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Feeding predictability as a cognitive enrichment protects brain function and physiological status in rainbow trout: a multidisciplinary approach to assess fish welfare

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ABSTRACT

Cognitive enrichment is a promising but understudied type of environmental enrichment that aims to stimulate the cognitive abilities of animals by providing them with more opportunities to interact with (namely, to predict events than can occur) and to control their environment. In a previous study, we highlighted that farmed rainbow trout can predict daily feedings after two weeks of conditioning, the highest conditioned response being elicited by the combination of both temporal and signalled predictability. In the present study, we tested the feeding predictability that elicited the highest conditioned response in rainbow trout (both temporal and signalled by bubbles, **BUBBLE + TIME** treatment) as a cognitive enrichment strategy to improve their welfare. We thus analysed the long-term effects of this feeding predictability condition as compared with an unpredictable feeding condition (**RANDOM** treatment) on the welfare of rainbow trout, including the markers in the modulation of brain function, through a multidisciplinary approach. To reveal the brain regulatory pathways and networks involved in the long-term effects of feeding predictability, we measured gene markers of cerebral activity and plasticity, neurotransmitter pathways and physiological status of fish (oxidative stress, inflammatory status, cell type and stress status). After almost three months under these predictability conditions of feeding, we found clear evidence of improved welfare in fish from BUBBLE + TIME treatment. Feeding predictability allowed for a food anticipatory activity and resulted in fewer aggressive behaviours, burst of accelerations, and jumps before mealtime. BUBBLE + TIME fish were also less active between meals, which is in line with the observed decreased expression of transcripts related to the dopaminergic system. BUBBLE + TIME fish tented to present fewer eroded dorsal fin and infections to the pathogen *Flavobacterium psychrophilum*. Decreased expression of most of the studied mRNA involved in oxidative stress and immune responses confirm these tendencies else suggesting a strong role of feeding predictability on fish health status and that RANDOM fish may have undergone chronic stress. Fish emotional reactivity while isolated in a novel-tank as measured by fear behaviour and plasma cortisol levels were similar between the two treatments, as well as fish weight and size. To conclude, signalled combined with temporal predictability of feeding appears to be a promising approach of cognitive enrichment to protect brain function via the physiological status of farmed rainbow trout in the long term.

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1751-7311/

Implications

The welfare of farmed animals has become increasingly important, with a growing interest in finding ways to improve their living conditions. However, solutions to improve farmed fish welfare are much more recent. One promising but understudied solution is cognitive enrichment. We report herein how feeding predictability as a cognitive enrichment strategy improves rainbow trout welfare (reduced aggressive behaviours, improved health status). Our multidisciplinary approach sheds new light on the neurobiological mechanisms involved in cognitive enrichment, emphasises the importance of considering this enrichment in animal husbandry and suggests that feeding predictability should be investigated for the welfare of other fish species.

Introduction

Cognitive (or occupational) enrichment emerges as a promising strategy to improve the welfare of farmed animals (Clark, 2017; Oesterwind et al., 2016; Zebunke et al., 2013). This form of enrichment aims to stimulate the cognitive abilities of the animals by providing them with more opportunities to interact with and control their environment, such as the possibility to predict events that may occur. However, cognitive enrichment, and its effects on welfare remain a recent and understudied topic in fish farming (review: Kleiber et al., 2023). Predictability (i.e., having information about the regularity of salient daily events), and in particular predictability of feeding, is currently one of the most studied cognitive enrichment strategies (review: Kleiber et al., 2023). Feeding predictability allows for food anticipatory activity (**FAA**), a possible indicator of positive emotions due to the activation of reward neural circuits (Bassett and Buchanan-Smith, 2007). FAA is also associated with the release of dopamine from the mesolimbic pathway known to facilitate learning and plays a role in the subjective perception of pleasure (Fife-Cook and Franks, 2019; O'Connell and Hofmann, 2011). In fish, few studies have investigated the effects on welfare of feeding anticipation, either using temporal predictability – feeding occurring at fixed time-intervals (Reebs and Lague, 2000; Sánchez et al., 2009) –, or using signalled predictability where a signal (i.e., the conditioned stimulus, **CS**) precedes feeding (i.e., the unconditioned stimulus, **US**) (Barretto et al., 2018; Bratland et al., 2010; Nilsson et al., 2008; Thomassen and Fjæra, 1991). Some of these studies showed positive effects of a fixed-time feeding schedule for one to two months, resulting in improved growth in gilthead seabream (*Sparus aurata:* Sánchez et al., 2009) and sea bass (*Dicentrarchus labrax* Sébastien et al., 2016), and in fewer severe fin damages in salmons (*Salmo salar:* Cañon Jones et al., 2012). However, others have shown that feeding predictability induced no effect on growth parameters and more agonistic behaviours in salmons (Cañon Jones et al., 2012), or resulted in less bold individuals in sea bass (Ferrari et al., 2016) and guppies (*Poecilia reticulata:* Chapman et al., 2010). By using a light as a signal for fish to anticipate feeding for a two-week period, the researchers found decreased cortisol levels and increased social interactions in gilthead seabream (Cerqueira et al., 2017). However, tilapia (*Oreochromis mossambicus*) that had the possibility to anticipate feeding using a visual cue (yellow-black stripped card) for three weeks tended to have higher plasma cortisol after an acute stress (Galhardo et al., 2011a). These discrepancies between studies may depend on the ecology of the species, the type or relevance of the stimulus used, the density, but also the duration of the experiment (Kleiber et al., 2023). For instance, sea bream showed an increase in mean weight after 30 days of temporally predictable feeding schedules, compared to unpredictable ones, but returned to similar weights after 60 days (Sánchez et al., 2009). Moreover, while most studies on feeding predictability focused on physiological, zootechnical or behavioural parameters separately, assessing multidisciplinary parameters within the same experiment would provide a more reliable indication of the welfare and health status of fish and may better reflect how they perceive their rearing conditions.

In a recent study, we aimed to determine the most appropriate feeding predictability for rainbow trout (*Oncorhynchus mykiss*), the main continental farmed fish species in Europe (FAO, 2022). We found that rainbow trout can predict daily feedings using time and/or bubble diffusion as predictors after two weeks of conditioning, as evidenced by their increased swimming activity before feeding (Kleiber et al., 2022). Temporal-only predictability resulted in more agonistic behaviours, jumps, and burst of accelerations before feeding, which are well-known behavioural indicators of poor welfare in rainbow trout (Martins et al., 2012). Signalled predictability using bubble diffusion led to fewer prefeeding agonistic behaviours, jumps, and burst of accelerations compared to temporal predictability (Kleiber et al., 2022). The combination of both temporal and signalled predictability elicited the highest FAA response, suggesting that this combination was more efficient and more likely to promote positive welfare. However, this study had a relatively short duration of exposure to the conditioning procedure (12 days) and requires a complete multidisciplinary approach to assess the effects on fish welfare and health. For instance, although previous studies have described the effects of environmental predictability on neural proliferation rates in fish brain (Cerqueira et al., 2020; 2017), the neural regulatory mechanisms underlying effects of cognitive enrichment on brain function and plasticity, neurotransmitters pathways and physiological status remain, to our knowledge, uninvestigated. Exploring the neural mechanisms underlying the behavioural effects of feeding predictability and potential protective effects on brain regulatory pathways and networks, which we previously reported on rainbow trout (Kleiber et al., 2022) may provide additional insight into the benefits of this practice for farmed fish.

In the present study, we aimed to analyse the long-term effects of an efficient feeding predictability condition on the welfare and health status of rainbow trout through a multidisciplinary approach including the modulation of brain function. For this purpose, we pursued our previous experiment (Kleiber et al., 2022) for two more months with the signalled + temporal feeding predictability condition, using the unpredictable feeding condition as a control. Fish activity and agonistic behaviours, burst of accelerations, and jumps were recorded for each condition to assess the impact of feeding predictability on *in situ* welfare indicators. Other parameters such as emotional responses to a novel-tank test, stress-induced anorexia and cortisol release, fin erosion, and growth were also considered as fish welfare indicators, widely used to evaluate the impact of a husbandry practice (Galhardo et al., 2011a; 2011b; Martins et al., 2012). The bactericidal lysozyme and microbe-clearing complement components (ACH50), known as innate immune markers (Brunet et al., 2022; Seibel et al., 2021; Tort, 2011), as well as bacteria colonies presence on the skin surface were evaluated as health indicators. Finally, in order to reveal the brain regulatory pathways and networks involved in the long-term effects of feeding predictability, we used a high-throughput microfluidics quantitative polymerase chain reaction (**qPCR**) system to measure the expression of selected genes in specific areas of the trout brain (forebrain, hypothalamus and hindbrain). These genes included markers of cerebral activity, neural plasticity (neurotrophic markers), neurogenesis, synaptogenesis, neurotransmitters pathways and physiological status of fish (oxidative stress, inflammatory status, cell type and stress status).

Material and methods

Animals and maintenance conditions

The animals were female triploid rainbow trout (*Oncorhynchus mykiss*), fertilised and reared at the INRAE-PEIMA fish farming facilities (Permit number D29-447-02, Sizun, France; https://www6.rennes.inrae.fr/peima/) from a delayed autumnal strain. In this study, we pursued for two more months a previous study that we conducted on four feeding predictability conditions (Kleiber et al., 2022) with the same fish. We kept only the condition for which they exhibited the strongest conditioned response (bubble-signalled + temporal predictability condition of feeding: **BUBBLE + TIME** treatment), as well as the condition with unpredictable feedings as a control (**RANDOM** treatment), to investigate longer-term effects of feeding predictability on fish welfare. After 19 days of conditioning (see previous study: Kleiber et al., 2022), fish from these two selected conditions were randomly netted, weighed (14 g mean weight) under anaesthesia (50 mg/L tricaine and 50 mg/L sodium bicarbonate) and equally distributed into uncovered rearing tanks (six tanks/treatment, 72 fish/tank) supplied with spring water (Fig. 1). Before beginning data collection, at 145 days postfertilisation (dpf), the fish underwent a 2-week acclimatisation period to their new environment under their respective rearing conditions. Fish thus had 34 days of rearing under their respective condition of feeding predictability before the experiment started. As for our previous experiment (Kleiber et al., 2022), fish were fed five times a day, in equal amounts, with extruded and commercial flowing pellets (1.9 mm pellets, BioMar, France) in accordance with fish growth rate. The feed was delivered by ARVOTEC feeders at precise schedules using the computer-controlled Imetronic software (version 2008). We also used the same top-view video recording equipment for continuous monitoring of fish behaviour (Full HD: 1 920 × 1 080 px, 105°, VIZEO – Adrien Alarme) and air diffuser (4 × 6 mm) for bubble diffusion. Two air pumps supplied six air diffusers (six tanks/condition) at the same time through a six-valve low-pressure air distributor. We used the same tanks as in our previous experiment (Kleiber et al., 2022), with uniform grey opaque walls, size (70 × 70 cm), volume of water (196 L) and water flow rate (one renewal per hour). Both sides of each tank were equipped with a yellow light bulb (Leds 4000 K, 9.6 W, ELVADIS) controlled by a programmer panel to provide a 12L:12D photoperiod, a 35% light intensity, and a progressive lighting over 15 minutes. Water temperature remained constant at 12 ± 0.4 °C, and fish density was maintained below 25 kg/m^3 throughout the experiment. Ammonia, nitrite and nitrate concentrations were checked at the beginning and at the end of the experiment and were always lower than 0.33, 0.00 and 41.60 mg/L, respectively.

