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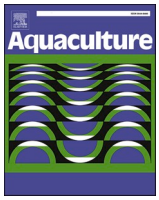

Air bubble curtain improves the welfare of captive rainbow trout fry and fingerlings

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ABSTRACT

Fish welfare is becoming a priority for the fish farming industry. The search for practical, easy-to-implement methods to promote farmed fish welfare is therefore essential. Environmental enrichment aims to improve the psychological and physiological needs of a captive animal by increasing the complexity of its environment. During previous studies, we observed that fish seemed to be positively affected by short diffusions of air bubbles. In this study, we evaluated the effects of an innovative enrichment strategy consisting of introducing into the tank at the earliest stages of life, a pipe generating a curtain of air bubbles. Using rainbow trout (*Oncorhynchus mykiss*) as a captive fish model, we compared the short- (~7 weeks) and long-term (~21 weeks) effects of this bubble curtain diffused for one hour four times a day (Bubble condition) to a standard condition without bubbles (Control) on fish growth, aggressive and abnormal behaviors, as well as on fish motivation to access a bubble curtain, their emotional responses and their learning abilities. We found that bubble diffusion decreased aggressive and abnormal behaviors during diffusions in both the short-term and the long-term experiments. In the long-term experiment, this decrease was also observed during feedings and neutral periods when no bubble was diffused. Bubbles were found to be attractive for young Control fish (bubble-naive fish) subjected to a motivation test in the short-term experiment. When subjected to the emotional reactivity test, Bubble fish seemed less fearful, exhibiting a lower maximum velocity than Control fish in the long-term experiment only. However, the other behavioral parameters measured during this test, appetite and plasma cortisol levels were similar between treatments, irrespective of the experimental period. The latency to consume the reward observed in the spatial learning test in the long-term experiment was decreased in Bubble fish compared with Control fish, showing enhanced learning abilities in fish that experienced bubbles for 21 weeks. Growth parameters and fin erosion index did not differ between treatments. We conclude that repeated bubble diffusions act as an environmental enrichment for fish, combining physical, occupational, and sensory enrichment via the tactile stimulations provided by the air bubbles. This type of enrichment had a positive impact on the behavior of farmed rainbow trout in the long term, and would make it possible to integrate the notion of "positive welfare" into fish farms, while guaranteeing easy technical maintenance.

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1. Introduction

The ongoing revision of European animal welfare legislation concerning farmed fish (Pavlidis et al., 2023) requires the review committees to be provided with objective scientific data and proposals for validated strategies to improve fish welfare particularly for rainbow trout (*Oncorhynchus mykiss*) farming, which represents the leading continental fish species produced in Europe (FEAP, 2022). Animal welfare, and especially positive welfare, can be defined as the physical and mental states that exceed what is strictly necessary for short-term survival (Mellor, 2016; Fife-Cook and Franks, 2019). Thus, it becomes essential to give captive fish opportunities to experience positive affects by generating various forms of comfort, pleasure, stimulation, interest, sense of safety and/or control in order to induce lasting positive affective states (Brunet et al., 2022). One strategy is to provide animals with environmental enrichment, defined as any technique designed to reduce maladaptive and aberrant traits of an animal, including fish, by increasing environmental complexity and the biological relevance of the captive environment (Newberry, 1995; Näslund and Johnsson, 2016; Arechavala-Lopez et al., 2022). Environmental enrichment can be subdivided into social, nutritional, cognitive/occupational, sensory and physical (Bloomsmith et al., 1991). These categories are not mutually exclusive. Beneficial effects of physical enrichments (mainly by the addition of structures and/or shelters) on welfare has been frequently demonstrated experimentally in farmed fish. For example, some studies showed better growth performance (rainbow trout: (Kientz et al., 2018)) and reduced aggressive behaviors linked to the hierarchy establishment (redbreast tilapia, *Tilapia rendalli*: (Torrezani et al., 2013)), due to limitations of visual contacts (Atlantic salmon *Salmo salar*: (Dolinsek et al., 2007)) and utilization of shelters by subordinate fish (brown trout *Salmo trutta*: (Höjesjö et al., 2004)). Recently, we found important behavioral differences between juvenile rainbow trout reared with enrichments (pipes, plastic plants, and stones) for three months and juveniles held in a barren environment (Brunet et al., 2022). When observed in their rearing tanks, enriched fish showed fewer aggressive behaviors, burst of accelerations, and jumps, known to be indicators of poor welfare in farmed fish (Martins et al., 2012). When subjected to social isolation in a novel environment, enriched fish explored more and exhibited fewer anxiety-related behaviors, which seemed to reflect a sense of safety in these fish despite the testing procedure (Brunet et al., 2022). In rainbow trout, also the presence of plants, gravel and shelter in the tank was shown to reduce human-induced stress recovery responses measured by plasma cortisol (Pounder et al., 2016).

The effects of cognitive (or occupational) enrichment, as well as sensory enrichment on the welfare of farmed fish are more scarcely described. For example, giving fish the opportunity to anticipate mealtimes, control their feeding using self-feeders or meet cognitive challenges are occupational enrichment strategies with promising consequences on fish welfare (Kleiber et al., 2023). Recently, we used signaled feeding predictability as a cognitive/occupational enrichment for rainbow trout (Kleiber et al., 2022). We showed that feeding predictability using bubble diffusion as a predictor of meals for two weeks resulted in fewer pre-feeding agonistic and abnormal behaviors than in a temporal predictability condition, where fish were fed at fixed times every day. We concluded that the use of bubbles as a predictor of feeding could represent an interesting approach to improve the welfare of farmed rainbow trout, acting as a cognitive, physical and/or sensory enrichments, these three modalities remaining to be deciphered, since we observed that bubbles were highly attractive for fish whether or not their diffusion was predictive of feeding (Kleiber et al., 2022). The positive effects of sensory enrichments have also been described. These are mainly auditory enrichment, through the provision of music (Papoutsoglou et al., 2013), and visual, by rearing fish in tanks of different colours (McLean, 2021) or using ambient lights with different wavelengths, blue light sources being recommended for the welfare of cultured rainbow trout (Güller et al., 2020). Tactile stimulations have been shown to decrease stress levels in the surgeonfish *Ctenochaetus striatus* (Soares et al., 2011), Nile tilapia *Oreochromis niloticus* (Gauy et al., 2021), or in zebrafish *Danio rerio* (Schirmer et al., 2013), but the long-lasting effects of tactile stimulation as sensory enrichment on farmed fish welfare represent an unexplored field of research.

Increasing the complexity of captive environments through enrichment also enhances fish cognitive abilities, especially spatial learning (Strand et al., 2010; Salvanes et al., 2013; Makino et al., 2015; Abreu et al., 2019; Zhang et al., 2021). Enrichment, in the form of objects that can be used to hide or serve as visual landmarks, provides animals with more variable sensory experiences, triggering brain plasticity (zebrafish: (von Krogh et al., 2010); rainbow trout: (Cardona et al., 2022)). In an experiment aiming to determine the effects of duration of exposure to environmental enrichment, rainbow trout that experienced enriched conditions for the longest duration (12 weeks) had better spatial learning abilities than fish exposed to an early period of enrichment of 5 weeks only (Ahlbeck Bergendahl et al., 2017). In addition, providing fish with a changing and stimulating environment at early stages increases cognitive abilities (Kotrschal and Taborsky, 2010) and adaptive shoaling behaviors later in life (Salvanes et al., 2007), which underlines the interest of studying the effects of enrichment when it is provided from the earliest stages.

While the use of enrichments already exists in terrestrial farmed animals, the fish farming industry has not yet adopted them, probably due to a lack of scientific knowledge and regulations specific to fish welfare. Moreover, physical enrichment by adding complex structures to the tank has several drawbacks: potentially abrasive to fish if poorly designed, they can also hinder the netting, thus increasing capture time. They may be also difficult to maintain, as they are suspected of constituting a bacterial reservoir (source of fish pathogens) and of harboring ectoparasites, which carry out part of their cycle on the bacterial veils of solid supports. These potential drawbacks may also explain the industry's reluctance to implement these practices.

An innovative and easily applicable enrichment strategy would be to introduce a curtain of bubbles into the tanks, since we showed in a recent study that rainbow trout were strongly attracted to areas where bubbles were diffused, whether or not bubble diffusion was predictive of feeding (Kleiber et al., 2022). The diffusion of bubbles in rearing tanks may act as (i) a physical enrichment by making the environment more complex, (ii) an occupational enrichment by limiting monotony if the diffusion is not continuous and encouraging physical activity, and as (iii) a sensory enrichment via tactile stimulations provided by the bubbles. In a preliminary study, we confirmed the positive valence of bubbles for fish exposed to a bubble curtain for an early period of 5 days, as evidenced by their spontaneous preference for a bubble area over bubble-naive fish in a choice test device where fish could see bubbles behind a transparent window without any possibility of physical contact (Kleiber et al., in preparation). However, this small-scale experiment did not investigate the long-term effects of this type of enrichment on classical indicators of fish welfare, including aggression, abnormal behaviors and fin erosion (Noble et al., 2020), in standard rearing conditions.

Here, we studied the extended effects on rainbow trout fry and fingerlings of this innovative enrichment strategy consisting in introducing a pierced pipe connected to an air pump into the tank, thus generating a curtain of bubbles. The diffusion of bubbles was provided at the earliest life stages and we evaluated the impacts at short term (~7 weeks) and at long term (~21 weeks) on zootechnical performance, fish aggressive and abnormal behaviors, and fin erosion. We also evaluated fish motivation to access into the curtain of bubbles, their physiological and behavioral emotional responses when subjected to a novel tank test, and their spatial learning performances. We hypothesized that repeated exposure to bubbles (for 1 h, 4 times a day) would act as an environmental enrichment, either physical, occupational, and/or sensorial, and would improve fish welfare.

2. Material and methods

All experimental procedures comply with the ARRIVE guidelines (Percie du Sert et al., 2020), and were carried out in strict compliance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental plan was approved by the Ethical Committee for Animal Experimentation of Finistère (CEFEA) and received the approval of French minister of national education, research and innovation under the authorization number: APAFIS #37362–2,022,061,516,449,420 v4.

