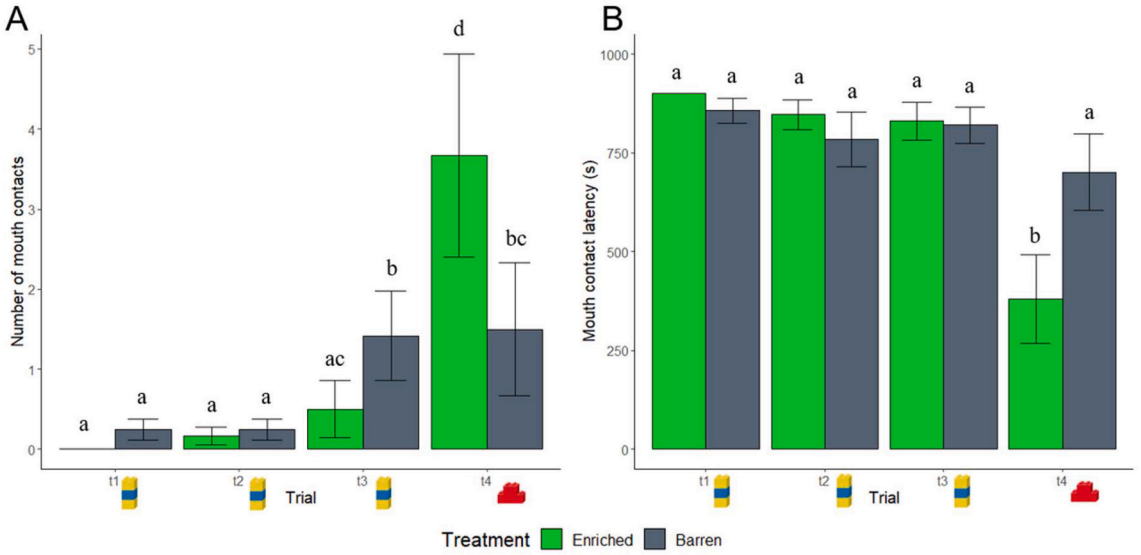


Fig. 4. Number of mouth contacts with objects (A) and mouth contact latency (B) after the introduction of the objects at each trial (t1, t2, t3, t4) of trout raised in enriched environment (E: green) or barren environment (B: grey) during the novel object test. Values are means and the mean standard error is represented. Post-hoc tests (Tukey's HSD) have been run for all variables except for mouth contact latency (GLM), and differences are represented by different letters ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

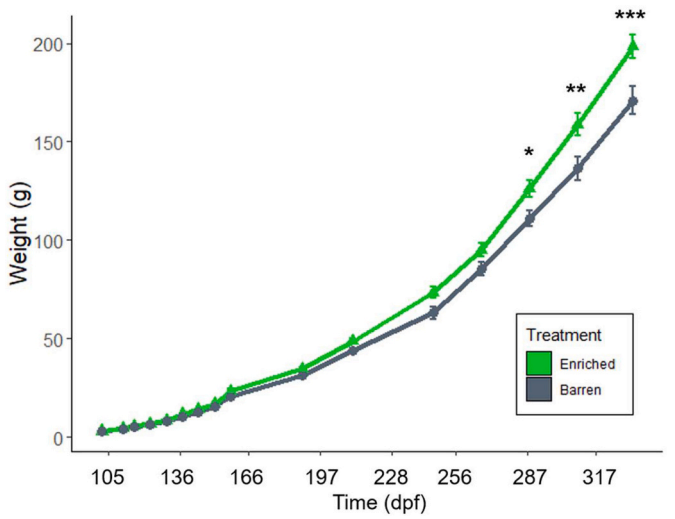


Fig. 5. Mean (± SEM) weight (g) of trout reared in enriched (E: green curve) and non-enriched (B: gray curve) environments according to the days post-fertilization (dpf) (between 102 and 333 dpf). Differences between treatments are represented by represented by *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$).