Conditions of fish raised under feeding predictability or feeding unpredictability

Fish were raised under two feeding predictability conditions:

(i) Treatment BUBBLE + TIME, where feeding occurred at fixed time schedules (10 am, 12 am, 2 pm, 4 pm and 6 pm each day) and announced by a signal (Conditioned stimulus, CS) of 15 seconds of bubble diffusion which was systematically followed 5 seconds later by feed delivery (Unconditioned stimulus, US).
(ii) Treatment RANDOM: neither bubbles nor time predicted feed delivery. For this treatment, daily feedings were randomly delivered each day between 0930 am and 0720 pm according to a manually randomised schedule with a minimum of one hour and a maximum of three hours between two feedings to avoid any starvation or digestion issues. Bubbles were systematically diffused at intervals of at least 15 minutes from feedings and on the same schedule as BUBBLE + TIME for practical reasons.

Prior to this study, fish were conditioned to these two predictability conditions for a total of 34 days, before starting the analyses for a longer-term period of 54 more days (Fig. 1). In each treatment, fish received five bubble diffusions (of 15 seconds each) and five feedings per day (total of 440 bubble and feeding sequences for a total of 88 days under both predictability conditions) (Fig. 1).

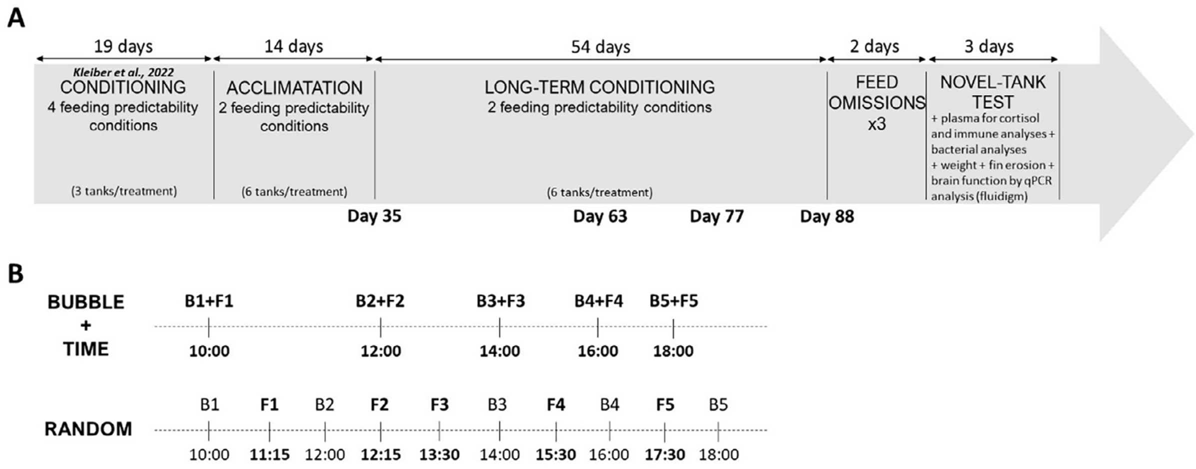

Fig. 1. (A) General schedule of the experiment included a preliminary conditioning period of 19 days with four predictability conditions of feeding (BUBBLE, TIME, BUBBLE + TIME, RANDOM) whose results are presented in our previous study (Kleiber et al., 2022); a 14-day acclimatisation period for fish from the two predictability conditions of feeding (BUBBLE + TIME, RANDOM) that we pursued in this study in their new environment (the initial three tanks for the BUBBLE + TIME treatment and three for the RANDOM treatment were equally distributed into six tanks each to respect fish density); a long-term conditioning of 54 days with days represented in bold (35, 63, 77 and 88) being the selected days to analyse the progression of fish behaviours over the experiment; then, three feed omissions trials were performed in both treatments followed by a novel tank-test after which physical and physiological parameters, and specific brain genes expression were scored. (B) Example of a day during conditioning for each treatment, with BUBBLE + TIME based on temporal + signalled predictability of feeding (based on time and bubble diffusion), and RANDOM corresponding to unpredictable feedings (random feeding delivery). B1 to B5 represent the five daily bubble diffusions, and F1 to F5 the five daily feedings.

Group behaviours during conditioning

Previous studies have shown that fish activity is a relevant behavioural indicator of food anticipation (Reebs and Lague, 2000). FAA in the rearing tank was automatically analysed using EthovisionXT® software, as described in our previous study (Kleiber et al., 2022). Briefly, activity was given as a proportion of pixels that changed intensity between two successive images (25 images per second). As a result, activity data represent global activity of the group of individuals and are given as a percent of activity (%). Behavioural recordings were made at two different sequences of feeding or bubble diffusion (1st, and 4th sequences of the day) on days 35, 63, 77, and 88 of conditioning to examine progression during the experiment (Fig. 1).

For each treatment, we analysed fish activity during the 15-second sequence of bubble diffusion and the 5 seconds that followed, before feed delivery. For FAA, we also analysed the 5 minutes preceding bubble diffusion for the BUBBLE + TIME treatment or feeding for the RANDOM treatment. Group fish activity was also analysed during neutral periods (i.e., 5-minute period without feed delivery or bubble diffusion). All analyses were performed in the whole tank area.

Previous studies have shown that agonistic behaviours are observed when fish anticipate feeding, using time as the main predictor (Heydarnejad and Purser, 2009; Kleiber et al., 2022). Therefore, we counted the number of agonistic behaviours (biting, chasing), as well as burst of accelerations, and jumps throughout the tank during the 5-minute period preceding feedings, and during neutral periods (see Kleiber et al., 2022 for the ethogram).

Feed omission tests

Three feed omission trials were conducted after 88 days of conditioning to assess whether fish could associate bubbles with feed delivery by measuring fish activity, aggression levels, burst of accelerations, and jumps: one on day 88 and two on day 89 (Fig. 1). During these trials, feed was not delivered at the expected feeding moment. The omission trials were interspaced by one conditioned trial to maintain the associative value of the CS and the US, similarly to what has been done in previous studies (Vindas et al., 2012, 2014a; 2014b).

During each omission trial, fish group activity was analysed during a period of 28 seconds that included the 15 seconds of bubble diffusion for both treatments, the 5 seconds before expected feed for the treatment BUBBLE + TIME, and the 8 seconds after corresponding to the moment when feed was supposed to be delivered for the treatment BUBBLE + TIME. Activity was also recorded during the 5 minutes that followed feed omissions.

The total number of agonistic behaviours, burst of accelerations, and jumps per minute were also scored during the 5-minute period that followed each omission (see Kleiber et al., 2022 for the ethogram).

Individual fish emotional reactivity

The emotional reactivity of an individual animal lies in the degree to which it responds to emotion-inducing stimuli like novelty and suddenness (Boissy et al., 2007). Therefore, it appears essential to evaluate how the animal perceives its environment, whether it finds it threatening or not, its capacity to adapt to environmental changes, and as a result, its overall welfare state. The emotional reactivity of fish was assessed after 88 days of conditioning on days 92, 93, and 94 (Fig. 1), using a novel-tank test based on the same procedure described in Kleiber et al. (2022). A total of ninety-six fish (48 fish/treatment, eight fish/tank) were randomly selected and individually placed into a novel tank (68 × 33 × 32 cm). Their behavioural responses were recorded on video over a period of 40 minutes. Analysis focused on the first 20 minutes, which were divided into 5-minute intervals using the Ethovision ® XT software. At the 35-minute mark, each individual automatically received 50 feed pellets (approximately 500 mg), and the remaining pellets were counted after 5 minutes to determine if there was any inhibition in feed intake after a stressful situation, known as stress-induced anorexia. The recovery of feed consumption following an acute stressor is a commonly used stress indicator in fish (Braithwaite and Salvanes, 2005). The same set of behavioural parameters used in Kleiber et al. (2022) were assessed by automated tracking for each individual, including the total distance moved (in cm), maximum swimming velocity (in cm/s), angular velocity (in °/s) (i.e., erratic swim), and the time spent (in %) in the peripheral area (i.e., thigmotaxis).

Physiological and zootechnical parameters

Plasma cortisol responses

Blood samples were taken from anaesthetised fish to assess plasma cortisol at basal levels – fish that were not subjected to the novel-tank test but were netted directly from their rearing tank and euthanised (200 mg/L tricaine and 200 mg/L sodium bicarbonate) – and after an acute stressor, i.e., the novel-tank test. Fish were left in social isolation in their test-tank for up to 40 minutes before being netted and euthanised. Forty minutes is the average delay required to observe a peak in 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). For both basal and acute stress levels, the blood of 48 fish was sampled (24 fish/treatment and four fish/tank) from below the lateral line into heparinised syringes and samples were stored on ice. After sampling, blood cells and plasma were separated by centrifugation (15 min at 3 000 G at 4 °C). Plasma was collected and frozen at −20 °C until basal cortisol analysis. Plasma cortisol assay was carried out by ELISA following manufacturer instructions (BioSource, Nivelles, Belgium), and following the same procedure as the one detailed in Brunet et al. (2022).

Immune responses

Lysozyme and alternative haemolytic complement (ACH50) activities were analysed from the same plasma samples collected for basal cortisol level but on 16 fish/treatment only (four fish/tank, four tanks sampled/treatment), to assess fish immunocompetence according to the condition of feeding predictability (Fig. 1). Indeed, the bactericidal enzyme lysozyme and microbe-clearing complement components are frequently used as innate immune markers to assess fish welfare (Seibel et al., 2021; Tort, 2011). Analyses were performed using the same protocols than reported in Brunet et al. (2022). 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). The ACH50 value was defined as the reciprocal of the plasma dilution inducing 50% of the rabbit red blood cells (RRBCs, Clinisciences) haemolysis.