2.1. Experimental animals

The animals were triploid female rainbow trout *Oncorhynchus mykiss* (autumnal strain) produced and reared at the INRAE experimental fish farm (PEIMA, INRAE, 2018, Fish Farming Systems Experimental Facility, France. DOI: 10.15454/1.5572329612068406E12). At 40 days post-fertilization (dpf), 10 batches of 250 fry were formed (Fig. 1) and distributed into 10 identical tanks (66 × 66 × 39 cm; 170 L; 2.8 ± 0.2 L/min), in a flow-through system supplied by spring water. Upstream the system, water was sterilized by UV light and CO_2 degassed mechanically. Parameters of the spring water were monitored every week: N-NH_4 = 0.016 ± 0.014 mg/L, N-NO_2 = 0.002 ± 0.003 mg/L, N-NO_3 = 8.62 ± 1.75 mg/L, Total Phosphorus = 0.025 ± 0.019 mg/L, O_2 = 10.13 ± 0.21 mg/L, pH = 6.71 ± 0.28. Water temperature was automatically monitored every day: 12 ± 0.4 °C. The water flow was adjusted to ensure a two tank volumes turnover per hour to allow a dissolved oxygen concentration >6 mg/l in all rearing tanks. Each tank was surrounded by two light bulbs (3000 K LEDs, 9.6 W, 1350 lm, Elvadis SASU, France; Fig. 2) controlled by a programmer panel allowing selection of the photoperiod (13 L:11D), light intensity measured on the tank surface (472.5 lm in the short-term experiment, 135 lm in the long-term experiment), and gradual switch-on to simulate dawn and dusk over 30 min. During acclimation to rearing tanks, between 40 and 61 dpf (Fig. 1), food was delivered continuously during the day by belt feeders (Scubla, Italy). After acclimation, from 62 to 111 dpf (short-term experimental period), trout were fed commercial pellets in quantities regularly adapted to fish growth (58% proteins, 33% lipids, Ø 0.8 mm, BioMar, France). Food was delivered by ArvoTec TD2000 dispensers (Arvo-Tec Oy, Finland; Fig. 2) at a rate of 8 feedings per day (4 in the morning and 4 in the afternoon) between 9 a.m. and 5 p.m. using a computer-controlled Imetronic® software (v1.0.0.0, Imetronic, France). From 112 dpf to 210 dpf (long-term experimental period), fish were fed 4 times per day at 9 a.m., 11 a.m., 2 p.m. and 5 p.m. with pellets regularly adapted to fish growth (from 112 to 132 dpf: 58% proteins, 33% lipids, Ø 1.1 mm, BioMar, France; from 133 to 146 dpf: 52% proteins, 17% lipids, Ø 1.4 mm, Le Gouessant Aquaculture, France; from 147 to 174 dpf: 52% proteins, 17% lipids, Ø 1.7 mm, Le Gouessant Aquaculture; from 175 to 210 dpf: 47% proteins, 17% lipids, Ø 2.5 mm, Le Gouessant Aquaculture). Fish density in the experimental tanks was between 0,42 kg/m^3 and 6,24 kg/m^3 with 250 fry per tank (from 62 dpf to 111 dpf for the short-term experiment), and between 8.68 kg/m^3 and 30.16 kg/m^3 (from 147 dpf to 210 dpf for the long-term experiment). Biomass was adjusted twice: at 111 dpf to 100 fry per tank, and at 174 dpf to 80 fingerlings per tank. The few fish that died during the 21 weeks of experimentation were regularly counted and removed from the tanks. Each rearing tank was equipped with an air diffuser (diameter: 3 cm, length: 51 cm, EPDM DY 1002–20, Aqualor, France; Fig. 2) connected to a single air pump (160 W, 0.64 kWh) to diffuse a curtain of bubbles in all "Bubble" tanks (see 2.2). Once a week, the diffusers were removed and the microporous membrane was washed to remove the bacterial film. The bubbles were then checked daily for homogeneity in density and size.

2.2. Experimental conditions

Two conditions were applied: a standard condition where bubble diffusers were present but never active (Control), and an enriched condition where bubble diffusers were active 4 times a day for one hour (Bubble). The bubbles were provided intermittently and scheduled at different hours for each day of the week, to avoid fish habituation since a prolonged exposure to physical enrichment devices can result in a loss of interest and exploitation from the animals (Kuczaj et al., 2002). For the short-term experiment, fish were reared under these two different conditions from 62 dpf to 111 dpf (for ~7 weeks; Fig. 1). We used 5 tanks per treatment as replicates. For the long-term experiment, fish were reared under the two different conditions from 62 dpf to 210 dpf (for ~21 weeks; Fig. 1) and 4 tanks per treatment were used as replicates. Video surveillance cameras (Full HD: 1920 x 1080px, 105°, Vizeo, ADRIEN ALARME, France) were positioned above each tank.

2.3. In situ behavioral observations

Video recordings were made once a week every Tuesdays. Day 0 (D0 in Fig. 1) corresponds to the day when bubble diffusions first occurred in the Bubble treatment (at 62 dpf). The videos began one week before Day 0 (D-7) and continued until 6 weeks after Day 0 (D42) for the short-term experiment, and from D84 to D147 for the long-term experiment (Fig. 1). On Tuesdays, bubble diffusions occurred at 10 a.m., 12 a.m., 3 p.m. and 6 p.m. (Fig. 3). The bubbling times for the others days of the week are given in the Supplementary Data (Fig. S1). We analyzed fish group behavior from sequences of 5 min before, during and after each

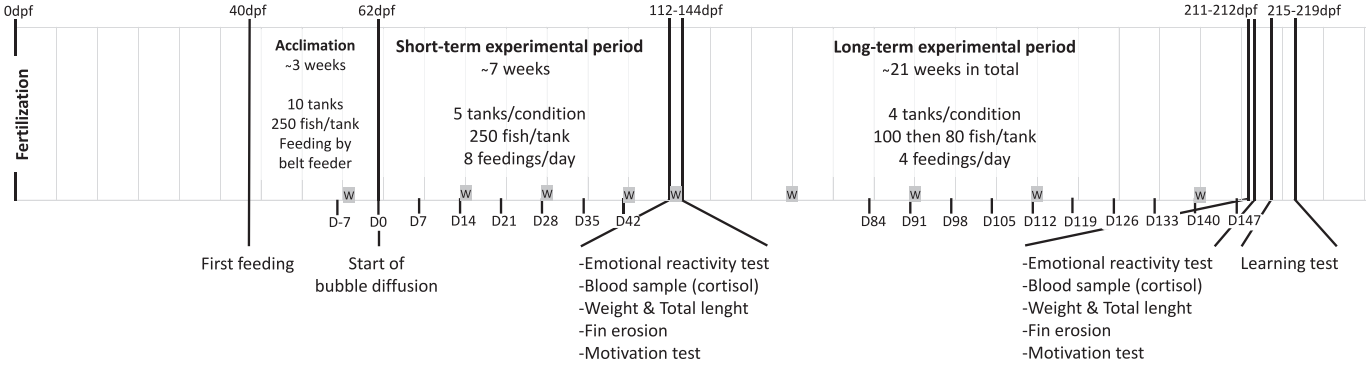

Fig. 1. Experimental schedule. Rainbow trout from the Bubble condition were exposed from 62 dpf to 211 dpf to 4 daily one-hour bubble sequences. Fish behaviors observed in the group of both conditions (Bubble and Control) were quantified from weekly video recordings from D-7 (one week before Day 0) to D42 in the short-term experiment, and from D84 to D147 in the long-term experiment, Day 0 (D0) being the day when bubble diffusions occurred for the first time in the Bubble condition. Behavioral tests and samplings were performed after 7 weeks of bubble diffusions (short-term experiment) and after 21 weeks of bubble diffusions (long-term experiment). dpf = day post-fertilization; W = Weighing groups of fish; DXX = Day of video recording.

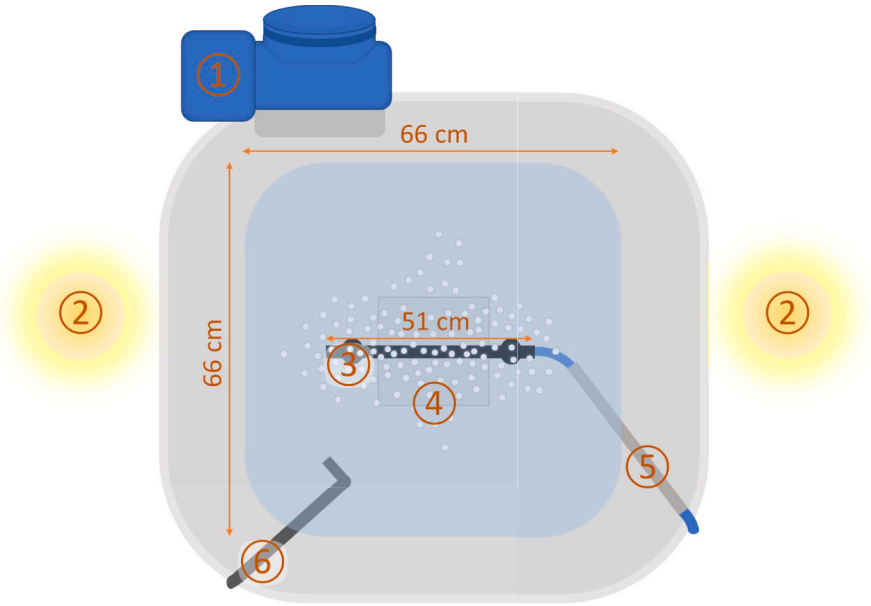

Fig. 2. Layout of the experimental tanks during the short- and the long-term experiments: ① Food dispenser; ② Lights; ③ Air diffuser; ④ Water outlet grid; ⑤ Pipe from the air pump; ⑥ Water inlet. The tanks from the Bubble condition were equipped with an active air diffuser emitting a curtain of bubbles for 1 h, 4 times a day, while the same diffuser in the Control tanks was not connected to the air pump (inactive air diffuser).

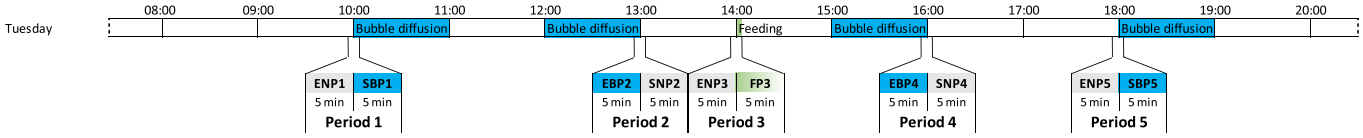

Fig. 3. Time schedule of five video recording periods for both conditions (Bubble and Control) performed each Tuesday for the short- (7 weeks of bubble diffusions) and the long-term (21 weeks of bubble diffusions) experiments with rainbow trout. The same 5-min sequences were chosen in both conditions despite the absence of bubble diffusions in the Controls. ENP1: End of Neutral sequence in Period 1; SBP1: Start of Bubbles sequence in Period 1; EBP2: End of Bubbles sequence in Period 2; SNP2: Start of Neutral sequence in Period 2; ENP3: End of Neutral sequence in Period 3; FP3: Feeding in Period 3; EBP4: End of Bubbles sequence in Period 4; SNP4: Start of Neutral sequence in Period 4; ENP5: End of Neutral sequence in Period 5; SBP5: Start of Bubbles sequence in Period 5.

event (the 4 bubble diffusions and the feeding distribution of 2 p.m.), resulting in 5 periods (P1 to P5, Fig. 3). Each 5-min sequence concerned: end of neutral period (ENP1), start of bubbles period (SBP1), end of bubbles period (EBP2), start of neutral period (SNP2), end of neutral period (ENP3), feeding (FP3), end of bubbles period (EBP4), start of neutral period (SNP4), end of neutral period (ENP5) and start of bubbles period (SBP5). Neutral periods (NP) refer to periods without bubbles or feeding (Fig. 3). We did not analyze FP3 for D-7 since fish were fed continuously by belt-feeders on that day.

For each 5-min sequence, we manually quantified the occurrence of aggressive interactions (bites, chases), burst of accelerations (when fish crossed more than the half of the tank with high speed) and jumps (see Kleiber et al. (2022) for the ethogram details) observed in the group. Burst accelerations and jumps are then referred to as "abnormal behaviors" throughout this study. These behaviors were indiscriminately grouped and counted in a standardized arena drawn on the computer screen of the center of the tank (70 × 39.5 cm). This arena, assumed to be representative of the entire tank, made it possible to reduce the observation zone and ensured that no behavior was missed.