Poisson et al., 2017; Sadoul et al., 2016). A perceived threat, here represented by isolation in a novel environment, triggers release of stress hormones - catecholamines and corticosteroids, mainly cortisol in fish species (Sadoul and Geffroy, 2019) - that precipitate immediate behavioral changes, i.e., freeze or escape (Ellis et al., 2012). Our results confirm previous published data reporting no difference in post-stress plasma cortisol levels in rainbow trout (Pounder et al., 2016) and Atlantic salmon (Naslund et al., 2013) held in enriched and barren environments. In general, differences between cortisol levels (either basal or after an acute stressor) are observed when comparing very contrasted treatments, for example a group subjected to a strong chronic stress and a non-stressed group. Basal plasma cortisol is higher in red porgy (*Pagrus pagrus*) subjected to 3 weeks of chronic stress by crowding compared to controls (Rotllant and Tort, 1997), and rainbow trout exposed to a 3-week hypoxia challenge are more sensitive to an additional acute stressor, showing higher cortisol levels than non-chronically stressed fish (Colson et al., 2019). In the present study, the control group was reared in standard conditions sharing similar rearing conditions (water quality, photoperiod, density) as those of the enriched group. Although aggression occurred more frequently than in enriched groups, we cannot consider that barren fish were chronically stressed to the point of elevating their cortisol levels higher than enriched fish after the novel-tank test. To resume, providing stimulations in the living environment seems to result in a positive mental state leading to low anxiety-related behaviors and a propensity to explore when individuals are subjected to a novel-tank test, accompanied with an elevation of cortisol concentrations reaching those attained by fish reared in non-enriched (standard) conditions.

Conversely to the emotional reactivity test, the novel object testing is a free-choice paradigm, where animals can show signs of preference for information gain when kept in a familiar environment (Hughes, 1997). In our novel object testing paradigm, fish were acclimated to the test-tank for 18 h before the first object was deposited, which is a shorter acclimation length than the 7 days used in other studies (Basic et al., 2012; Frost et al., 2013). However, the low baseline levels for time spent not moving and angular velocity observed at trial 1, which considerably increased in both treatments when object 1 was firstly introduced (comparison "before" vs "after"), suggest that fish had recover from their introduction in the test-tank and were still highly responsive when an external event occurred. This also indicates a first fear response to the introduction of an object into the test-tank for all treatments, which confirms that rainbow trout, either enriched or not, are firstly naturally neophobic towards a novel object (Sneddon et al., 2003). The total period length used for object familiarization was 9 h (3 times 3 h), which is shorter than the 32 h used for rainbow trout in a previous study (Sneddon et al., 2003), but longer than the lengths used in guppies (*Poecilia reticulata*) (1 and 3 h: (Lucon-Xiccato and Dadda, 2016)). Indeed, we found that 3 trials of 3 h of familiarization was long enough for rainbow trout to acquire information on object 1's features since time spent not moving decreased over trials (from t2 to t3) in both treatments, and responses of enriched fish towards the novel object (object 2) at trial 4 were very pronounced and even differed from barren fish. Under these experimental conditions, we observed an increased interest towards object 2 in fish reared in enriched tanks, suggesting

bold phenotypes in these animals. This point is especially noticeable with the higher number of entries into the object zone and the higher number of mouth contacts with the object. The latency to get in contact with object 2 was also significantly lower in enriched fish. These results show that environmental enrichment by using plants, rocks and pipes leads to a behavioral plasticity, reducing fearfulness and shyness in rainbow trout. Similarly, guppies reared in an enriched environment showed a preference for a novel object over a familiar one (Lucon-Xiccato and Dadda, 2016), as well as enriched Nile tilapia which were less stressful and explored more a novel object when they were previously reared in an environment provided with structures (colored balls and pipes) (Tatemoto et al., 2021). Our hypothesis to explain these results is that trout raised in a barren environment would be more sensitive to habits changes, here the unexpected shape/color of an initially familiar object. This is confirmed by the anxiety-related behaviors observed in barren fish when object 2 was presented, spending more time not moving and displaying a higher angular velocity than enriched fish. A hypothesis along these lines formulated by Tatemoto et al. (2021) submits the idea that a lack of stimuli in the living environment could cause hypersensitivity to novel stimuli, and thus a more accentuated neophobia. Conversely, curiosity towards the novel features of the object, and motivation to seek out new information without exhibiting anxiety-like behaviors suggest positive affects in the enriched fish, and thereby a good welfare state from the feeling-based approach, which is in accordance with the results obtained from the novel-tank test.

5. Conclusions

Our findings demonstrate that providing physical structures in the rearing environment of rainbow trout decreases aggression levels in the group, inducing more opportunities for resting and exploring in shoal cohesion close to the structures. The lower level of aggression probably contributed to improve growth, without any effect on health parameters. These housing conditions seem to give captive fish opportunities to experience positive affects by generating various forms of stimulation, interest, and sense of safety, inducing long-lasting positive affective states, as observed by lower anxiety-related behaviors, higher exploration, and decreased neophobia towards novelty (environment or object), meeting the positive welfare concept. To our knowledge, this is the first experiment demonstrating the positive effects of physical enrichments by using the different approaches to assess animal welfare in an aquaculture species. If environmental complexity positively affects emotional states and also leads to positive welfare from the nature- and functions-based perspectives, physical enrichment should be encouraged in rainbow trout's farms as a routine husbandry practice to increase animal welfare.

Credit author statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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