Bacterial analysis

Aeromonas spp. and *Flavobacterium psychrophilum* were researched in external with mucus sampling and in internal organ with blood renal sampling on the same 16 fish/treatment (four fish/tank, four tanks sampled/treatment) studied for immune analyses. *Flavobacterium psychrophilum* (causing rainbow trout fry syndrome or cold-water disease) and *Aeromonas salmonicida* (causing furunculosis) are ones of the most widely distributed and devastating infectious pathogens of wild and farmed freshwater salmonids (Cipriano and Bullock, 2001; Duchaud et al., 2007). Specific media were inoculated, Glutamate Starch Phenol red agar (**GSP**) for

Aeromonas spp. and FLavobacterium Psychrophilum (**FLP**) agar for *Flavobacterium psychrophilum* and were incubated at 22 °C for 48 hours and at 17 °C for 48–72 hours, respectively. On each plate, presumptive colonies were yellow, *Aeromonas* spp. on GSP and *F. psychrophilum* on FLP. To confirm their identity, they were tested for their Gram-negative reaction, catalase test and oxidase test (Liofilchem, Italy). After growth on FLP and GSP media, the plates were also observed for the presence of any other colonies, such as yeast, gram and bacillus or cocci, in addition to the specific bacteria (*F. psychrophilum* and *Aeromonas* spp.).

Growth

Fish BW (**W**) and length (**L**) were measured at the end of the two-month conditioning period, on the same fish sampled for cortisol and immune analyses and/or that passed the novel-tank test on days 92, 93, and 94, i.e., on 72 fish/treatment in total (12 fish/-tank) (Fig. 1). For each fish, the condition-factor was calculated as followed: K-factor = 100 (W/L^3). Measurements were made under anaesthesia (50 mg/L tricaine and 50 mg/L sodium bicarbonate).

Fin erosion

We analysed fin erosion (dorsal and caudal) from photographs taken when fish were weighed (72 fish/treatment, 12 fish/tank) (Fig. 1). 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 or inferior to 30% of the fin surface damaged; score 2: between 30 and 70% of the fin surface damaged; score 3: more than 70% of the fin surface damaged.

Brain marker measurement by real-time quantitative PCR

From the same fish sampled for basal cortisol, total RNA for eight fish per treatment (two fish/tank, four tanks sampled/treatment) (Fig. 1) was extracted from the brain divided into three areas (forebrain, hindbrain, and hypothalamus) using the TRIzol® reagent method (Invitrogen, Carlsbad, CA, USA) with Precellys® (Bertin technologies, Montigny le Bretonneux, France) following Trizol manufacturer's instructions as previously described (Roy et al., 2021). The quantities of extracted RNA were analysed using a spectrophotometer (ND-1000, Nanodrop, Thermo, Vantaa, Finland), and samples with an OD 260 nm/280 nm ratio > 1.8 were used for analysis. 2 μg of RNA was reversed-transcribed into cDNA using the Super-Script III RNAse H-Reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) along with random primers (Promega, Chartonniéres-les-bains, France). High-throughput real-time quantitative PCR (RT-qPCR) analysis was conducted using the Biomark microfluidic system from Fluidigm® (Fluidigm, San Francisco, USA). Each gene combination was accomplished using 96.96 Dynamic Array™ IFCs (BMK-M-96.96, Fluidigm) as previously described in Cardona et al. (2022). The obtained results were meticulously analysed employing Fluidigm real-time PCR analysis software v.4.1.3. Data were derived from standard curves and then normalised to a validated housekeeping gene.

Notably, among 18 s and actin, the elongation factor 1α gene (eef1α) demonstrated the most consistent expression across diverse brain areas and tissues, making it the preferred reference gene. The relative expression of the target genes was determined utilising the $\Delta\Delta$CT method (Pfaffl, 2001; Roy et al., 2022). The fold changes relative to the BUBBLE + TIME treatment group are presented as mean ± SEM values for each group. Gene sequences for RT were identified through in silico analysis using Genomicus software program, version 100.01 (https://www.genomicus.biologie.ens.fr) and Ensembl (https://www.ensembl.org, Ensembl Release 102; November 2020, RT genome available). These sequences were then compared against mammal or fish genomes using the BLAST tool in both Ensembl and NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their identification, as detailed in Table 1. The primer sequences employed to amplify these genes, along with their accession numbers, are provided in the same table (Table 1). In cases where gene targets had not been previously validated, the primers were tested on a cDNA pool, and the amplified products were systematically sequenced. Specific primer pairs for each gene were designed using Primer3 software (Rozen and Skaletsky, 2000). To ensure optimal annealing temperature for each gene, PCR experiments were carried out with these specific primer pairs, and the resulting PCR products were analysed through electrophoresis on a 1.5% agarose gel.

Statistical analysis

All tests and graphs were performed using the 1.4.1717 version of RStudio and were plotted using the packages ggplot2, ggthemes, and effects.

Group activity analysis

Group activity was analysed by performing linear mixed-effects models (LMMs), with the lmerTest package (n = 6 tanks per treatment). Group activity (given in % by Ethovision) was analysed with the treatment (BUBBLE + TIME, RANDOM) and the period of analysis (Neutral periods, Before feedings, During bubbles) and their interaction, as fixed factors. "Neutral periods" correspond to 5-minute periods without feed delivery or bubble diffusion, "Before feedings" to the 5 minutes that preceded bubble diffusion for the BUBBLE + TIME treatment or feed delivery for the RANDOM treatment, and "During bubbles" to the 15 seconds of bubble diffusion + the 5 seconds that followed just before feed delivery. Rearing tanks were considered as random factors. The following LMM was used: lmer(Mean.activity ~ Treatment*Period + (1|Rear ing tank)). During feed omissions tests, group activity was analysed with treatment, period of analysis (During omissions, After omissions), omission number (1, 2 or 3), and their interaction, as fixed factors. "During omissions" corresponds to the 15 seconds of bubble diffusion + the 5 seconds that followed + the 8 seconds when feed was supposed to be delivered for the treatment BUBBLE + TIME, and to the 15 seconds of bubble diffusion + the 13-second period after the end of bubbles for the treatment RANDOM. "After omissions" corresponds to the 5-minute periods after the feed omissions. The following LMM was used: lmer(Mean.acti vity ~ Treatment*Period*Omission.number + (1|Rearing tank)). Similar to the previous model, rearing tanks were added as random factors.

Day (day 35, day 63, day 77, or day 88) was not included as a fixed factor in the models because the data provided by Ethovision did not account for fish growth between days 35, 63, 77, and 88 of the experiment, resulting in variable pixel changes between days of analysis.

All data were checked for normality by observing the dispersion of the model's residuals and variances homogeneity. If data did not meet the assumptions for parametric statistics, data transformation was applied else by inverse, log- or log10-transformation. For each model, the effects of fixed factors on each variable were evaluated using the analysis of deviance table with ANOVA of type III. When significant, only the effect of the interaction of fixed factors is presented in the results section.

Analysis of agonistic behaviours, burst of accelerations, and jumps

The analysis encompassed the occurrences of agonistic behaviours, burst of accelerations, and jumps during the two-month conditioning period. To do this, we used generalised linear mixed-effect models (GLMMs) with a Poisson family and logarithmic function. These models took into account group behaviours,

Table 1
Nucleotide sequences of the PCR primers used to evaluate mRNA abundance of transcripts by RT (Reverse Transcriptase)-PCR in the brain of rainbow trout.