2.4. Emotional reactivity test

At the end of the short- (at 112 dpf, i.e., 16 weeks post-fertilization) and long-term experimental periods (at 211 dpf, i.e., 30 weeks post-fertilization, Fig. 1), fish emotional reactivity was assessed using a test of social isolation in a novel environment, also known as the open-field test in terrestrial animals (Forkman et al., 2007). An animal's emotional reactivity refers to its propensity to react to emotion-provoking stimuli and depends on its perception of the environment (Boissy et al., 2007). We assessed fish emotional reactivity in the novel-tank test by using our established protocol (Colson et al., 2019). Fish were not fed for 24 h before being tested. Four fish were simultaneously netted arbitrary in their rearing tanks and introduced into 4 separate test tanks (68 × 33 × 32 cm) for a total of 40 min before being netted and euthanized for blood sampling (see following paragraph). This operation was repeated by the same experimenter in 3 rearing tanks to obtain 12 individuals per rearing condition. The fish were filmed using the cameras placed above the tanks and behaviors were analyzed with Ethovision® XT software (v. 17.0.1630, Noldus, The Netherlands). In the software, the peripheral zone of the tank was delimited (one-third fish body size from the perimeter of the ground on each side) in order to measure thigmotaxis, defined as time spent near the walls, a secure zone for an animal, including fish (Sharma et al., 2009). During the first 10 min the following behavioral responses were analyzed by a 5-min time step: maximum swimming velocity (cm/s), total distance moved (cm), time spent in thigmotaxis (% of the total duration), mean angular velocity (°/s) (i.e., erratic swimming, a well-known anxiety-related behavior observed in various fish species, mainly described in zebrafish (Kalueff et al., 2013)), and number of rotations (>90°). Fish were left for a further 30 min in the test tank to be sampled for plasma cortisol (see next paragraph), since peak cortisol release occurs between 30 and 60 min after an acute stress in rainbow trout (Auperin and Geslin, 2008; Gesto et al., 2015; Sadoul et al., 2016). After 35 min, individuals were given 20 pellets and the remaining pellets were counted after 5 min to measure

any inhibition of food intake (anorexia) following a stressful situation (Braithwaite and Salvanes, 2005).

2.5. Plasma cortisol assay

At the time of the emotional reactivity test, 12 other fish per treatment were netted in their rearing tank (4 fish per tank netted simultaneously by another experimenter) in order to measure basal cortisol concentrations without having been subjected to the emotional reactivity test. These fish were first anesthetized by placing them in a bucket containing circuit water supplemented with 50 mg/L tricaine methanesulfonate and 50 mg/L sodium bicarbonate to buffer the medium. They were then euthanized using lethal dose of tricaine (200 mg/l + 200 mg/l sodium bicarbonate) prepared in another bucket. Blood was immediately collected from the caudal vessels using heparinized 1 mL syringes (Terumo Europe NV, Belgium) and 25G x $^{5}/_{8}$" AGANI™ needles (Terumo Europe NV, Belgium). Then, blood samples were transferred into 1.5 mL microtubes (Trefflab, Switzerland) and kept on ice. After collection, blood was centrifugated (30 min at 3000 G at 4 °C) and plasma was collected and frozen at −20 °C until analysis. To measure plasma cortisol concentrations after an acute stress (final levels), the 24 fish subjected to the emotional reactivity test (12 Bubble and 12 Control fish) were netted after 40 min, then anesthetized and euthanized as described above. As for fish sampled for basal cortisol, blood was immediately collected, centrifuged and the plasma stored at −20 °C until cortisol analysis.

Plasma cortisol assay was carried out by ELISA following manufacturer instructions (BioSource, Europe, Belgium), and the detailed protocol is described in our recent study (Brunet et al., 2022). The plasma cortisol assay was performed with two replicates per fish (20 µl of plasma per replicate, 40 µ in total), except for 14 fry whose sample was too small to be replicated (20 µ in total for these fry). The number of samples analyzed ranged between 9 and 12 per treatment (Control vs Bubble), per stressor (basal vs final) and per period (short- vs long-term experiment).

2.6. Motivation test

The psychological motivation testing evaluates a fish's propensity to overcome an aversive obstacle to reach a particular resource or environmental condition (Maia et al., 2017). Here we tested fish motivation to leave a shelter and enter a lighted area to access a bubble curtain. Fish psychological motivation to enter in contact with bubbles was assessed at 114 dpf (~7 weeks of bubble diffusion in their rearing tank for the short-term experiment) and at 212 dpf for the long-term experiment (~21 weeks of bubble diffusion). A new cohort of individuals was used for this test. Four fish were simultaneously netted from the same rearing tank and individually isolated in a holding tank (35 × 35 × 40 cm) for 24 h to acclimatize to social isolation. No food was delivered during acclimation. We used a test tank (60 × 40 cm) surrounded by two white light bulbs (0.96 W, 135 lm) illuminating two different zones (Fig. 4). Each zone contained an air diffuser (one active and one inactive), and the individual had to leave a dark start-box covered by an opaque lid (representing a shelter for fish) in order to access the diffusers (Fig. 4). On the day of the test, the fish were introduced into the dark start-box separated from the testing device by a transparent Plexiglas divider, allowing the individual to see the bubble curtain formed by the active diffuser during habituation. After 20 min of habituation, the divider was gently removed and the test lasted for 40 min. The four fish were tested simultaneously in 4 testing devices (2 with the active air diffuser on the right and 2 with the active diffuser on the left side). This operation was repeated 6 times to obtain a total of 12 individuals per treatment. All tests were video recorded using cameras placed above each tank, and the videos were manually analyzed from the moment the divider was removed until the end of the test. For each fish, we measured the time spent in the "bubble zone" (diffuser active), the time spent in the "control zone" (diffuser inactive), the latency to exit from the shelter and the latency to reach the bubble zone (in seconds). For the short-term experiment, due to poor visibility on videos or malformed individuals involuntarily netted, several individuals were withdrawn from the analyses, resulting in 8 fish for the Bubble condition and 10 fish for the Control condition. For the long-term experiment, 12 individuals per

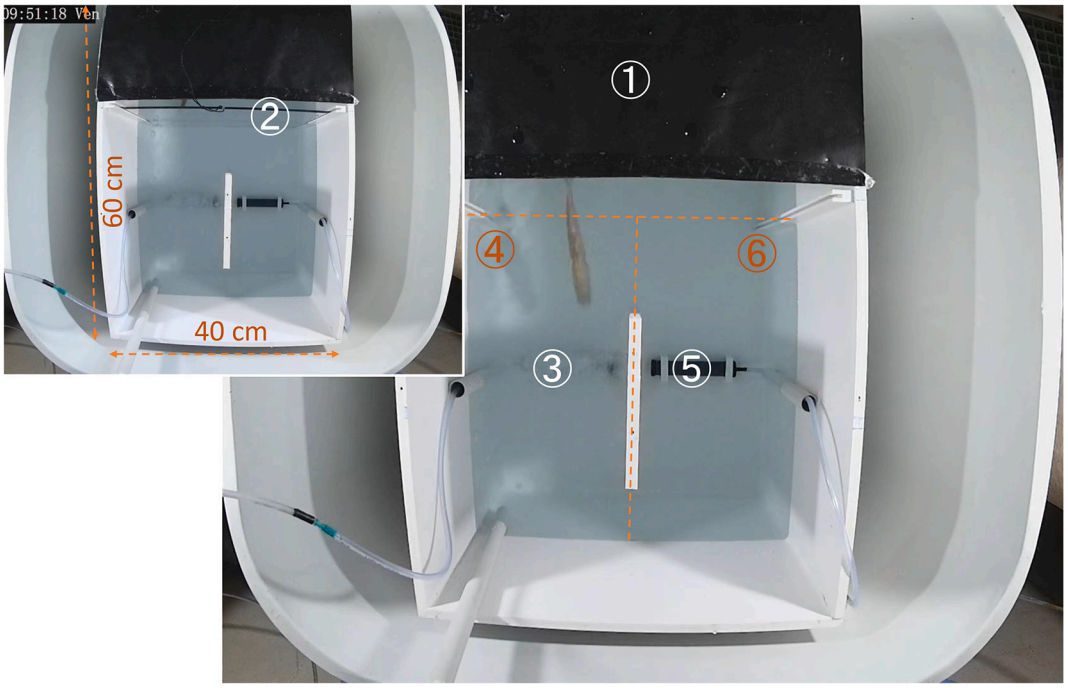

Fig. 4. Experimental device used for the motivation test performed after the short-term experiment (7 weeks of bubble diffusions) and after the long-term experiment (21 weeks of bubble diffusions) in rainbow trout fry and fingerlings: ① Star-box/shelter; ② Removable transparent divider; ③ Active air diffuser; ④ Bubble zone; ⑤ Inactive air diffuser; ⑥ Control zone.

treatment were analyzed.

2.7. Learning procedure

At the end of the long-term experiment (between 215 and 219 dpf), we assessed fish learning abilities using a spatial discrimination test. A new cohort of fish had to associate a zone of the testing device (right or left side) with a food reward, and the decreased latency to reach this zone across trials indicated learning. Prior to testing, fish were placed individually for 24 h in the same holding tanks as those previously used for the motivation test, where no food was delivered. Learning abilities were tested for 5 fish from the Bubble condition and 8 fish from the Control condition.

Two identical devices (60 × 40 × 60 cm) were used (Fig. 5), allowing to test two fish simultaneously. An opaque pipe (Ø 2 cm) was placed at the front of the device, enabling the experimenters to manually distribute food pellets in the reward area. A visual cue (grey plastic panel, 40.4 × 4 cm) indicated to the fish where the food would be released. Half of the fish were conditioned to receive the food reward on the right side and the other half on the left. A black square measuring 20 × 20 cm was marked on the tank bottom with a permanent marker from the edges of the device to delimit the location of the reward area. The cameras placed above the two test tanks enabled two experimenters to observe live on two directly connected monitors (13.3″ screen, 2 K, 100% sRGB, Full HD, ARZOPA, China). Each test tank was equipped with a triangular-shaped start-box (SB) made up of two folding PVC plates to hold the fish before the start of each trial.

2.7.1. Habituation phase (Day 1)

After 24 h in their holding tank, fish were habituated to the device with 3 first successive trials. At trial 1, the fish was netted from its holding tank and placed into the SB for a first 5-min period. The SB was then removed by the experimenter, who immediately distributed 5 pellets (Ø 2.5 mm) into the reward area through the pipe. The first habituation trial lasted 30 min and the two other trials lasted 15 min. If the trout did not spontaneously move to the reward area after 15 min (trial 1) and after 10 min (trials 2 and 3), another 5-pellet reward was released into this area in order to encourage them to move there. At each fish entrance into the reward area, pellets were distributed. After each trial, the SB was reinserted into the device by gently guiding the fish inside and folding back the walls. Uneaten pellets were removed from the device. The inter-trial intervals (ITI) when fish returned to the SB lasted 3 min. At the end of trial 3, the fish was netted and returned to its holding tank until the day after.