Transcript	Forward Primer	Reverse Primer	Accession Number
Reference			
eef1a1	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	AF498320
Anti-oxidant			
sod-1	CGTAGTCGTGGCTCAATGGT	CCAAACCAACCCTAGCCACT	NM_001124329.1
sod-2	CTGGGCTTCGACAAGGAGAG	GTAGGCATGCTCCCACACAT	XM_021612540
sod-3	GGCGGCCATTACAACCCTTA	CCGAACAGAGTGGCCTTTGA	XM_021619043.1
cat	GCCTCCAAGATGTGATGCCT	TCTATCGCATTCACGGACGG	XM_021564294
gsr	TCATTGCCACAGGAGGGTCC	CTATGACGCTGCGCTTAGGA	HF969248.1
Cytokines/chemokines			
IL-1β	ACATGGTGCGTTTCCTTTTC	CCGTGCTGATGAACCAG	NM_001124347
IL-4	GACAATCTTGGCCTCCGTGA	CCACCTGGTCTTGGCTCTTC	NM_001246341
IL-6	CCCTACTCCCCTCTGTCACA	AACACGCTTCCTCTCACTGG	NM_001124657.1
IL-8	ATTGAGACGGAGAGCAGACG	AATCTCCTGACCGCTCTTGC	NM_001124362.1
IL-10	CGCCTTCTCCACCATCAGAG	CTGTCCATAGCGTGACACCC	NM_001245099
IL-11	ACCACCCTGCTCTCCCTAAA	GGAGAGAGACGCTGAGCATC	AJ535687.1
tnf-α	AGTGGAGAAAGGATGACGGCCAGG	AACGAAGCCTGGCTGTA	NM_001124357
nfkpb1	CCAACCTGAAGATTGTGCGG	TAGAAGCGCACCTGGATGTC	XM_021561045.1
ikk-α	GGAGAATGAGGAGAAGGCGG	CTCTGCAGCTCCACGATCTC	FR915835.1
ikk-β	CCAGACGGATCCTAAGCACG	TGCTGTAACCTCTGCCAGTC	FR911637.1
caspase 3	GTGGCTCTGATCTGGACTGT	AGTCTGCCTCCACTGGAATC	NM_001246335
Cell markers			
gfap	CAGAGCTTCTCCAACCTGCA	CCCCGTCTCTAGTCTCCACA	XM_021558456
tmem119	GTCTCTGACGGGCCTGATTT	TTCGGTTTGGGCTCCTTACC	XM_021621681
rbfox3	ACGCCACTGTCAACTGTATGA	TCACTCTGCATGCTGTCACA	XM_021556260
pdgfra-1	TCAGTGGAGAGATCAGAGCCA	CTGACAAACTCCACCACTCCA	Scaffold_1131 GSONMG00048607001
pdgfra-2	ATGCTGGAGATGAGTGACGC	CAGCATGTTGTCCATCTCACT	Scaffold_669 GSONMG00023327001
Stress (cortisol)			
crf	GCAACAGTCTCTTCCCCTCC	CTTGGGGTGCATGACTTTCG	NM_001124286.1
crhr	ACAACCATGCCCTGAGAGTG	AGGTCAAACAGCTGTGGTCT	XM_021613637
mc2r	CGCAGAGCTACGAAACACCT	TGCAGACAAAGCCCTTCAGT	NM_001124680.1
mrap-1	CCCGTTCAGTCAGTCTACCC	CAGAGGGGTTTGGGGATCAA	FR837908.1
mrap-2	GAGGGTCTCAAGGCTCATCG	GTCTTGGTCAGCAGGGTGAG	XM_021611508
Neuronal activity			
delta-fosb	TGCAGCCAACTCTCATCTCG	GAGGAATAACTGGGCCCTGG	XM_021587380.1
npas4a	GAGATGGTGTTGCAGGTGGA	TAGTTGTGGCAGCTGATGGG	XM_021618560.2
npas4b	AGCGAGAGAGGGAGGACATT	GTGGGTGGGGTTATTCTGGG	XM_036967710.1
syngr1	CTTCCCACAGATCAGCTCCG	CAGGAAACAGAACCCCACGA	XP_036805055.1
pcna	GTGGACAAGGAGGAGGAAGC	ACTGTCTTGGAGAGGGGTGT	XM_036936092.1
egr1b	CCCAACATGTCTCTGCCCAT	GCTCTGACACTGGAAAGGCT	XM_021617535.1
Neurotrophic factor			
c-fos	AACAGACTCTCCATGGCAGT	TGCTGATGTGATGACGGTGG	XM_021611391.1
bdnf	GCTGCCGTGGAATAGACAAG	TCCTTATAAACCGCCAGCCA	GU108576.1
ncam	GCTAACGTCACCAAAGCCAA	GGCAGCAGTACAGTTGTAGC	XM_021582629.1
neurabin-1	AGGAGAGAGAGGAGACAGCA	TTCCTCCGCCTGTTTCTCAT	XM_021572886.1
neurod1	AACCATGAGTAAGGACGGCG	TTCTCCCGACCCTCCTTCTT	XM_021608264.2
ntkr2b	ACTATCCTGGAGCTGCTGGA	CTGTTCGTGGAGCTCTTGGT	XM_021602993.2
stxbp5	GTCCTCCAAGTCACACCCTG	TCAGATCCCACAACACCACG	XP_021441002.2
stx12	ACAACTTCCAGGCCGTACAG	CAGAGGCACCATCTTCAGCA	XM_036970689.1
stx1b	TGACCGAGTACAACACCACG	GCATATCCTCCAGCTCCTCG	NP_001117929.1
Plasticity/synaptogenesis factor			
mapk1	CCTGCTCATCAACACCACCT	AGCCACGTACTCTGTCAGGA	XM_036937960.1
mapk4	GGCAGGCTCTAAACCCTTGT	AGAGAGGAGTGGGAGTGGTC	XM_021620877.2
mapk-erk	AGTCCATCTCCACGACCATC	GAAAGCCTCCAGACGTTTCC	NM_001124424.1
mtor	CCAAGGACTTCGCTCACAAG	GCTCCTTGATGTCTTGCTGG	XM_021615845.1
creb	CAGATTGACAGCTGCCCCTA	TGGTGTTCTGGTGTAGTGCT	MG310160.1
egr1a	CGATCACCTGACCACACACA	TCAGGTGGATCTTGGTGTGC	ENSOMYT00000041426.1
egr1c	CTCGTACCCCTCTCCCTCAA	AGATGGAGGCTACGGAGGAG	ENSOMYT00000036541.1
camk1a	AGAGGACGGGAATGGATGGA	CTAGCACCACCTCCGAGAAC	XM_036950122.1
camk1b	GTTGGCCCAGAAACCCTACA	AGAGCTTGGCGTCATTCTCA	NM_001124638.1
camk2b	GCCGCTGTGTCAAACTTTGT	GCTTCCCTCTCCAGCTTCTG	XM_036976944.1
camta1b	CTACTGCCCTGCCCATGAAA	GGGCAAGTCTCGAGCTTTA	XM_036947092.1
Dopamine markers			
th	ACGCTCTCTCAAGGTGTTCG	AAAGTACTCCAGCCCCTCCA	XM_021564247.1
drd1	GGAGGAGCTGCAGAAGAAGG	TTTCCAGTGACACATCGGCA	XM_021617454.1
drd2	CCTCCAGTCCACCACCAATT	CCACTCTCCCACCACCTCTA	NM_001124372.2
dat	CTACCTCAGCGTCGACTTCC	TAGCACACCAAACCCGACTC	XM_021592557.1
Serotonin markers			
tph1a	ACACCAGAGCCAGACACATG	TCATCTGAAGCTCCGAGGGA	XM_021598622.1
tph1b	AGCGTCCGTTTACAGTGAGG	GCCCACGATGTCCAGTTCAT	XM_021598622.1
tph2	AGCACCTCAAAGACCACGTC	ACTGGTCGAGCTCTGCAATC	MG015698
5ht1aa	CCCAACACTCCACAGTCCTC	ACCGAGCGTCTTTACCGTTT	XM_021622104
5ht1ab	GAGGACCAACGGGGACCCGA	AATCGCCGTGCTTGACCGCA	CCAF0100015582
sert	CCTGCTGCCCTACATGTTGA	GGGGCAGATGTGTTTCCAGA	M_021582096.1

Table 1 (*continued*)

Transcript	Forward Primer	Reverse Primer	Accession Number
Gabaergic markers			
gabarap	CAGATGCACTTTCCCTCCCC	TCAACCGAAATCCCCATCTCG	NM_001165091.1
gbrl2	AGAGAGAGATGGGGATGGCT	AGGATGCAAGGGTTGTGTCA	NM_001165109.1
gbrap	CTCACAGTGGGCCAGTTCTA	GAGGTGGGAGGAATGACGTT	NM_001165091.1
gabat1	GGTGATGGAGTTTTGGGAGC	TAAACCAGGACCCAAGCGAT	XM_021615563.1
chat	CATCATCGTGGCATGCAAGA	AGTTCTCCGCCATCTTCACT	XM_021581165
Glutamatergic markers			
grin1a	AACAAGCGAGGACCTAAGGC	CTGGCGGAGAGGATGATGAC	XM_021602512.2
grin2ca	ACCCTCTGCCTTTCTTGAGC	CACAGGGCTGCAGTACTCAA	XM_036938636.1
grik5a	GCAGATCAGGGTCCAGTCAC	AGTCAAAATACCCTCCCGCG	XM_036969398.1
grik5b	TGAAGAGGAGGTGGTGGGAA	GATGATGAGGCCGCAGATCA	XM_036952756.1
grin3bb1	CTACTTCAGTGAGCGTGCCA	TACTCGAAGCGCATGTCCTC	XM_021614590.2
grin3bb2	GGATCCAGAATAGGCCTGCC	GAACACCCTCTTCCCACAGG	XM_036977945.1
gria1b1	GCCTTTCAGAACCTCCGGAA	CTGGATGTCGATACCCTGGC	XM_036989628.1
gria1b2	GCGTATTGACATTTCCCGGC	CCTCAATCCGAACCTGCTGT	XR_005035026.1
grm1a	GCTGATCGAAAGTGTGGGGA	ATGTTGGGGAGCAGGAAAGG	XM_021600740.2
grim2a	TGCATCGCCACTTCAGCTAA	TGCGTGTGAAGAGGATGACC	XM_036950357.1
grim2b	GTGAGGGGAAGTGAGACAGC	GGGGTTCCGTGTGTTAGTGT	XM_021568769.2
grm4a	CCATTTCATCTGGGTGGGCT	CCTCTGATGGACTGGCGTTT	XM_021609206.2
grm4b	GTGCCAGAGACCTTCAACGA	GACTGCGAGGTCCCAAAGAA	XM_036988394.1
grim5b	GGGCATCCTGTTTGACGAGA	TGTCCCAGCTCCCTACGTTA	XM_036961649.1

with treatments (BUBBLE + TIME, RANDOM), analysis periods (Neutral periods, Before feedings), days (day 35, day 63, and day 88), and their interactions as fixed factors. The tanks in which the fish were reared were treated as random factors. The GLMM used was glmer(Mean.activity ~ Treatment*Period*Day + (1|Rearing tank), family = poisson(link=“log”)).

Regarding the feed omission tests, we analysed occurrences of agonistic behaviours, burst of accelerations, and jump occurrences during the 5 minutes following feed omissions, taking into account treatment, omission number (1, 2 or 3), and their interactions as fixed factors. Once again, rearing tanks were treated as random factors. The following GLMM was used: glmer(Mean.activity ~ Group*Omission.number + (1|Rearing tank), family = poisson(link=“log”)).

Emotional reactivity

Concerning the assessment of emotional reactivity test, we tested the effect of the treatment on the total distance moved, swimming velocity, angular velocity, and the time spent in thigmotaxis. LMMs were used to test all these parameters, with the rearing tank considered as a random factor. We assessed treatment effects on the number of pellets consumed at the end of the novel-tank test using a Chi-square test.

Zootechnical and physiological parameters

Cortisol data were log-transformed and analysed using LMM, with the treatment and the sampling period (concentration at basal level or after an acute stress), and their interaction as fixed factors. The rearing tank was defined as a random factor.

For each model, we investigated how fixed factors influenced each variable by using the Type III ANOVA analysis of deviance table. The results section presents the significant effects of the fixed factors and their interactions. If the effect of the interaction of the fixed factors was significant, only this effect is presented in the results section. When significant effects were detected, post-hoc analyses were conducted using HSD-Tukey tests.

Fish weight, length, K-factor, fin erosions, and complement (ACH50) and lysozymes activities were compared between treatments using Student’s *t*-tests (independent explanatory variables, normal distribution) or a Wilcoxon’s tests when data did not meet normal distribution, after having checked the absence of a rearing tank effect.

Data for bacterial analyses were analysed by chi-square tests as a presence or an absence of colonies on each fish sampled, after having checked the absence of a rearing tank effect.

Data from one tank in the treatment RANDOM were removed from all analyses from day 88 due to a technical issue that suppressed bubble diffusion in this tank.

Brain markers measurement

Analyses were carried out on untransformed data as criteria for normality and homogeneity of variances were met (Shapiro-Wilk’s and Levene’s test respectively). Values of mRNA level of all transcripts were analysed by welch’s *t*-tests. If the criteria (normality and homogeneity) were still not met, a non-parametric test was used for the analysis (Kruskall Wallis test).

P-values <0.05 were considered statistically significant for all statistical analyses.

Results

Group behaviours during the conditioning

For each conditioning day analysed, fish group activity was dependant on both period (Neutral periods vs Before feedings vs During bubbles) and treatment (interaction treatment × period: day 35 LMM: χ^2 = 40.84, df = 2, $P < 0.001$; day 63 LMM: χ^2 = 59.77, df = 2, $P < 0.001$; day 77 LMM: χ^2 = 90.79, df = 2, $P < 0.001$; day 88 LMM: χ^2 = 344.26, df = 2, $P < 0.001$; Fig. 2).

During bubble diffusion, fish group activity was higher in BUBBLE + TIME than in RANDOM treatment on each day (Tukey, day 35: $P = 0.03$; day 63: $P = 0.02$; day 77: $P = 0.01$; day 88: $P < 0.001$; Fig. 2). For the BUBBLE + TIME treatment, activity was systematically higher during bubble diffusion than during Neutral periods (i.e., 5-minute period without feed delivery or bubble diffusion) and Before feedings (Tukey, $P < 0.05$, Fig. 2). The same increasing effect of bubble diffusion on fish activity was also observed for the fish in the RANDOM treatment, but only on day 63 (Tukey, during bubble vs neutral period: $P < 0.01$, and During bubbles vs Before feedings $P = 0.002$; Fig. 2B).

During the five-minute periods preceding feed delivery (i.e., Before feedings), BUBBLE + TIME fish were as active as RANDOM fish, irrespective of the day (Tukey, $P > 0.05$; Fig. 2). However, on days 63 and 88, BUBBLE + TIME fish were significantly more active

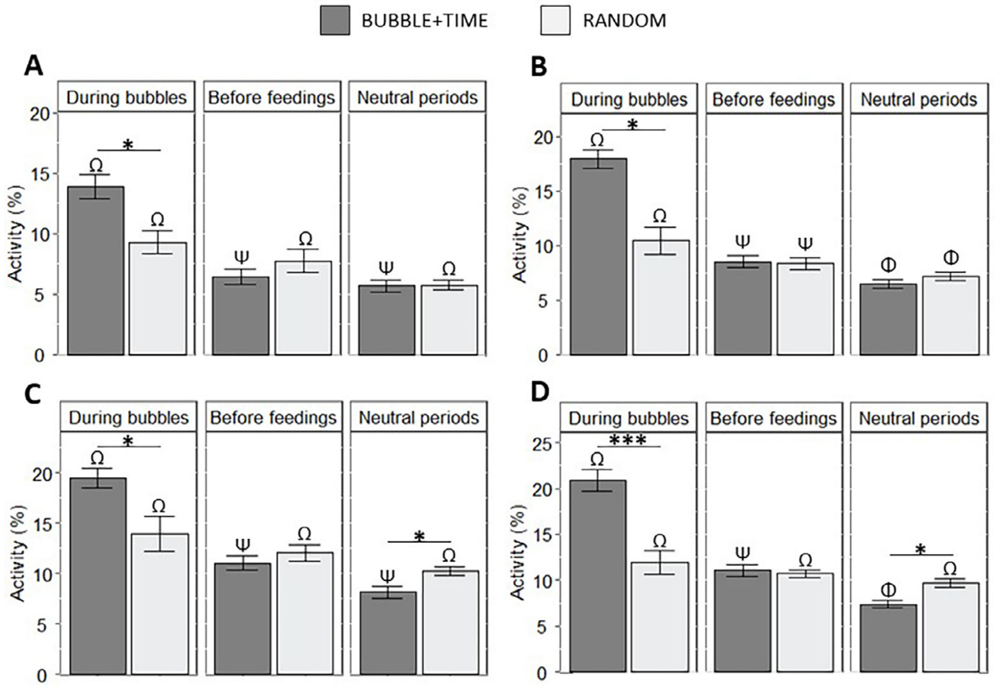

Fig. 2. Percentage of fish group activity (% of differing pixels between two successive images) in the whole tank recorded for three different periods on each day of conditioning analysed, with "During bubbles" corresponding to the 15 seconds of bubble diffusions + the subsequent 5 seconds, "Before feedings" to the 5 minutes preceding feed delivery, and "Neutral periods" to the 5-minute periods without bubble diffusion or feeding. Results are given for each treatment on (A) day 35, (B) day 63, (C) day 77, and (D) day 88 of conditioning. Values are mean, and the SEM is represented. Significant differences between treatments are represented by asterisks, and different greek letters indicate differences between periods within the same treatment (*$P < 0.05$; ***$P < 0.001$; Tukey posthoc tests).

during these periods than during the Neutral periods (Tukey, $P = 0.01$ and $P = 0.04$, respectively; Fig. 2B and D). The same tendency was observed on day 77 (Tukey, $P = 0.095$; Fig. 2C), while this was not the case for RANDOM fish (Neutral periods vs Before feedings: Tukey, $P > 0.05$), except on day 63 (Tukey, $P = 0.02$).

During the Neutral periods, at the end of the conditioning on days 77 and 88, RANDOM fish showed higher activity than BUBBLE + TIME fish (Tukey, $P = 0.046$ and $P = 0.02$, respectively; Fig. 2C and D).

Analysing the frequency of agonistic behaviours, burst of accelerations, and jumps on all conditioning days combined revealed an interaction between treatment, day, and period (GLMM: $\chi^2 = 22.76$, df = 3, $P < 0.001$; Fig. 3). Before feedings, BUBBLE + TIME fish exhibited less agonistic behaviours, burst of accelerations, and jumps than RANDOM fish on all days except day 63 (Tukey, day 35: $P < 0.001$, day 77: $P = 0.01$, and day 88: $P = 0.002$, Fig. 3). During Neutral periods, no difference between treatments was observed, except on day 88 (Tukey, $P = 0.07$, and

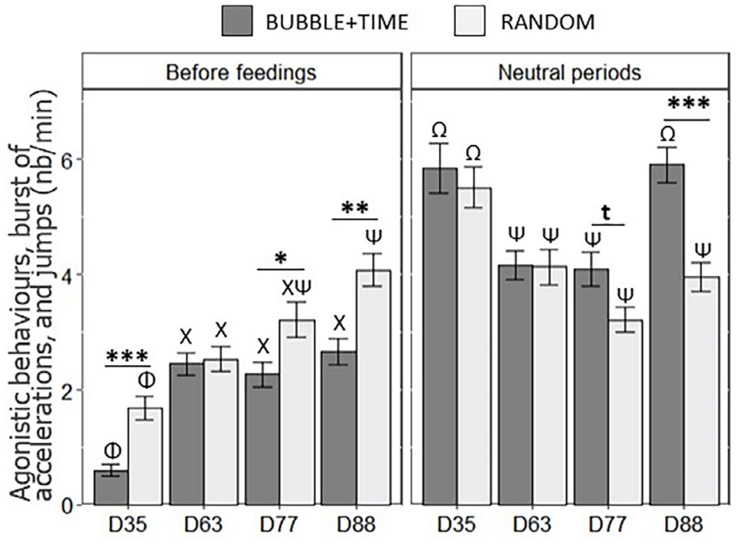

Fig. 3. Number of fish agonistic behaviours, burst of accelerations, and jumps scored per minute in the whole tank over the 5 minutes preceding feed delivery (Before feedings) and the 5-minute periods without bubble diffusion or feeding (Neutral periods) for each day of conditioning analysed. Results are given for each treatment, period of analysis, and for each conditioning days analysed, with D35 = day 35, D63 = day 63, D77 = day 77, and D88 = day 88. Values are mean and the SEM is represented. Significant differences between treatments are indicated by asterisks, and significant differences between periods within the same treatment are indicated by Greek letters (*t*: $0.05 < P < 0.10$; *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$; Tukey posthoc tests).

$P = 0.001$, respectively; Fig. 3) for the BUBBLE + TIME fish, which showed more of these behaviours. A comparison between the two periods on each day revealed that these behaviours were systematically less frequent in BUBBLE + TIME fish Before feedings than during Neutral periods (Tukey, all $P < 0.001$; Fig. 3). This did not apply to RANDOM fish, except on day 35 and day 63 (Tukey, $P < 0.001$ for both days; Fig. 3).

Feed omission tests

Analysis of group activity during the three feed omissions trials revealed an interaction between period (during omission vs after omission) and treatment (LMM: $\chi^2 = 47.43$, df = 1, $P < 0.001$; Fig. 4). As expected, group activity was higher for BUBBLE + TIME than for RANDOM fish, in both the "During omissions" and "After omissions" periods (Tukey, $p < 0.001$ and $P = 0.005$, respectively, Fig. 4). These results confirm that fish in the BUBBLE + TIME treatment learned that bubbles predicted feed delivery.

During the five-minute periods that followed feed omission ("After omissions"), agonistic behaviours, burst of accelerations, and jumps also differed between treatments and feed omissions trials (interaction treatment × feed omission trial: GLMM: $\chi^2 = 17.91$, df = 2, $P < 0.001$; Fig. 5). Fish from the BUBBLE + TIME treatment exhibited significantly less agonistic behaviours, burst of accelerations, and jumps during the first and third feeding omissions compared to fish from the RANDOM treatment (Tukey, OM1&OM3: $P < 0.001$; Fig. 5).

Individual fish emotional reactivity

There were no differences between treatments in any of the assessed measures during the novel-tank test (total distance moved, maximum swimming velocity, angular velocity, time spent in thigmotaxis, number of pellets eaten) (LMM: $P > 0.05$, and $\chi^2 = 1.10$, df = 1, $P > 0.05$ for all measured behavioural parameters).

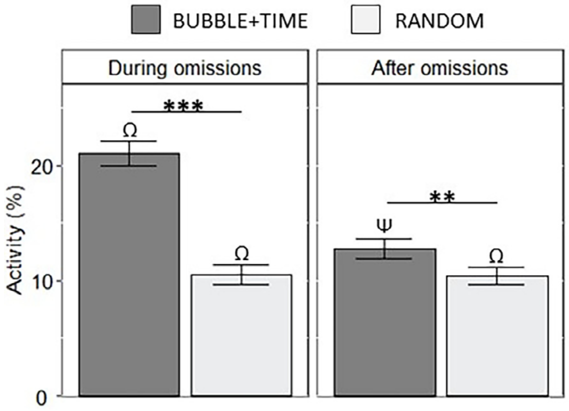

Fig. 4. Percentage of fish group activity (% of differing pixels between two successive images) during and after feed omissions recorded in the whole tank. For the period "During omissions" activity is measured from the onset of bubble diffusion to 8 seconds afterwards (total analysis duration of 28 seconds: 15 seconds of bubbles, the subsequent 5 seconds before the expected feeding for treatment BUBBLE + TIME, and 8 seconds after the omission. For treatment RANDOM, the period of 8 seconds started 5 seconds after the end of bubbles). For the period "After omissions" activity is measured during the 5-minute period following feed omissions. Values are mean of the three feed omissions and the SEM is represented. Significant differences between treatments are indicated by asterisks, and different Greek letters indicate differences between periods within the same treatment (**$P < 0.01$; ***$P < 0.001$; Tukey posthoc tests).

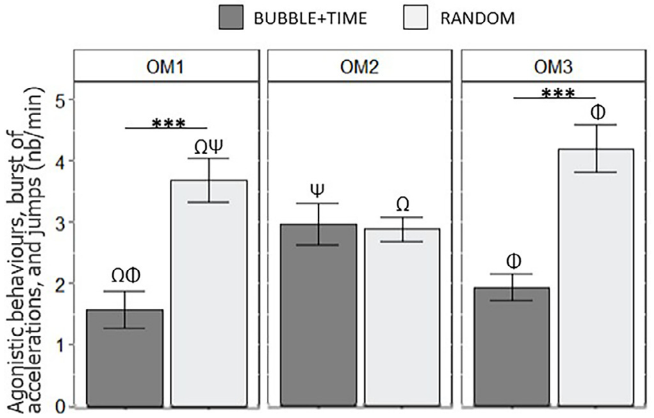

Fig. 5. Number of fish agonistic behaviours, burst of accelerations, and jumps scored per minute in the whole tank during 5-minute periods following feed omissions. OM1, OM2 and OM3 correspond to the three omission trials analysed. Values are mean and the SEM is represented. Different Latin letters indicate significant differences between treatments, and different Greek letters indicate differences between periods within the same treatment (***$P < 0.001$; Tukey posthoc tests).