2.7.2. Training/test phase (Days 2 and 3)

On Days 2 and 3, the fish had 5 successive training trials per day, 15 min each, reaching a total of 10 training trials. Between Day 2 and Day 3, individuals were returned to their respective holding tanks for the night. Each day began with an initial 5-min stay into the SB. The SB was then removed by the experimenter but no pellet was distributed at this step. Once the fish entered into the reward area, 5 pellets were released and the fish had a maximum of 3 min to consume the pellets before being gently guided to the SB for an ITI of 3 min. If the fish did not consume any pellet during the 3 min after being entered into the reward area, it was also returned to the SB. Day 3 (trials 6 to 10) was considered as the test phase. After trial 10, the fish were netted and returned to their respective rearing tank.

The latency (in seconds) to enter the rewarded area was manually recorded for each trial. The fish was considered to be into the area when at least half its body was within the area delimited by the ground mark. The experimenters also noted the latency (in seconds) required before the first consumption after distribution of the food reward (latency to eat). For each trial, we noted whether the fish entered (or not) the reward area (go/no go response), and whether the first entrance was correct (or not) at Day 3 (first correct enter).

2.8. Fin erosion

We measured the erosion of the caudal and dorsal fins of the fish whose blood was sampled at 112 dpf and 211 dpf (24 fish/treatment/period). A photograph of each individual was taken and fins erosion was evaluated on the basis of the key identification established by Noble et al. (2020), using an erosion index ranging from 1 to 3. Score 1: no lesion; score 2: 0 to 50% of the fin surface damaged; score 3: >50% of the fin surface damaged.

2.9. Growth parameters

The fish were weighed by tank prior to the experiment at 58 dpf.

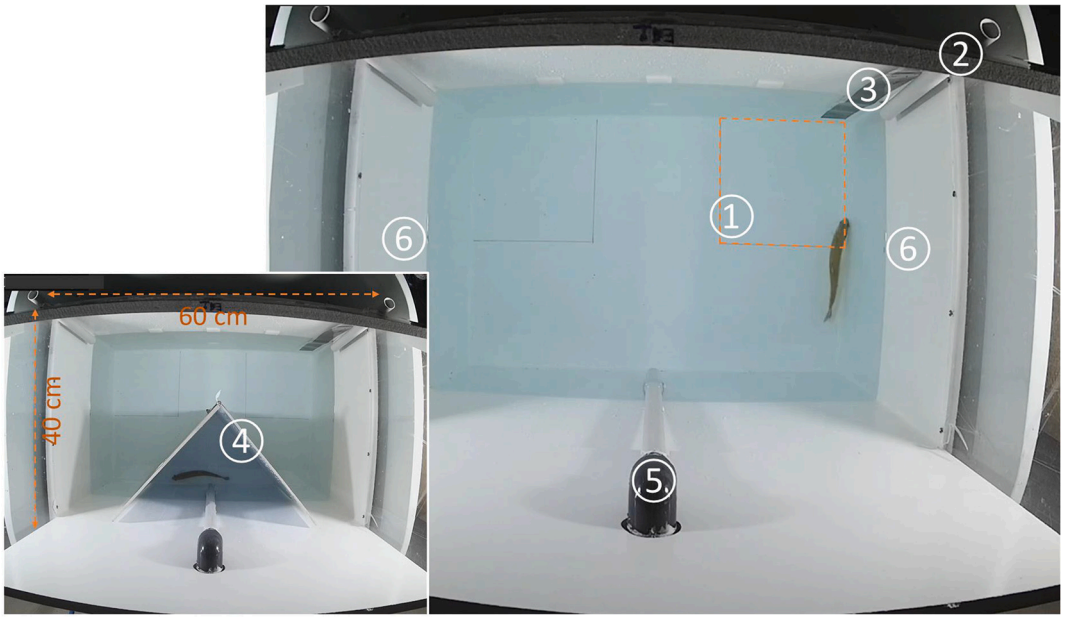

Fig. 5. Experimental device used for the learning test performed on rainbow trout fingerlings after the long-term experiment (21 weeks of bubble diffusions): ① Square mark delimiting the rewarded zone; ② Pipe releasing pellets in the rewarded zone; ③ Visual cue; ④ Removable start-box; ⑤ Water inlet; ⑥ Water outlet.

Then, weighing occurred every 2 weeks until the end of the short- and long-term periods. The Specific Growth Rate (SGR) and Coefficient of Body Weight Variation (CV_{BW}) were calculated.

$$SGR\ \left(\%.day^{-1}\right) = 100 \times \left[ln\left(W_f\right) - ln\left(W_i\right)\right]/T,$$

$$CV_{BW}\ (\%) = 100\ x\ \left(SD_{Wf}/W_f\right).$$

where Wi (g) = mean initial body weight at 58 dpf; W_f (g) = mean final body weight (at 105 dpf in the short-term, and at 203 dpf in the long-term experiment); T = number of rearing days between final and initial measurements; and SD_{wf} = standard deviation of W_f. Mean body weights W_i and W_f were calculated from total biomass (g ± 0.1 g) in each tank.

After blood samplings (at 112 and 211 dpf), weight and total length (from head to tail tip) were also measured individually. Individual weight was measured in g ± 0.1 g and length in cm ± 0.1 cm, and the condition factor was calculated as follows: K factor = 100 (weight (g) / length (cm) ^3).

2.10. Statistics

All analyses were carried out using R software version 4.2.0 (R-Core-Team, 2022) and graphs were drawn with the ggplot2 package. Homogeneity of variances was tested using a F test. When equality of variances was met and when data were normally distributed or could be transformed to meet a Gaussian distribution as assessed graphically, parametric tests were performed with general linear models (GLM) (R package car) for unrepeated data, or mixed linear models (MLM) (package nlme) using Type "marginal" ANOVAs for repeated data needing to include random factors in the model. When data did not meet a normal distribution, generalized linear models (GLIM) (package mass) for unrepeated data, or generalized linear mixed models (GLMM) (package lme4) including random factors, were used using Type "III" ANOVAs for the latest model. Post-hoc tests for pairwise comparisons (package emmeans) were run in case of significant effects of the fixed factors and/or their interaction. *P*-values <0.05 were considered statistically significant for all statistical analyses.

For the occurrence of aggressive and abnormal behaviors collected in situ (total occurences/number of fish/5 min), the short- and long-term experimental periods were analyzed separately, given the change of fish numbers per tank and rearing density.

These data were log-transformed before performing MLMs. Before analyses, we performed MLMs to check whether the behavioral data were explained by the fixed factor sequence, when the different periods were taken into account (ENP1, SBP1, EBP2, SNP2, ENP3, FP3, EBP4, SNP4, ENP5, SBP5). Thus, in the short- and long-term experiments, sequences at the start (SBP1, SBP5) did not differ significantly between P1 and P5, and sequences at the end of bubbles diffusion (EBP2, EBP4) did not differ either, allowing us to group them together into a single start of a bubble sequence named StartBub, and a single end of a bubble sequence named EndBub. In the short-term experiment, we found a significant effect of the sequence on behavior ($F_{4,335} = 4.912$, $P < 0.001$), but the end neutral periods ENP1, ENP3, ENP5 did not differ between them as evidenced by post-hoc tests ($P > 0.05$). Therefore, the three end neutral periods were grouped into a single sequence called EndNeutral. The start neutral periods (SNP2, SNP4) did not differ between them and were grouped into a single StartNeutral sequence. In the long-term experiment, we found a sequence effect ($F_{4,346} = 4.700$, $P < 0.01$), and post-hoc tests revealed a significant difference between the neutral period ENP5 and the two others ($P < 0.01$). Thus, ENP5 was removed from the analyses. The other end neutral periods (ENP1, ENP3) were grouped into a single EndNeutral sequence. The start neutral periods (SNP2, SNP4) did not differ and were grouped into a StartNeutral sequence.

Behaviors were then analyzed over time using MLMs. The following fixed explanatory factors were included in the models: treatment (Bubble and Control) and sequence (StartBub, EndBub, FoodP3, StartNeutral, EndNeutral), as well as the treatment*sequence interactions. Then, we focused on Bubble sequences, Food sequences and Neutral sequences separately, and we run models with treatment and day (from D-7 to D42, and from D84 to D147 for the two respective experiments) as fixed explanatory factors. Rearing tank number was considered as a random factor in the models to take into account the repeatability of the data over time.

For the 10-min emotional reactivity tests performed at the end of each experimental period, effects of the fixed factors treatment (Bubble and Control) and experimental period (short- and long-term) on each variable were analyzed using GLMs or GLIMs. Total distance moved and angular velocity data were sqrt- and log-transformed respectively, before running GLMs. Data from maximum velocity and time spent in the peripherical area (thigmotaxis) were analyzed with a GLIM using a Gamma family distribution (inverse link-function), and run with a Fisher test. Numbers of uneaten pellets at the end of the anorexia test, and numbers of rotations data followed a Poisson distribution and were analyzed using GLIMs (log link-function) run with a Chi^2 test.

For plasma cortisol analyses, data did not meet Gaussian distribution and were square-transformed. Then, we run GLMs with treatment, experimental period and stressor (basal levels before, and final levels after the emotional reactivity test) as the fixed factors.

To analyze the data of the motivation tests, we compared the time spent in zones "bubble", "control" and "shelter" between treatments using a MLM. Data were square-transformed before running the model. Treatment, zone and experimental period (short- and long-term) were the fixed factors and individuals were considered as random factors. We also compared the two treatments for the latency to exit the start-box and the latency to enter the bubble zone using GLIMs run with a Fisher test (Gamma family, inverse link-function).

Regarding the learning test performed in the long-term period, the binary data (go/no go response), and first correct enter were analyzed with a GLMM using a binomial family (logit link-function). After removing the trials when individuals did not enter the rewarded area (no go responses), the latency to enter the rewarded area and the latency to eat the reward were sqrt-transformed and analyzed with a GLMM using a Gamma family (inverse link-function). For each GLMM, treatment and day (D1 to D3) were the fixed factors and the individual was entered into the model as a random factor. For the variable first correct enter, the day factor was not included into the model since this variable was observed at D3 only.

Growth parameters were compared between treatments using Student's *t*-tests. The proportions of dead fish (mortality rates) in each treatment, and the distribution of fin erosion scores between treatments were analyzed manually using χ^2 tests.

3. Results

3.1. In situ behavioral observations

When analyzing aggressive and abnormal behaviors according to the sequences (StartBub, EndBub, FoodP3, StartNeutral, EndNeutral) and the treatments (Bubble and Control), there were significant sequence*-treatment interactions (MLMs: short-term experiment: $F_{4,837} = 171.414$, $P < 0.001$, Fig. 6A; long-term experiment: $F_{4,630} = 57.021$, $P < 0.001$, Fig. 6B).