Physiological and zootechnical parameters

Analyses of plasma cortisol levels revealed that the interaction between treatment and sampling period (basal versus after acute stress exposure) tended to be significant (LMM, $\chi^2 = 2.93$, df = 1, $P = 0.09$). Confirming this, BUBBLE + TIME fish had higher basal cortisol concentrations than RANDOM fish (Tukey, $P = 0.02$; Table 2). After an acute stress exposure (i.e., novel-tank test), plasma cortisol concentrations were similar between groups (Tukey, $P = 0.63$; Table 2).

Of the different zootechnical measures assessed at the end of the 88-day conditioning period (fish weight, body size, K-factor, fin erosion), the dorsal fin of fish in the BUBBLE + TIME treatment tented to be less eroded than that of fish in the RANDOM treatment (W = 1 732, $P = 0.07$; Table 2).

Fish health measures (lysozyme and complement activity, number of bacterial colonies) showed that the number of *Flavobacterium psychrophilum* colonies on the skin surface and in the kidney tended to be higher in fish from the RANDOM treatment than in fish from the BUBBLE + TIME treatment ($\chi^2 = 3.43$, df = 1, $P = 0.06$; $\chi^2 = 2.87$, df = 1, $P = 0.09$, respectively; Table 2).

Genic markers of brain function, regulatory pathways and networks and physiological status of rainbow trout

Measures taken to assess the brain status of fish (Fig. 6) showed that markers of oxidative status were decreased for *cat* (catalase) in forebrain and *sod3* (superoxide dismutase) in both forebrain and hypothalamus in fish from the BUBBLE + TIME treatment compared to fish from the RANDOM treatment ($P < 0.05$; Fig. 6A). For mRNA level of inflammatory markers, interleukins *il-6*, *il-8* and *tnf-α* (tumoral necrosis factor) were decreased in forebrain and hypothalamus, *il-1β*, *il-4* and *il-11* in forebrain and *nfkb1* (nuclear factor kappa B subunit 1) and *ikk-β* (nuclear factor kappa-B kinase subunit beta) in hindbrain in fish from the BUBBLE + TIME treatment compared to fish from the RANDOM treatment ($P < 0.05$; Fig. 6B). The mRNA level of *gfap* (Glial fibrillary acidic protein), the astrocyte marker, *tmem119* (transmembrane protein 119), the marker of microglial cell, and *pdgfra-1* (platelet-derived growth factor receptor A), the marker of oligodendrocyte, were decreased in the forebrain and *pdgfra-1* and *2* decreased in hypothalamus in fish from the BUBBLE + TIME treatment compared to fish from

Table 2
Statistical effects of the treatment (BUBBLE + TIME: predictable feeding condition, RANDOM: unpredictable feeding condition) for each variable analysed relating to fish zootechnical and physiological parameters, as well as the interaction between treatment and sampling period for cortisol measures.

Item	Measures	Variables	BUBBLE + TIME		RANDOM		Treatment		
			Mean	SEM	Mean	SEM	χ^2/t/W	df	*P*-values
Zootechnical parameters	Growth	Weight (g)	67.08	1.39	65.30	1.40	0.90	127.82	0.37
		Body size (cm)	17.30	0.13	17.04	0.12	1.48	127.88	0.14
		K-factor	1.29	0.02	1.31	0.01	1 745		0.13
	Fin erosion (mean score)	Dorsal fin erosion	1.68	0.08	1.92	0.10	1 732		0.07#
		Caudal fin erosion	1.75	0.07	1.85	0.08	1 923.5		0.37
Physiological parameters	Innate immune markers	Lysozyme activity (U/mL)	64.03	3.19	58.21	1.67	1.62	20.75	0.12
		ACH50 activity (U)	120	4.50	126.83	9.16	0.35	18.04	0.73
	Bacterial infections on skin surface (presence/absence of colonies)	*Aeromonas*	0.75	0.11	0.83	0.11	0.28	1	0.59
		Pseudomonas	0.56	0.13	0.67	0.14	0.31	1	0.58
		Flavobacterium psychrophilum	0.06	0.06	0.33	0.14	3.43	1	0.06#
		Gram-positive cocci	0.125	0.09	0	0	1.62	1	0.20
		Gram-negative bacilli	0.69	0.12	0.5	0.15	1.01	1	0.31
		Yeasts	0.125	0.09	0	0	1.62	1	0.20
	Bacterial infections in kidney (presence/absence of colonies)	*Aeromonas*	0	0	0.08	0.08	1.38	1	0.24
		Flavobacterium psychrophilum	0	0	0.17	0.11	2.87	1	0.09#
							Treatment × sampling period		
	Cortisol concentration	Basal (ng/mL)	27.70*	4.32	14.64	1.60	2.93	1	0.09#
		After novel tank-test (ng/mL)	134.03	9.15	125.99	12.03			

* $P < 0.05$.
\# Tendency: $0.05 < P < 0.10$.

the RANDOM treatment ($P < 0.05$; Fig. 6C). Concerning markers of stress status, *crf* (corticotropin releasing factor) was decreased in forebrain, *mrap1* (melanocortin 1 receptor-associated protein) was decreased in both the forebrain and hypothalamus and *mrap2* in hindbrain in fish from the BUBBLE + TIME treatment compared to fish from the RANDOM treatment ($P < 0.05$; Fig. 6D).

Based on measures of brain activity (Supplementary Fig. S1A), neurotrophic markers were found to be decreased for *neurod1* (neuronal differentiation 1, marker of neurite formation) in both the forebrain and hypothalamus and for *ncam* (neural cell adhesion molecule, marker of neural cell migration and adhesion) in hindbrain and *stx1b* (Syntaxin-1b, marker of synaptogenesis) was increased in BUBBLE + TIME fish compared to RANDOM fish ($P < 0.05$; Supplementary Fig. S1A). For neurotrophic factors, only *npas4b* (neuronal PAS domain protein 4, early markers of excitatory-inhibitory balance of neural circuits) was decreased in forebrain in fish from the BUBBLE + TIME treatment compared to fish from the RANDOM treatment ($P < 0.05$; Supplementary Fig. S1B). For mRNA level of brain plasticity markers, *mapk4* (mitogen-activated protein kinase 1, marker of synaptic plasticity and cell proliferation) and *mapk1* were decreased in the forebrain and hindbrain respectively of fish from the BUBBLE + TIME treatment ($P < 0.05$; Supplementary Fig. S1C).

Measures concerning neurotransmitter pathways (Supplementary Fig. S2) showed that for dopamine pathways, *th* (tyrosine hydroxylase enzyme) and *dat* (dopamine carrier) were decreased in the hindbrain of fish from the BUBBLE + TIME treatment compared to fish from the RANDOM treatment ($P < 0.05$; Supplementary Fig. S2A). For serotonin pathways, *tph2* (precursor isoenzyme of serotonin) was increased in the hypothalamus of fish in the BUBBLE + TIME treatment while *sert* (serotonin carrier) was decreased compared to fish in the RANDOM treatment ($P < 0.05$; Supplementary Fig. S2B). For GABAergic pathways (GABA, main inhibitory neurotransmitter of the nervous system), *chat* (enzyme of GABA) and *gabarap* (Gamma-aminobutyric acid receptor-associated) were decreased in the forebrain and hindbrain respectively in BUBBLE + TIME fish ($P < 0.05$; Supplementary Fig. S2C) while *gabarap*, *chat* and *gabaT1* (GABA carrier) were increased in the hypothalamus compared to RANDOM fish ($P < 0.05$ Supplementary Fig. S2C). For glutamatergic pathways (glutamate main excitatory neurotransmitter of the nervous system), *grik5a* (glutamate receptor, ionotropic, kainite 5) in the forebrain, and *grik5b* and *gria1b1* (ionotropic AMPA receptor) in the hindbrain were decreased in fish from the BUBBLE + TIME treatment compared to fish from the RANDOM treatment ($P < 0.05$; Supplementary Fig. S2D). In the hypothalamus, *grin1a* (glutamate receptor, ionotropic, N-methyl D-aspartate 1a) and *grin1ab1* were increased in BUBBLE + TIME fish ($P < 0.05$; Supplementary Fig. S2D).

Discussion

The present study reveals that after 3 months of conditioning to feeding, rainbow trout from the BUBBLE + TIME treatment still rely on bubbles to predict feed delivery, as shown by increased activity during bubble diffusion compared to the RANDOM treatment. Fish in the BUBBLE + TIME treatment could also temporally anticipate feedings, as suggested by increased activity before feeding compared to neutral periods, which was not the case for fish in the RANDOM treatment. During neutral periods, RANDOM fish were more active than BUBBLE + TIME fish. Following feed omissions, BUBBLE + TIME fish showed less agonistic behaviours, burst of accelerations, and jumps than RANDOM fish. In addition, the BUBBLE + TIME treatment tended to reduce dorsal fin erosion and the frequency of *Flavobacterium* type bacteria in the kidney and on the skin surface. Cortisol concentrations were higher at basal level in trout from the BUBBLE + TIME treatment but similar after acute stress and we found no effect of the 3-month conditioning on the emotional reactivity or immune responses of the fish. Fish were similar in weight and body size between treatments at the end of the 3-month conditioning period., Consistent with the observed upward trends in fin erosion and bacterial infection, genic markers related to brain function and physiological status revealed a strong increase in most genes involved in oxidative stress and inflammatory responses in RANDOM fish.