For both experiments, post-hoc tests revealed fewer aggressive and abnormal behaviors in the Bubble treatment from the start to the end of bubble diffusions ($P < 0.001$; Fig. 6 A and B). In the long-term experiment, tendencies appeared during the food sequence ($P = 0.050$) and at the start of neutral periods ($P = 0.051$; Fig. 6B). In the Bubble treatment, bubble diffusions decreased significantly the level of these behaviors, compared with the food distribution and the neutral sequences ($P < 0.001$). In the short-term experiment, aggression and abnormal

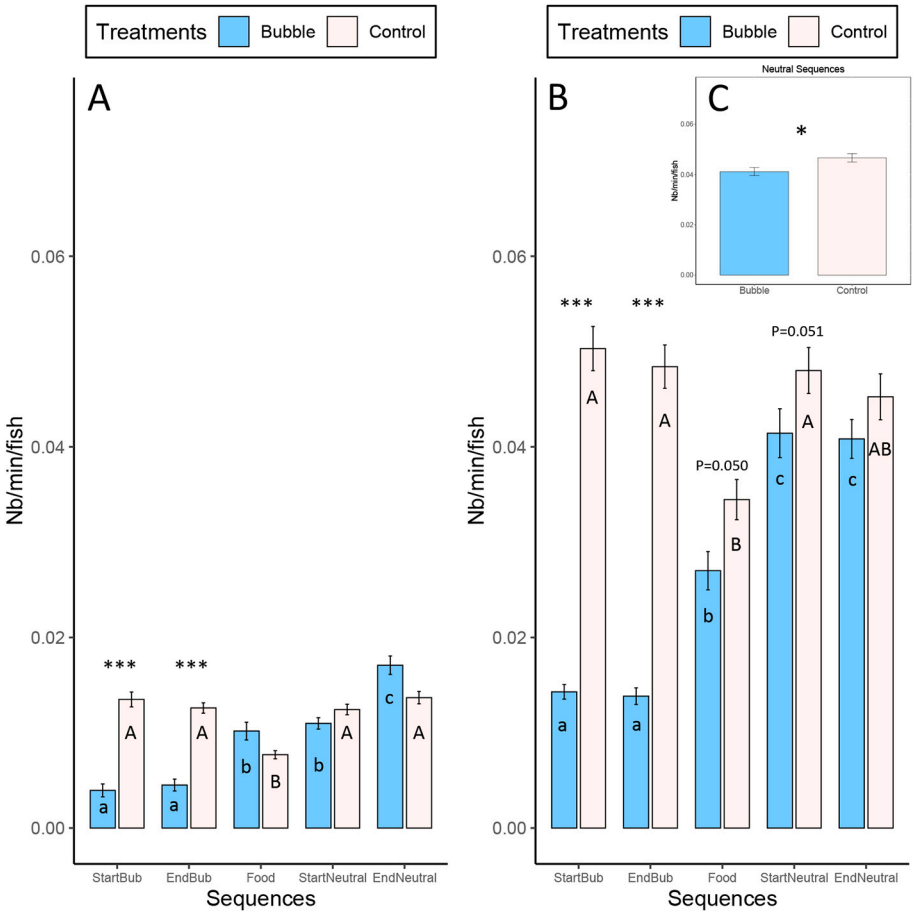

Fig. 6. Mean (± SEM) number of aggressive and abnormal behaviors per minute and per fish observed over sequences when pooling the days in the Bubble and in the Control tanks of rainbow trout, (A) for the short-term experiment (7 weeks of bubble diffusions, $n = 5$), and (B) for the long-term experiment (21 weeks of bubble diffusions, $n = 4$). StartBub: Start of Bubble sequence; EndBub: End of Bubble sequence; Food: Food distribution; StartNeutral: Start of Neutral sequence; EndNeutral: End of Neutral sequence. Asterisks represent significant differences between treatments at each sequence (post-hoc tests, *** $P < 0.001$). Different lowercase letters represent significant differences between sequences within the Bubble treatment (post-hoc tests, $P < 0.001$), and different capital letters represent differences between sequences within the Control treatment ($P < 0.01$). (C) Mean (± SEM) number of aggressive and abnormal behaviors per minute and per fish observed during the neutral sequences when start and end were pooled in the Bubble and in the Control tanks, during the long-term experiment (* $P < 0.05$).

behaviors levels were lower after bubble diffusions (start neutral periods) than at the end of the neutral periods ($P < 0.001$). In the Control treatment, food distribution reduced aggression and abnormal behaviors compared with the other sequences ($P < 0.01$), but not compared with the end of neutral periods in the long-term experiment ($P = 0.068$; Fig. 6B). In the long-term experiment, when pooling sequences from the start and the end of neutral periods which did not significantly differ, we found fewer aggressive and abnormal behaviors in the Bubble treatment than in the Control ($F_{1,6} = 7.832$, $P = 0.031$; Fig. 6C).

In the short-term experiment, we found significant effects of the factor day on the occurrence of aggressive and abnormal behaviors during Bubble sequences (MLM: $F_{8,323} = 51.25$, $P < 0.001$; Fig. 7A), Food sequences ($F_{7,56} = 6.09$, $P < 0.001$) and Neutral sequences ($F_{8,400} = 31.62$, $P < 0.001$). Significant treatment*day interactions were found for each sequence ($F_{8,323} = 21.90$, $P < 0.001$; $F_{7,56} = 3.69$, $P < 0.01$; and $F_{8,400} = 3.12$, $P < 0.01$, respectively; Fig. 7A). Statistical results for day-by-day changes within each treatment are given in the Supplementary Data (Table S2). During Bubble sequences, we observed fewer aggressive and abnormal behaviors in the Bubble treatment than in the Control treatment on each day from D0 (post-hoc tests: $P < 0.001$; Fig. 7A). During Food sequences, the occurrence of aggressive and abnormal behaviors was higher in the Bubble treatment on D2 ($P < 0.05$), but lower at D21 ($P < 0.05$). During the Neutral sequences, this occurrence was lower in the Bubble treatment at D35 ($P < 0.05$, Fig. 7A).

In the long-term experiment, there were significant effects of the factor day on the occurrence of aggressive and abnormal behaviors during Bubble sequences (MLM: $F_{8,262} = 12.38$, $P < 0.001$; Fig. 7B), Food sequences ($F_{8,56} = 16.53$, $P < 0.001$) and Neutral sequences ($F_{8,264} = 38.68$, $P < 0.001$). Significant effects of the factor treatment were found during Bubble sequences ($F_{1,6} = 120.00$, $P < 0.001$) and Food sequences ($F_{1,6} = 8.34$, $P < 0.05$). Significant treatment*day interactions were found for Bubble sequences ($F_{8,262} = 2.46$, $P < 0.05$) and Neutral sequences ($F_{8,264} = 2.29$, $P < 0.05$). Post-hoc tests revealed fewer aggression and abnormal behaviors in the Bubble treatment during Bubble sequences on each day ($P < 0.001$), and during Neutral sequences on D112 and D126 ($P < 0.05$, Fig. 7B). During Food sequences, the treatment*day interaction was not significant and fish displayed globally fewer aggression and abnormal behaviors in the Bubble treatment than Control fish ($P < 0.05$, Fig. 7C).

3.2. Emotional reactivity test

3.2.1. Behavioral responses

There were significant effects of the experimental period on each variable (GLMs or GLIMs: $P < 0.05$, Table 1) except for the number of uneaten pellets (GLIM: $\chi^2 = 0.245$, $P = 0.621$). Maximum velocity, distance moved and number of rotations were higher when observed at an early stage (112 dpf) than at 211 dpf ($P < 0.05$). Time spent in

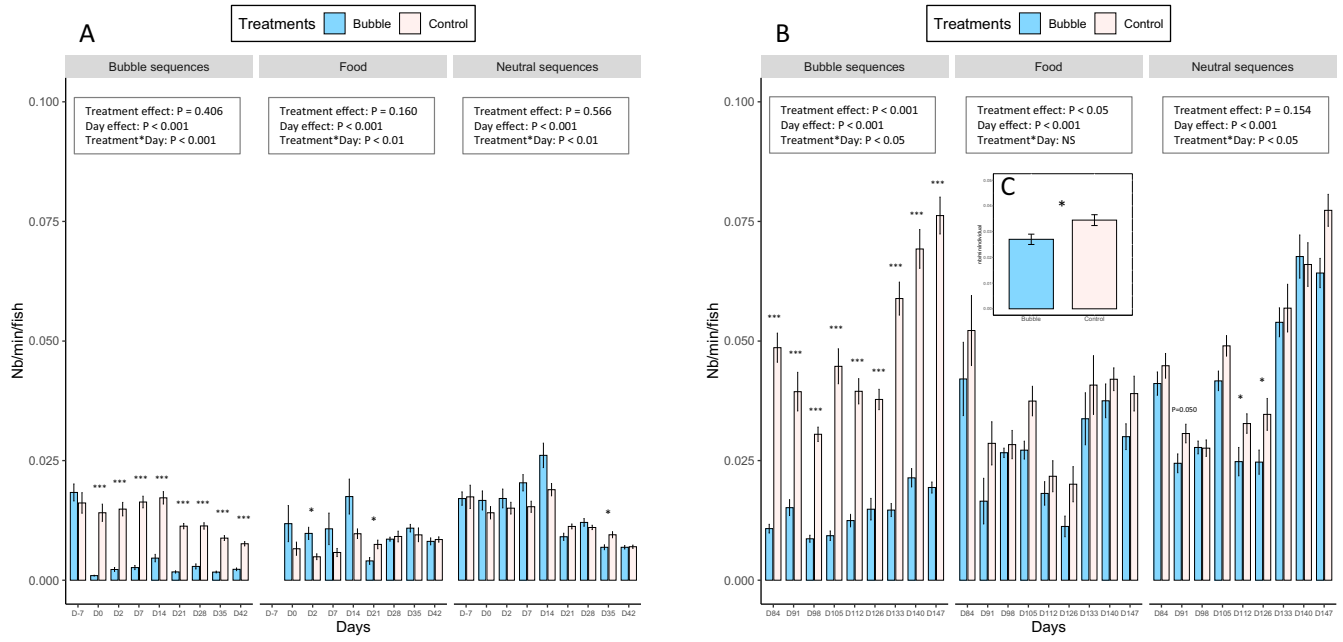

Fig. 7. Mean (± SEM) number of aggressive and abnormal behaviors per minute and per fish observed during Bubble, Food and Neutral sequences, over days in the Bubble and in the Control tanks of rainbow trout, (A) in the short-term experiment (7 weeks of bubble diffusions, n = 5), and (B) in the long-term experiment (21 weeks of bubble diffusions, n = 4). Asterisks represent significant differences between treatments within each day (post-hoc tests, * P < 0.05, *** P < 0.001). (C) Mean (± SEM) number of aggressive and abnormal behaviors per minute and per fish observed during Food sequences in the Bubble and in the Control tanks, during the long-term experiment. No significant treatment*day interaction was found and the treatment effect is represented (* P < 0.05).

Table 1
Statistical results of the fixed factors treatment (Bubble and Control), experimental period (short term period: at 112 dpf, ie. after 7 weeks of bubble diffusions in the Bubble treatment; long term period: at 211 dpf, ie. after 21 weeks of bubble diffusions in the Bubble treatment) and their interaction on the behavioral variables observed in rainbow trout subjected to a 10-min emotional reactivity test. The significant effects are highlighted in bold. When treatment*period interaction was significant, differences between treatments and between periods given by the post-hoc tests are represented with different letters (P < 0.01, *n* = 12). The df-value is equal to 1 for each statistical result.