Feeding predictability effects on growth parameters

Fish weight, body-size, and condition-factor were similar between treatments at the end of 88 days as after 12 days of

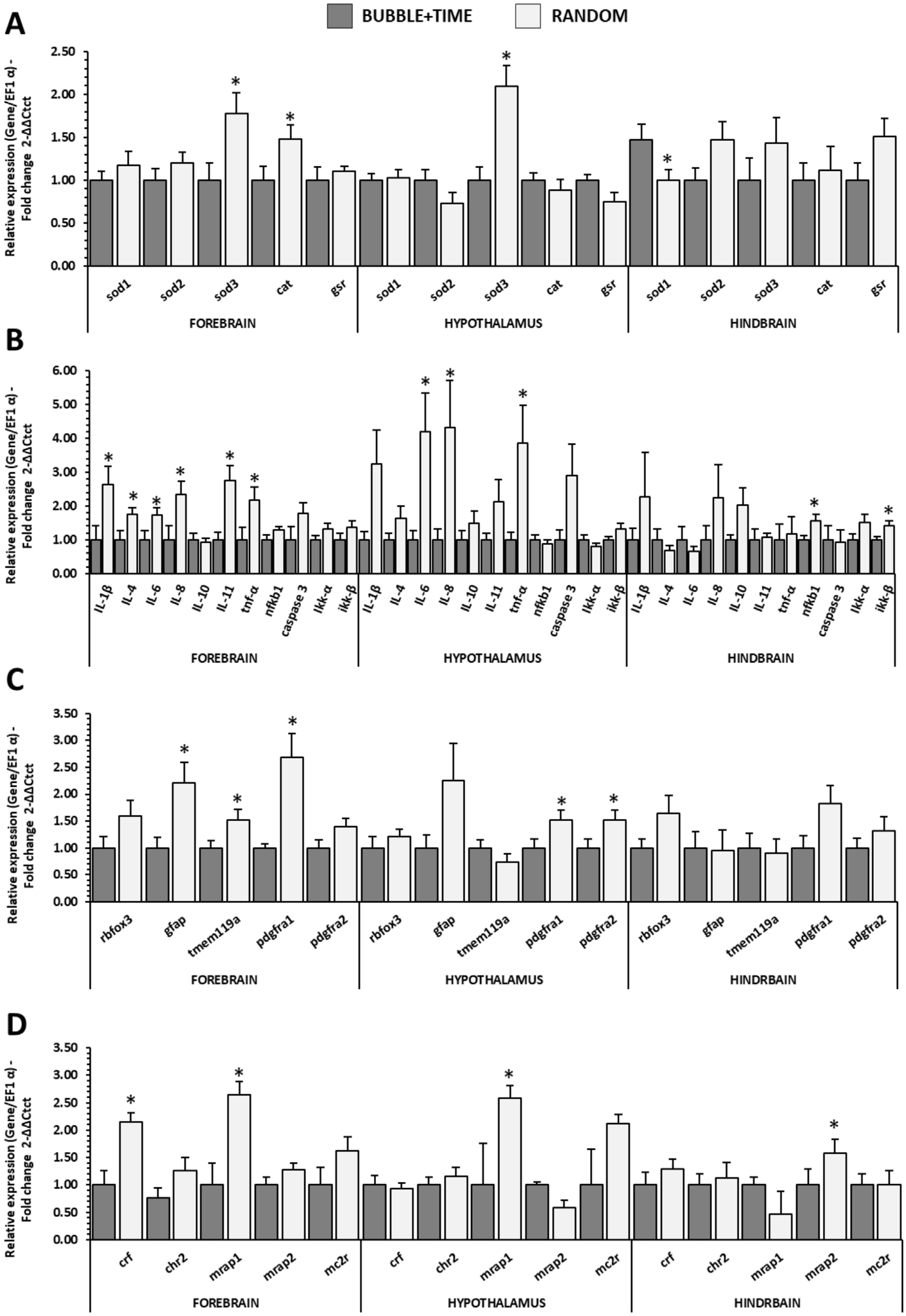

Fig. 6. Effects of treatment in antioxidant (A), inflammatory (B), cell type markers (C) and stress (D) status related mRNA level in forebrain, hypothalamus, and hindbrain of rainbow trout. Values are expressed as group mean and the SEM is represented. Fold change *versus* treatment is reported for all genes. Replicates (n = 8) correspond to different individual fish. Differences between treatments are represented by *($P < 0.05$; Welch's t-test).

conditioning (Kleiber et al., 2022). This result is consistent with sea breams (Sánchez et al., 2009), salmons (Cañon Jones et al., 2012; Thomassen and Fjæra, 1991), guppies (*Poecilia reticulata:* Chapman et al., 2010), and three-spined sticklebacks (*Gasterosteus aculeatus:* Ali and Wootton, 2001), which had similar growth whether provided with predictable or unpredictable feedings for 20–60 days.

Conditioning to bubbles

Prefeeding swimming activity results showed that after 3 months of conditioning, BUBBLE + TIME fish still relied on bubbles to predict feed delivery, as evidenced by their higher activity during bubble diffusion compared to RANDOM fish, regardless of the day. In addition, feed omissions confirm the effectiveness of conditioning, as revealed by the higher activity maintained by bubble diffusion up to 5 minutes after feed omissions in the BUBBLE + TIME treatment only. These results are consistent with those obtained in our previous study for a shorter conditioning period of fourteen days (i.e., 60 trials) (Kleiber et al., 2022). This is also in line with sharks that required 10 days (i.e., 60 trials) to be successfully conditioned to feeding after bubble diffusion (Guttridge and Brown, 2014). Bubble diffusion as a CS appears to be a good candidate for effective conditioning in fish, given the specific behavioural responses it produces – movements and bites towards the bubbles in sharks, and attraction and aggregation in rainbow trout – compared to other and perhaps less salient stimuli, such as a light signal, which did not produce as strong conditioned responses as bubble diffusion in sharks (Guttridge and Brown, 2014).

Conditioning to time

In addition to bubbles as a predictor of feeding, the BUBBLE + TIME fish also appeared to rely on time to predict the occurrence of feeding, but their FAA was less obvious than after twelve days of conditioning (Kleiber et al., 2022). Contrary to what was found in our previous study for the same conditioning conducted for a shorter period, we found no difference from 35 to 88 days of conditioning between BUBBLE + TIME and RANDOM fish in swimming activity before feedings. After 35 days of conditioning, the fish may also have strategically stopped to temporally anticipate feedings to rely solely on bubbles - a highly reliable signal - rather than fixed times that require energy to synchronise (López-Olmeda et al., 2012). In line with this, previous studies have reported that conditioning with conditioned stimuli composed of two predictors can result in one predictor being overshadowed by the second (Mackintosh, 1976; Pavlov, 1927; Wasserman and Miller, 1997). According to Mackintosh (1976), such overshadowing can occur when animals fail to attend to or deliberately ignore a given stimulus, raising concerns about the efficacy of the overshadowed predictor in conditioning processes. However, when the time was paired with bubble diffusion as predictors of feeding for rainbow trout, the conditioning was stronger than with time or bubble diffusion as the sole predictors (Kleiber et al., 2022). In the present study, we did not observe FAA within the chosen time frame, but this does not necessarily mean that they no longer temporally anticipate feedings. On days 63, 77, and 88, the swimming activity of BUBBLE + TIME fish differed between the neutral and before feedings periods, with more activity before feedings, suggesting that they likely distinguish between these two periods. However, the hypothesis that the time predictor is overshadowed in the long term by the reliable bubble signal also seems plausible, but it deserves to be confirmed by further studies.

Feeding predictability effects on the markers of fish brain function

Given the successful conditioning response exhibited by fish in the predictable condition, one might expect an improvement in memory and learning-associated functions in the brain in response to a stimulating environment, as previously demonstrated in fish reared with physical enrichments (Ebbesson and Braithwaite, 2012; Salvanes et al., 2013), including rainbow trout (Cardona et al., 2022). Surprisingly, genes involved in neuronal plasticity, neurogenesis and synaptogenesis, in particular immediate early genes (IEGs), were down-regulated in the feeding predictability condition. IEGs are rapidly up-regulated following neural stimulation in brain and have been widely used as markers of neuronal activity (Kawashima et al., 2014). Our results are consistent with those obtained for seabream (Cerqueira et al., 2017) and seabass (Cerqueira et al., 2020), which also exhibit down-regulated IEGs when subjected to predictable environments. In line with these results, the expression of *npas4b* - an IEG involved in contextual learning - is also decreased following feeding predictability in the forebrain (brain area central to decision-making, including the hippocampus for memory processes and the amygdala for learning emotional components in mammals), suggesting a role for contextual learning in predictability, as stated by Cerqueira et al. (2020). Several hypotheses can explain the down-regulation of these genes under a predictable environment. It could reflect an adaptive response to a predictable environment and thus to a lower workload for brain cells. In a stable and predictable environment, fish may not need to maintain heightened vigilance, observable by a higher expression of IEGs, as their response to events becomes familiar. IEGs have also been shown to be down-regulated in unstressed fish compared to stressed fish (Johansen et al., 2012; Ponzoni et al., 2021; Sadoul et al., 2018). Thus, rainbow trout raised under unpredictable condition of feeding could be more subjected to stress than fish that have predictable feeding.

Feeding predictability effects on fish activity during neutral periods and markers of fish brain function

Indeed, the higher activity of RANDOM fish recorded during neutral periods could be the result of a constant state of expectation of feeding. This result is in accordance with other studies that showed that random feeding schedules led to a continuous active state, which was correlated with increased plasma cortisol in sea bream (López-Olmeda et al., 2012; Sánchez et al., 2009) or goldfish (*Carassius auratus*) (Vera et al., 2007) compared to fish that had predictable feedings. When fish face a fasting period, the same increase in activity is encountered and is associated with an increased expression of *th* (tyrosine hydroxylase, an enzyme converting tyrosine to L-DOPA, markers of dopamine synthesis) in the brain (Wall and Volkoff, 2013). In RANDOM fish, we also observed an up-regulation of *th* in the hindbrain. Authors explain this to be linked to the increase in locomotor activity as the fish are actively looking for food and is consistent with studies showing that TH knockout mice exhibit low locomotor activity (Szczypka et al., 1999). In fish, tyrosine is involved in the synthesis of dopamine which controls physical movement, learning and eating behaviours (Szczypka et al., 1999). The dopamine transporter (*dat*) was also down-regulated in the hindbrain following feeding predictability. In fish, the hindbrain is namely responsible for motor activity and is known to control some cognitive abilities such as the ability to learn from preceding events through classical conditioning, by dopamine metabolite turnovers (de Abreu et al., 2018; Terry, 2018). The dopaminergic system in the hindbrain may thus play a role in regulating food anticipatory behaviour and the formation of associative memory in rainbow trout. During learning, there is increased neuronal activity, particularly of dopaminergic neurons, which decreases over time once the conditioned response has been learned, which could be even truer after omissions of the expected reward (Schultz, 2010). As for the reduced IEGs expression, the reduced expression of *th* and *dat* in the hindbrain of BUBBLE + TIME fish could therefore result in an adaptive response as a consequence of their successful and rapid conditioning learning. Consistently, the downregulation of the dopaminergic system could lead to reduced foraging stress under predictable feeding conditions. A constant state of alertness, as suggested by the high fish activity and increased *th* expression in RANDOM fish, may involve a high waste of energy, and thus potentially induce poor welfare (Fureix and Meagher, 2015). In line with this, brain mRNA expression involved in GABAergic and glutamatergic pathways, acting as inhibitory and excitatory neurotransmitters respectively (Horzmann and Freeman, 2016), were up-regulated in the hypothalamus of BUBBLE + TIME fish. In fish, the hypothalamus was namely found to play a key role in regulating appetite and satiety by controlling the release of hormones that signal hunger and fullness (Volkoff, 2016) and also the general homeostasis control of the body. These up-regulations could thus explain the observed differences in activities between the two predictability conditions. The observed decreased expressions of the genes *pdgfra-1* and *pdgfra-2* – which promote proliferation of oligodendrocyte progenitor cells and inhibit premature differentiation (Domingues et al., 2016) – in the hypothalamus and forebrain of trout are also consistent, as these genes are associated with myelin production, known to be promoted by excitatory-neural activity in mammals (Gibson et al., 2014). In other words, the increase of oligodrendrogenesis and thus of myelination markers in brain of RANDOM fish could suggest that fish are constantly on the lookout and in a feeding expectancy state, as suggested by their higher activity during neutral periods. For BUBBLE + TIME fish, the lower activity during neutral periods is in accordance with other studies observing that adding physical structures into the tanks leads to reduced swimming activity in rainbow trout (Brunet et al., 2022), Atlantic cod (*Gadus morhua:* Salvanes and Braithwaite, 2005) and zebrafish (*Danio rerio:* von Krogh et al., 2010). Thus, the same behavioural patterns observed between groups of fish provided with either physical enrichments or a signalled, predictable feeding schedule over time could support the hypothesis that feeding predictability represents a cognitive enrichment for fish. However, these results should be approached with caution. A thorough neurochemical analysis of all these neurotransmitters using HPLC would provide a more precise assessment of their turnover rates. Gene expression in the fish brain remains an indirect measure of potential protein activity. Moreover, while our study was designed for a global perspective, it is important to exercise caution in interpreting the results, as specific micro-zones or specific neuron populations can be isolated within the telencephalon in fish, including in rainbow trout (Folgueira et al., 2004; Pérez et al., 2000), which can differ in their expression profiles. Future studies could, therefore, explore the differences within these more specific zones, providing a richer understanding of the intricacies of fish neuroanatomy in relation to feeding predictability.