Variables	Periods	Bubble (Mean ± SEM)	Control (Mean ± SEM)	Treatment		Period		Treatment*Period	
				F or χ2	P-value	*F* or χ2	P-value	*F* or χ2	P-value
Maximum velocity (cm/s)	Short term	**1018 ± 115** a	**914 ± 116** a	*F* = 1.65	0.205	*F* = 20.71	**< 0.001**	*F* = 16.37	**< 0.001**
	Long term	**250 ± 20** b	**666 ± 174** a						
Total distance moved (cm)	Short term	5802 ± 962	5649 ± 638	*F* = 1.64	0.207	*F* = 7.15	**0.010**	*F* = 1.58	0.215
	Long term	3164 ± 383	4624 ± 503						
Time (s) spent in thigmotaxis (/600 s)	Short term	315 ± 36.6	313 ± 40.6	*F* = 0.40	0.528	*F* = 9.81	**0.003**	*F* = 0.25	0.619
	Long term	421 ± 56	480 ± 37.5						
Angular velocity (°/s)	Short term	1037 ± 162	750 ± 121	*F* = 3.28	0.077	*F* = 20.82	**< 0.001**	*F* = 0.13	0.715
	Long term	1618 ± 114	1369 ± 143						
Number of rotations	Short term	**50.5 ± 6.4** a	**46.1 ± 4.9** a	χ2 = 2.84	0.092	χ2 = 208.92	**< 0.001**	χ2 = 26.57	**< 0.001**
	Long term	**18.4 ± 2.8** b	**28.7 ± 4.0** c						
Number of uneaten pellets (/20)	Short term	11.1 ± 2.2	13.9 ± 1.7	χ2 = 0.33	0.564	χ2 = 0.245	0.621	χ2 = 4.92	**0.027**
	Long term	12.8 ± 2.6	11.2 ± 2.7						

thigmotaxis and angular velocity were lower in the short-term experiment ($P < 0.01$). When treatment*period interactions were significant, post-hoc tests revealed differences in the long-term experiment, with Bubble fish displaying fewer rotations ($P < 0.001$) and lower maximum velocity (P < 0.01) than Control fish (Table 1).

3.2.2. Plasma cortisol responses

There were significant differences between basal and final cortisol concentrations (GLM, F = 45.53, df = 1, P < 0.001), but no significant interactions between the three fixed factors treatment, period and stressor (F = 2.36, df = 1, $P = 0.128$, Table 2). There was no significant effect of the treatment (F = 0.04, df = 1, $P = 0.844$) or the period (F = 0.28, df = 1, $P = 0.597$).

3.3. Motivation test

No significant treatment*period interaction was found for the latency to exit the start-box (Mean ± SEM: Bubble: 365 ± 228 s and 334

Table 2
Mean (± SEM) plasma cortisol concentrations (ng.mL^{-1}) measured before (Basal) and after (Final) the emotional reactivity test in different rainbow trout from the Bubble and Control treatments, for each experimental period (short term period: at 112 dpf, ie. after 7 weeks of bubble diffusions in the Bubble treatment; long term period: at 211 dpf, ie. after 21 weeks of bubble diffusions in the Bubble treatment).

a		Short-term experimental period		Long-term experimental period	
		Bubble	Control	Bubble	Control
Cortisol concentrations (ng.mL-1)	Basal	67.3 ± 16 (n = 12)	52.6 ± 12.9 (n = 10)	39.6 ± 14.8 (n = 11)	52.8 ± 13.6 (*n* = 11)
	Final	144 ± 34 (*n* = 10)	201 ± 49.6 (*n* = 9)	176 ± 19.2 (*n* = 11)	149 ± 25.3 (n = 11)

± 202 s; Control: 210 ± 82.3 s and 372 ± 207 s, for the short- and long-term experiment, respectively; GLIM: $F_{1,38} = 0.38$, $P = 0.543$), and there was no effect of the fixed factors treatment ($F_{1,40} = 0.07$, $P = 0.794$) and period ($F_{1,39} = 0.16$, $P = 0.692$). Similarly, no significant treatment*-period interaction was found for the latency to enter in the bubble zone (Bubble: 365 ± 229 s and 403 ± 206 s; Control: 245 ± 89.8 s and 509 ± 241 s, for the short- and long-term experiment, respectively; $F_{1,38} = 0.40$, $P = 0.533$), and there was no effect of the fixed factors treatment ($F_{1,40} = 0.00$, $P = 0.995$) and period ($F_{1,39} = 0.70$, $P = 0.408$).

For the time spent in each zone of the testing device, there were significant treatment*zone (MLM: $F_{2,76} = 3.22$, $P = 0.046$; Fig. 8) and treatment*period interactions ($F_{1,38} = 6.52$, $P = 0.015$). The zone*-period interaction was not significant ($F_{2,76} = 0.64$, $P = 0.532$). The interaction treatment*zone*period was just above significance ($F_{2,76} = 2.61$, $P = 0.080$). During the short-term experiment, post-hoc tests revealed that Control fish spent more time in the bubble zone than Bubble fish ($P < 0.01$), and they spent more time in the bubble zone than in the control zone ($P < 0.001$). Control fish spent also more time in the bubble zone in the short-term experiment than in the long-term experiment ($P < 0.01$). During the short-term experiment, Bubble fish and Control fish spent more time in the shelter than in the control zone ($P < 0.05$ and $P < 0.001$, respectively). In the long-term experiment, time spent in the shelter was higher than in the other zones for both treatments ($P < 0.01$; Fig. 8).

3.4. Spatial learning test

There was no significant treatment*day interaction for the latency for fish to reach the rewarded area (GLMM: $\chi^2 = 1.55$, df = 2, $P = 0.461$), and there was no effect of treatment ($\chi^2 = 0.94$, df = 1, $P = 0.331$) and day ($\chi^2 = 3.59$, df = 2, $P = 0.166$).

Considering the latency for fish to eat the reward, the treatment*day interaction tended to be significant ($\chi^2 = 5.64$, df = 2, $P = 0.059$; Fig. 9) and the factor day was highly significant ($\chi^2 = 63.24$, df = 2, $P < 0.001$). The factor treatment was not significant ($\chi^2 = 2.22$, df = 1, $P = 0.136$).

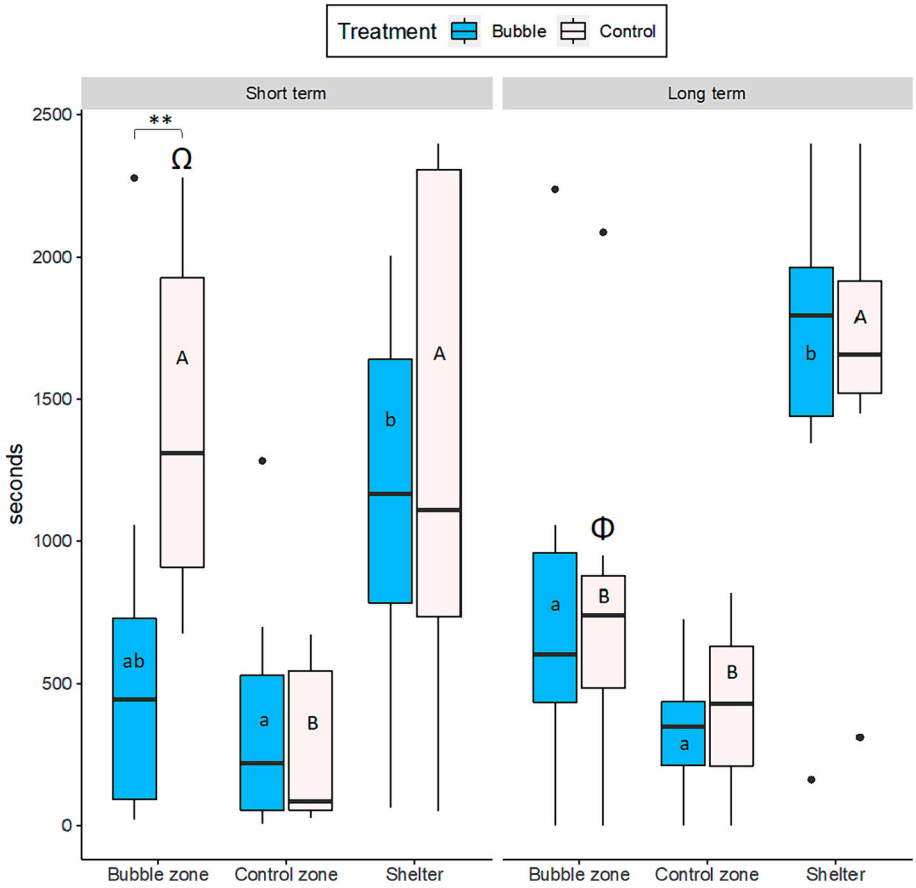

Fig. 8. Median (quartiles: 25 and 75%) time spent by Bubble and Control rainbow trout in each zone of the motivation test setup (bubble zone: diffuser active, control zone: diffuser inactive, and shelter) in the short- (7 weeks of bubble diffusions) and the long-term (21 weeks of bubble diffusions) experimental periods. Different lowercase letters represent significant differences between zones in the Bubble treatment (post-hoc tests, $P < 0.05$; short-term experiment: $n = 8$, long-term experiment: $n = 12$), and different capital letters represent differences between zones in the Control treatment ($P < 0.001$; short-term experiment: $n = 10$, long-term experiment: n = 12). In the short-term experimental period, significant differences between treatments within zones are represented with asterisks (** $P < 0.01$). Two different Greek letters mean significant differences between periods within treatments and within zones ($P < 0.01$).

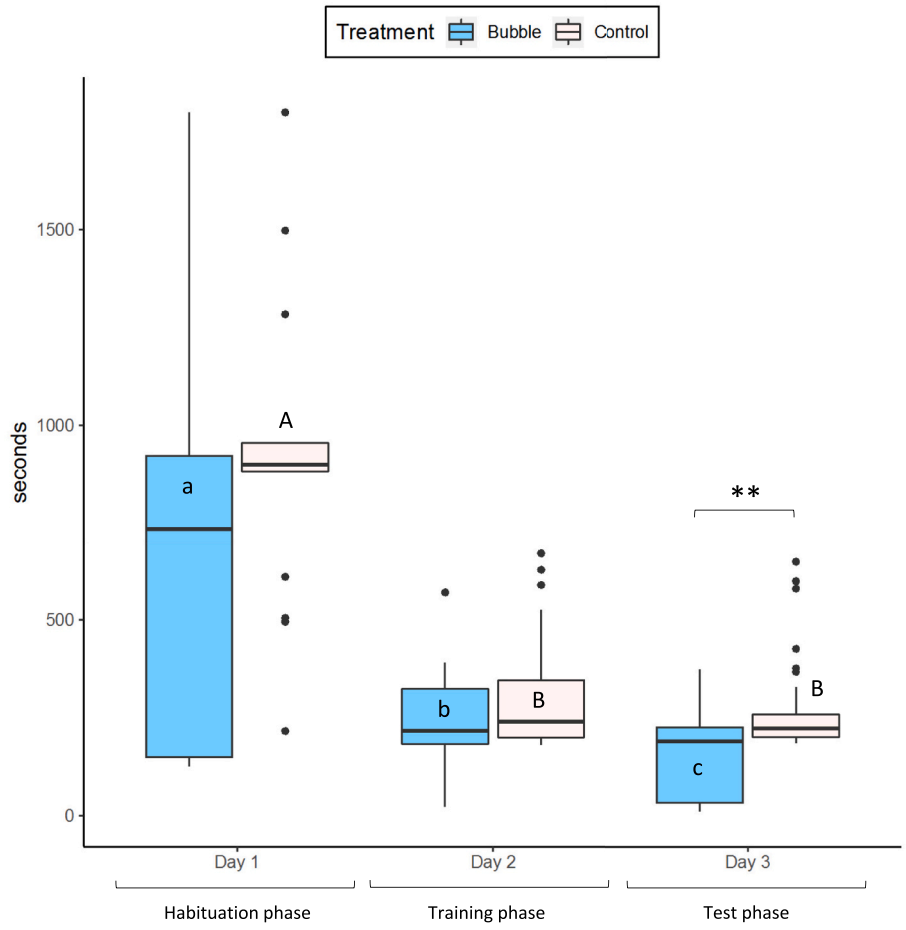

Fig. 9. Median (quartiles: 25 and 75%) latency to eat the reward for rainbow trout fingerlings from Bubble and Control conditions in the spatial learning test performed after 21 weeks of bubble diffusions (long-term experiment). Different lowercase letters represent significant differences between days in the Bubble treatment (post-hoc tests, $P < 0.01$, $n = 5$), and different capital letters represent differences between days in the Control treatment ($P < 0.001$, $n = 8$). Significant differences between treatments within days are represented with asterisks (** $P < 0.01$).

Post-hoc tests showed that the latency to eat the reward decreased from Day 1 (habituation phase) to the other days in both treatments ($P < 0.001$). In the treatment Bubble, this latency decreased also between Day 2 (training phase) and Day 3 (Test phase) ($P = 0.005$). On Day 3, the latency to eat the reward was lower for Bubble fish than for Control fish ($P = 0.009$; Fig. 9).

The go/no go response of the fish was not influenced by the treatment (GLMM: $\chi^2 = 0.02$, df = 1, $P = 0.899$) and no significant treatment*day was found ($\chi^2 = 1.25$, df = 2, $P = 0.535$). We found a significant effect of the day ($\chi^2 = 6.93$, df = 2, $P = 0.031$), with an increase of go responses between Day 1 and Day 2 (29 and 55 trials with a go response, respectively; post-hoc test on binary data: $P = 0.032$). On Day 3, we observed 53 trials with a go response. The total number of performed trials were 39, 60 and 60 on Days 1, 2 and 3, respectively.

On Day 3, we observed whether the first entrance was correct or not (first correct enter). There was no effect of the treatment for this binary variable ($\chi^2 = 1.42$, df = 1, $P = 0.233$).

3.5. Fin erosion

There was no effect of treatment on dorsal and caudal fin erosion scores observed at the end of each experimental period. In the short-term experiment, dorsal fin erosion scores 1, 2 and 3 were equally distributed between Bubble (14, 10 and 0 individuals with the respective scores) and Control fish (18, 6 and 0 individuals with the respective scores; $\chi^2 = 1.50$, df = 2, $P = 0.472$). Similarly, caudal fin erosion scores were equally distributed between Bubble (10, 14 and 0 individuals with scores 1, 2 and 3 respectively) and Control fish (10, 14 and 0 individuals with the respective scores; $\chi^2 = 0$, df = 2, $P = 1$).

In the long-term experiment, dorsal fin erosion scores 1, 2 and 3 were equally distributed between Bubble (11, 13 and 0 individuals with the respective scores) and Control fish (13, 10 and 1 individuals with the respective scores; $\chi^2 = 1.29$, df = 2, $P = 0.524$). Similarly, caudal fin erosion scores were equally distributed between Bubble (2, 22 and 0 individuals with scores 1, 2 and 3 respectively) and Control fish (1, 23 and 0 individuals with the respective scores; $\chi^2 = 0.36$, df = 2, $P = 0.837$).

3.6. Mortality rates and growth parameters

Total mortality rates calculated at the end of the 21-week experiment were low in both treatments: 1.76% in Bubble tanks (22/1248 fish) and 2.26% in Control tanks (33/1245 fish; $\chi^2 = 2.28$, df = 1, $P > 0.05$).

The treatment had no effect on the growth parameters measured in the tank as a whole during each experimental period (Table 3). In addition, there was no effect of treatments on fish weight, length or K-factor, when measured individually after the emotional reactivity tests performed at the end of each experimental period.

4. Discussion

This study investigated the effects of an intermittent curtain of bubbles as a new enrichment strategy on several welfare indicators measured for a short-term experimental period (~7 weeks) and a long-

Table 3
Mean (± SEM) growth parameters of rainbow trout from the Bubble and Control treatments measured in the whole tank during each experimental period (short term period: during 7 weeks; long term period: during 21 weeks).

Experiments	Variables	Bubble	Control	*P*-value
Short-term (n = 5)	Initial Weight (g)	0.37 ± 0.00	0.36 ± 0.00	0.405
	Final Weight (g)	4.36 ± 0.03	4.35 ± 0.02	0.741
	Specific Growth Rate (%. day^{-1})	5.24 ± 0.01	5.28 ± 0.05	0.461
	Coefficient of Body Weight Variation (%)	0.69	0.46	-
Long-term (n = 4)	Initial Weight (g)	0.37 ± 0.00	0.36 ± 0.00	0.405
	Final Weight (g)	56.95 ± 0.26	56.73 ± 1.04	0.849
	Specific Growth Rate (%. day^{-1})	3.47 ± 0.01	3.49 ± 0.02	0.510
	Coefficient of Body Weight Variation (%)	0.46	1.83	-

term period (~21 weeks) in captive rainbow trout fry and fingerlings. One of the most important results obtained in this study was the reduction in aggression and abnormal behaviors observed in the Bubble tanks, not only during bubble diffusions, but also, in the long-term experiment, during feeding or neutral periods, when bubbles were not diffused. During bubble diffusions, we observed the same behavioral pattern as that already described in Kleiber et al. (2022), with fish stopping their activity to aggregate in the bubble curtain. The majority of fish showed sustained locomotor activity, crossing the curtain and looping back to the water surface, while other individuals remained motionless, standing close to the curtain and exposing part of their body to the bubbles. More rarely, some fish chose to avoid the bubble-induced turbulence and aggregated motionless in a quiet area of the tank. In all cases, negative inter-individual interactions ceased, and individuals' burst accelerations, often observed in farmed rainbow trout and considered as indicators of poor welfare (Martins et al., 2012), also stopped. This phenomenon could have been observed only at the moment when bubbles started to be diffused since the novelty of the event may have been the reason of such interest. However, the low level of aggression and abnormal behaviors was still observed at the end of the bubble period after one hour of diffusion, and was still lower than the level observed in the control tanks. Air bubbles were sequentially diffused at four occasions each day to prevent any habituation phenomenon (Thompson and Spencer, 1966; Kuczaj et al., 2002), which would have decreased the fish's initial interest in bubbles. Therefore, the one-hour period we have chosen appears to be long enough to generate differences in the occurrence of abnormal behaviors between treatments even when bubbles are not diffused, and short enough to maintain fish interest from the beginning to the end of each bubble diffusion.

Increased aggression and burst accelerations, often accompanied with physical damage, may be a result of lack of occupation in the tanks. In many fish species, it has been shown that increasing the complexity of the environment (by the addition of structures, plants, shelters…) reduces these behaviors (zebrafish: (Basquill and Grant, 1998); redbreast tilapia: (Torrezani et al., 2013); black rockfish *Sebastes schlegelii*: (Zhang et al., 2020); rainbow trout: (Brunet et al., 2022)) and can also limit fin erosion (Atlantic salmon: (Näslund et al., 2013); rainbow trout: (Berejikian and Tezak, 2005; Brunet et al., 2022)). In our study, we found no difference in fin erosion score between the two treatments. This may be due to the low density we used in this experiment for both treatments (from 0.42 kg/m^3 at the start of the fry stage studied to 30.16 kg/m^3 at the end of the experiment). However, we observed the same reduction in aggressive and abnormal behaviors as those observed with more classical physical enrichment strategies. Thus, as supposed by Kleiber et al. (2022), the addition of a bubble curtain to the tanks can be considered as an environmental enrichment for rainbow trout, but it remains to be determined whether this is sensory, occupational and/or physical.

Air bubbles may provide visual, acoustic and especially tactile stimulations, which have been shown to decrease stress levels in the surgeonfish *Ctenochaetus striatus,* as evidenced by lower cortisol levels when physically touched by moving models of cleaner fish (Soares et al., 2011). Studies on Nile tilapia suggested that, although tactile stimulation did not lower blood cortisol levels in the short term, it could reduce aggressive behaviors (Gauy et al., 2021), just as we observed here, and may also reduce the overall stress associated with social interactions in the long term. Moreover, a study reported that koi carp *Cyprinus rubrofuscus* showed interest in tactile interactions with humans, suggesting that touching a new texture like human skin, could be a source of sensory enrichment (Fife-Cook and Franks, 2019, 2021). Here, the contact of bubbles that some individuals seemed to seek by exposing part of their body in the curtain, may represent a form of sensory enrichment for rainbow trout fry and fingerlings.

Providing fish with a regularly active curtain of bubbles during the day may also be likened to occupational enrichment, designed to introduce a variety of challenges into the rearing environment to avoid monotony and, consequently, boredom (Kleiber et al., 2022). The sustained swimming activity that the majority of fish displayed during the bubble diffusions, foregoing aggressive and abnormal behaviors, may have avoided the monotony of barren rearing tanks. An important point was that diffusions were interrupted after one hour, and only diffused four times a day to maintain fish interest and avoid habituation, but also to give those fish which swam actively through the curtain opportunities for resting. In this way, physical activity was neither forced, nor excessive, avoiding fish exhaustion which can result in the collapse of cardiac scope, as reported when rainbow trout are subjected to enforced swimming activity (Brijs et al., 2019).

Lastly, air bubbles might have increased the structural complexity of the environment, and be therefore considered as a physical enrichment, by creating a visual obstruction known to reduce negative social interactions (Dolinsek et al., 2007) as we also observed here, or by representing small objects to manipulate as has been observed in some fish species (Burghardt, 2005; Burghardt, 2015; Burghardt et al., 2015). These authors have suggested that repeated interactions with an object can be related to play behavior. The repeated loops we also observed in the tanks when bubble diffusions occurred resemble locomotor play (Fagen, 2017), but further studies using immersive cameras and a detailed ethogram containing the five minimal criteria which identify play of any type in any animal (Graham and Burghardt, 2010) would help to conclude for any play behavior in fish exposed to air bubbles.

The various behavioral observations obtained during bubble diffusions led us to consider this repeated event as a sensory, occupational and physical enrichment for rainbow trout, but sensory enrichment deserved to be confirmed. In the motivation test performed after either 7 or 21 weeks of bubble exposure in Bubble fish, we aimed to assess the "price fish was willing to pay" to reach a curtain of bubbles, giving an indication of how valuable this resource was to fish (Maia and Volpato, 2016). To access an area where bubbles were diffused or an adjacent area where the diffuser was inactive, the fish had to leave a dark shelter and cross an open, illuminated area, which is known to be a stressful situation for other salmonids (McCrimmon and Kwain, 1966; Migaud et al., 2007). The willingness to cross the aversive zone was taken as a measure of their psychological motivation (Maia et al., 2017; Gauy et al., 2021) to be in physical contact with bubbles, whether or not they had experienced bubbles before. We found no difference in the latency to exit the shelter and to reach the bubble area between treatments, neither at short term, nor at long term. It is possible that the light intensity was not as aversive as expected, since the brightness was similar to that provided in the rearing tanks. We had to avoid a situation that was too stressful for the fish to remain responsive, and we relied on the contrast between the dark shelter and the open area to create this "price

to pay". After leaving the shelter, Control fish, which were bubble-naive, spent more time in the bubble area than Bubble fish in the short-term experiment, and this difference disappeared in the long term.

The motivation test represented the first experience of tactile stimulation provided by bubbles for Control fish. Tactile stimulations were shown to reduce fish stress (Soares et al., 2011; Bolognesi et al., 2019), and fear (Schirmer et al., 2013). Thus, Control fish fry may have been more motivated to overcome the aversive zone in order to benefit from new tactile sensations and reduce the stress generated by the testing situation. However, when subjected to social stress, Nile tilapia do not choose to use tactile stimulation to relieve the effects of stress (Gauy et al., 2021). Another hypothesis could be that fish fry are less neophobic (i.e., fearful of novelty) than fingerlings, since all fish from the short-term experiment reached the bubble area, whereas two individuals (one per treatment) did not exit the shelter in the long-term experiment. This would explain the difference between treatments observed in the short-term but not in the long-term experiment. Then, once they entered the bubble area for the first time, their naivety about bubbles may have encouraged the Control fish fry to stay within the bubble curtain in order to benefit from the new tactile stimuli, whose positive valence seems confirmed through tactile modalities. At the same time, the lower motivation of Bubble fish fry to remain in the bubble zone in the short-term experiment may be explained by a phenomenon of habituation to bubbles, which would have reduced their sensitivity to this known stimulus (Thompson and Spencer, 1966). Outside their rearing tank, Bubble fish subjected to a motivation test may not be willing to pay such a high price for access to a stimulus that may have lost its initial sensory appeal, and preferred to remain in the shelter. The sensory enrichment that air bubbles also seem to represent for rainbow trout would make it possible to integrate into fish farming systems the notion of "positive welfare" proposed by Mellor (2016).

Another aim of the study was to observe fish emotional responses after 7 and 21 weeks of bubble exposure compared to Control fish. The rearing conditions of an animal can have an impact on its emotional responses (behavioral and physiological), either negatively or positively, depending on the long-lasting affective state generated by these rearing conditions (Doyle et al., 2011; Boissy and Erhard, 2014). The test consisting in isolating a fish in a novel tank (i.e., namely the emotional reactivity test), known to be highly stressful for fish (Overli et al., 2005), allows us to assess emotional responses of fear and anxiety expected to be influenced by rearing conditions. For example, a captive environment provided with physical enrichments reduces fish anxiety-related behaviors when individually introduced in a novel tank (zebrafish: (Collymore et al., 2015); rainbow trout: (Brunet et al., 2022)). In our study, this test revealed interesting behavioral differences between treatments, but also between developmental stages. Active behaviors (maximum velocity, total distance moved and number of rotations) were higher in the short-term experiment (16-week post-fertilization fry) than in the long term (30-week-old fingerlings), while anxiety-related behaviors (thigmotaxis and angular velocity (Kalueff et al., 2013)) were lower in the fish fry than in fingerlings. When paralleling these two behavioral patterns, we consider that active behaviors while exhibiting a low level of anxiety represent a greater motivation to explore the novel tank in the early life stages, a tendency that we also observed in the motivation test.

Considering differences between treatments, we found differences only in the long-term experiment, where Bubble fish showed fewer rotations and lower maximum velocity than Control fish, but with similar distance moved. Maximum velocity reflects startle responses which are frequently observed in zebrafish (Kalueff et al., 2013) and rainbow trout (Colson et al., 2015) when subjected to fear-eliciting situations. However, thigmotaxis and angular velocity did not differ between treatments. In the absence of difference in these two anxiety-related behaviors, we cannot claim with certainty that the lower maximum velocity observed in the Bubble fish (accompanied by fewer rotations), not simply being a concomitant expression of active behaviors since they covered the same overall distance as the Control fish, represented a lower fear response in the Bubble fish of the long-term experiment, even though this behavioral pattern may suggest so. This would, however, be consistent with the aforementioned studies on the effects of physical enrichment on the reduction of fear responses in fish (Collymore et al., 2015; Brunet et al., 2022). Giving captive fish the opportunity to experience positive affects by generating comfort, stimulation, and interest such as air bubbles may induce lasting positive affective states, essentially measured here by lower maximum velocity, which reflects a certain sense of safety despite the stressful situation. Other behavioral tests, the judgment bias test for example, recently used to assess the perceived valence of the environment and subsequent emotional states in fish (Espigares et al., 2022; Buenhombre et al., 2023; Epping et al., 2023), would deserve to be implemented in the future to confirm the positive long-lasting affective state of fish generated by repeated bubble diffusions.

As a measure of appetite inhibition induced by the novel tank test, we counted the uneaten pellets distributed into the test tank five minutes earlier. Appetite inhibition is known to be a prominent behavioral response to stressors and aversive experiences in fish (Overli et al., 1998). Individuals consumed pellets (even a small amount, i.e., less than half the ration in average) whatever their previous rearing condition, and whatever their age. This indicates that all fish were not as highly stressed as expected at the end of the emotional reactivity test, likely preventing any clear difference in fear behavioral responses between treatments, except the lower maximum velocity measured in Bubble fish. However, the final concentrations of plasma cortisol significantly increased compared to basal levels, showing that the stressor represented by the novel tank test had an influence on physiological responses, as frequently reported (Sadoul et al., 2016; Colson et al., 2019; Brunet et al., 2022). As for appetite inhibition, we did not find any effect of the treatment on post-stress plasma cortisol concentrations. Environmental enrichment rarely influences this physiological parameter in salmonids when experimental treatments share similar controlled rearing conditions (Näslund et al., 2013; Pounder et al., 2016; Brunet et al., 2022), questioning its relevance for enrichment studies. Fish recovery, such as the latency to return to basal cortisol levels or to recover initial opercular beats following stressors as reported in (Pounder et al., 2016), seem to be more suitable parameters for answering the question of the effect of enrichment on fish stress and welfare.

A physically enriched rearing environment, which in our case would be similar to air bubble diffusion, by providing animals with more variable sensory experiences, especially when provided from the earliest stages (Kotrschal and Taborsky, 2010), is known to improve the cognitive abilities of fish (review: Näslund and Johnsson, 2016), triggering brain plasticity (von Krogh et al., 2010; Salvanes et al., 2013; Cardona et al., 2022). Moreover, an accumulation of emotions modifies an animal's cognitive functions in a long-lasting manner (Boissy et al., 2007). In the spatial learning test performed in the long-term experiment, latencies to reach the reward area were similar across treatments. For 17.7-cm trout fingerlings in average, a test device longer than ours (which was 40 cm long) might have revealed more subtle differences in the latency to reach one of the two ends. In our case, the latency to consume the reward is undoubtedly a more instructive parameter of the fish's understanding of the zone-reward association. We found that the latency to consume the reward was decreased in Bubble fish compared with Control fish, indicating improved learning abilities in fingerlings that experienced bubbles for almost five months. Our results confirm those observed in our preliminary experiment with rainbow trout fry reared with short bubble diffusions periods, performing better in a similar spatial learning test after only 3 weeks of bubble exposure than fish reared under standard conditions (Kleiber et al. in preparation). These results coincide, for example, with those of other studies which showed that an enriched environment confers better spatial orientation and higher learning abilities in gilthead bream (*Sparus aurata*) (Arechavala-Lopez et al., 2020), goldfish (*Carassius auratus*) (Abreu et al., 2019), and rainbow trout (Ahlbeck Bergendahl et al., 2016). Enhanced

cognitive abilities can be extremely useful for farmed fish to limit the chronic stress caused by an unpredictable environment (Jones et al., 2012), for example by quickly habituating to repeated, fearful, but harmless stimuli (e.g., repeated cleaning related to aquaculture practices), or by being able to anticipate specific events (e.g., feed delivery). Therefore, our results confirm that offering captive rainbow trout the opportunity to live from the earliest stages in a variable and complex environment by diffusing bubbles at random times, allowing occasional visual obstructions, providing sensory experiences (particularly tactile), and encouraging moderate physical activity improves their cognitive abilities later in life, which may help them better cope with stressful events, as appeared to be the case in the emotional reactivity test.

Repeated bubble diffusion in the tanks had no effect on fish growth parameters. These results are in line with other studies carried out with structural enrichments (Roberts et al., 2011; Barnes and Internationals, 2018; Arechavala-Lopez et al., 2019; Anderson et al., 2022), but other works report positive effects of enriched rearing conditions on growth, attributed to a reduction in aggression (Kientz et al., 2018; Brunet et al., 2022). Here, we did observe reduced aggression but without any concomitant positive effect on growth. In enrichment studies, growth parameters are often influenced by the duration of exposure to enrichment (Anderson et al., 2022). Our enrichment exposure duration (50 days in the short- and 149 days in the long-term experiment) might be too short to make agonistic behaviors influencing growth parameters. (Brockmark et al., 2007) found no improvement in Atlantic salmon growth after 123 rearing days of structural enrichment exposure, but did observe improvement by 311 rearing days. Longer-term studies are needed to verify whether bubble diffusions during rainbow trout rearing have an impact on long-term growth.

5. Conclusions

We observed that random air bubble diffusions act as an environmental enrichment for rainbow trout, combining physical enrichment by making the environment more complex, occupational enrichment by limiting the monotony of the environment and encouraging physical activity, and sensory enrichment via the tactile stimulations provided by the bubbles. These additive factors had several positive effects on the welfare of fry and fingerlings, measured after 7 or 21 weeks of bubble exposure, which were even more pronounced when the fish were exposed to bubbles for the longest duration. In both the short- and long-term experiments, repeated exposure to the bubbles led to a reduction in aggressive and abnormal behavior, even - in the case of the long-term experiment - when the bubbles were not present, for example during food distribution. This is an important advantage to consider since food distribution can elicit negative interactions in fish farms if rations or food accessibility are suboptimal (Gianluca et al., 2010; Vindas et al., 2014). However, growth parameters were not influenced by the treatment even at long term. We found a greater motivation to stay in physical contact with the bubble curtain in the bubble-naive fry, highlighting the role played by tactile stimulations whose valence seems to be positive. In the long-term experiment, the emotional reactivity test elicited a lower maximum velocity in Bubble fish than in Control fish suggesting reduced fear-related behaviors in trout fingerlings exposed to bubble diffusion for 21 weeks. The affective state of rainbow trout seemed therefore to be positively influenced by repeated bubble diffusions from the earliest stages, as suggested by the sense of safety displayed by Bubble fish despite a stressful situation. We also demonstrated enhanced learning abilities in fish that experienced bubbles for 21 weeks, suggesting better abilities to cope with stressful situations in these fish. Overall, bubble diffusions as enrichment had a positive impact on the behavior of captive rainbow trout fry and fingerlings, but this enrichment strategy still needs to be investigated in the on-growing production phase. This would make it possible to integrate the notion of "positive welfare" into fish farming systems, while guaranteeing easy technical maintenance.

CRediT authorship contribution statement

Océane Amichaud: Writing – review & editing, Resources, Investigation, Formal analysis. **Thomas Lafond:** Writing – review & editing, Resources, Investigation, Formal analysis. **Georgina Lea Fazekas:** Investigation, Formal analysis. **Aude Kleiber:** Writing – review & editing, Methodology. **Thierry Kerneis:** Writing – review & editing, Resources, Methodology, Investigation. **Axel Batard:** Resources, Investigation. **Lionel Goardon:** Resources, Methodology. **Laurent Labbé:** Resources, Funding acquisition. **Sophie Lambert:** Investigation, Formal analysis. **Sylvain Milla:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Formal analysis. **Violaine Colson:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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.

Data availability

The data that support the findings of this study are available upon request.

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Appendix A. Supplementary data

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