Feeding predictability effects on aggression and fin erosion

Surprisingly, lower activity during neutral periods did not prevent BUBBLE + TIME fish to exhibit more agonistic behaviours, burst of accelerations, and jumps than RANDOM fish on days 77 and 88. This result seems to be specific to the end of the 3 month-conditioning period since no difference between treatments was found after twelve days of conditioning (Kleiber et al., 2022), and neither on days 35 and 63. However, the higher occurrence of these behaviours in BUBBLE + TIME treatment was limited to neutral periods, since they occurred less frequently before feedings and following feed omissions in this treatment. Moreover, dorsal fin erosions measured at the end of the experiment tended to be lower in this treatment. This result is consistent with salmons provided with temporally predictable feeding that were more aggressive but that presented lower frequency of dorsal fin erosion compared to salmons that were subjected to random feeding schedules (Cañon Jones et al., 2012). Fin erosion is a robust indicator of poor fish welfare and is often used as an indirect approach to quantify aggression and/or evaluate the quality of the environment (Cañon Jones et al., 2012; Ellis et al., 2008). When combined with injuries and fin damage, aggression is a strong indicator of poor welfare (Martins et al., 2012). Our results therefore suggest that aggression was of greater concern in the RANDOM treatment, which combined both a tendency towards greater dorsal fin erosion and higher levels of aggression before feedings. Even though protein quantification would have been relevant in addition to gene expression results, this is consistent with the observed up-regulations of *th* and *dat* for rainbow trout subjected to random feedings. Indeed, the dopaminergic system is known for its stimulatory role in aggression, with higher *th* and *dat* brain expression in mice subjected to repeated aggression experiences (Filipenko et al., 2001), and with increased *th* in fish brain associated with higher aggression levels (Filby et al., 2010).

Feeding predictability effects on the brain markers of physiological status

BUBBLE + TIME fish tended to present fewer *Flavobacterium psychrophilum* bacteria – a widely distributed and devastating pathogen of wild and farmed salmonid fish (Duchaud et al., 2007) – in the kidneys and on the skin surface, suggesting that they could have presented a better immune response during the conditioning period as fish from both treatments were provided with the same spring water. The lower number of *Flavobacterium* type bacteria isolated could result from the lower dorsal fin erosion found in the BUBBLE + TIME fish, as injuries can be a point of entry for many pathogens (Goede and Barton, 1990; Noble et al., 2020). Enriched environments have been shown to enhance immunocompetence, as salmons reared with small gravels and shelters had enhanced survival rates and disease resistance to parasitic infections or bacterial exposure to *Flavobacterium columnare* compared to salmons reared in barren environments (Karvonen et al., 2016; Räihä et al., 2019). If no difference was found at the protein level concerning innate immune responses (lysozyme and complement activities), numerous differences were observed between the two feeding predictability conditions at the brain level in genes involved in oxidative stress and inflammatory status. Feeding predictability attenuated most of the genes that we studied for oxidative stress reactions and triggered anti-oxidative defence mechanisms, as indicated by decreased expressions of numerous pro-inflammatory (cytokines and chemokines) and pro-oxidative mediators and by the upregulation of *sod1* (superoxide dismutase 1), a detoxifying enzyme, mainly located in the forebrain and hypothalamus. Same down-regulations were encountered for rainbow trout fed with a diet enriched with ω-3 long-chain polyunsaturated fatty acids, which is known to have strong anti-inflammatory properties (Roy et al., 2020a; 2020b). Similar results were also found for zebrafish provided with auditory enrichment (classical music) (Barcellos et al., 2018), but in a lesser extent than with feeding predictability in rainbow trout, suggesting that this strategy as cognitive enrichment have a strong anti-oxidative and anti-inflammatory effect. The mRNA expression of both *tmem119* - microglial cell marker – and *gfap* - astrocyte marker – was also decreased in the forebrain, two genes that mediate neuroinflammation with proliferating glial cells, which can release inflammatory cytokines such as TNF-α and IL-1β, and thus increase the inflammatory response (Douglass et al., 2017). This further suggests that rainbow trout with predictable feedings exhibited fewer inflammatory episodes. Gene expression results are consistent with the lower levels of aggression during the repetitive feeding periods, probably resulting in the tendencies observed for fewer fin erosions and fewer bacterial infections encountered with feeding predictability. This reinforces the hypothesis of a lower stress experienced by rainbow trout in an environment where feeding is predictable.

Surprisingly, basal plasma cortisol was higher in BUBBLE + TIME fish compared to RANDOM fish. This result differs from other studies which showed that goldfish (Vera et al., 2007) and sea bream (Cerqueira et al., 2017; Sánchez et al., 2009) subjected to predictable feeding schedules had lower cortisol levels than fish raised under random feeding schedules. When considering brain gene expression involved in the cortisol pathway, three (*crf, mrap-1, mrap-2*) of the five investigated genes were down-regulated in forebrain and/or hypothalamus. This is in accordance with a recent study showing that rockfish (*Sebastes schlegelii*) subjected to environmental enrichment have reduced brain expression of *crf* and of several genes associated with glucocorticoid receptors (Zhang et al., 2023). The higher basal plasma cortisol levels observed in our study may therefore be due to our fish sampling procedure, which was conducted prior to the novel-tank test and required fish to be fed in a single feeding the previous day. This may have caused stress in these fish, which had always been fed on a predictable feeding schedule of five daily meals, unlike the RANDOM fish, which were used to unpredictability. Furthermore, poststress cortisol levels and behavioural responses of the fish when subjected to the novel-tank test, including their stress-induced anorexia, were similar between the two treatments. The large inter-individual variations may mask any differences between treatments. In future studies, the use of dorsal aorta cannulation and/or physiological tags allowing measurement of indicators of systemic stress and swimming activity in the same fish before and after a stressor would avoid such variations and may result in more consistent data. This would however require the use of older fish capable of withstanding surgery.

Genic expression of *tph2* (tryptophan hydroxylase 2) – a rate-limiting enzyme in the biosynthesis of serotonin from tryptophan – was increased in the hypothalamus of rainbow trout reared on predictable feedings compared to fish reared on unpredictable feedings. TPH2 has been found to be a reliable indicator of chronic stress, as suggested by Shimomura et al. (2019) in medaka (*Oryzias latipes*), which showed a decrease in the mRNA expression of both *tph1* and *tph2* after repeated heat stress exposures. Furthermore in rainbow trout, low levels of catecholamines/indolamines have been observed for fish reared at low stocking densities, in addition to altered survival, growth, feed efficiency, social behaviour and increased stress, suggesting that the alteration of their brain levels as possible indications of "psychological" stress (Roy et al., 2021). All of these previous studies suggest that fish in our study may have been chronically stressed under the unpredictable feeding condition, which is consistent with our previous conclusions. However, although our findings are novel, it would be prudent in a future study to examine further the physiological status of rainbow trout through protein analyses using HPLC for brain neurotransmitters, ELISA kits for cytokines, and kits for oxidant enzymes. Additionally, the use of immunohistochemical staining would enable the visualisation of microglia, which play a significant role in inflammation resolution.

Conclusions

We found that bubbles are a prominent conditioned stimulus for predicting feeding, as indicated by the strong anticipatory activity of BUBBLE + TIME fish during their diffusion, which persisted throughout 88 days of conditioning. The increased activity of BUBBLE + TIME fish prior to feeding periods compared to neutral periods is also consistent with temporal anticipation of feeding. However, fish group activity before feedings did not differ between treatments, indicating a potential overshadowing of the time predictor by the reliable bubble signal when rainbow trout are subjected to long-term conditioning to feeding. Overall, more evidence supports improved welfare and health status in BUBBLE + TIME fish. Conversely, rainbow trout subjected to random feeding schedules were in a constant state of expectation of the upcoming feeding, whereas BUBBLE + TIME fish showed reduced swimming activity at neutral periods. This might have caused the more frequent agonistic behaviours, burst of accelerations, and jumps observed before feedings and after feeding omissions in RANDOM fish and the decreased brain mRNA expression of the dopaminergic system and the increased expression of GABAergic and glutamatergic pathways. Fish from the BUBBLE + TIME treatment tended to present a less eroded dorsal fin and fewer colonies of *Flavobacterium psychrophilum* isolated from the kidneys and skin surface. These results align with the down-regulations observed in brain mRNA expression involved in inflammatory and immune responses. The cortisol-related stress response in the brain of BUBBLE + TIME fish appeared to be less pronounced compared to RANDOM fish. These results imply that feeding predictability plays a major role in the health status of fish and that unpredictable feedings may cause chronic stress in fish. The growth parameters were unaffected after 88 days of conditioning. According to the multidisciplinary results of this study, the combination of both the signalled and the temporal predictability of feeding appears to be a promising cognitive enrichment strategy to protect brain function and enhance the welfare of farmed rainbow trout in the long term. Given the non-negligible beneficial effects on the welfare and health status of rainbow trout, including the prevention of flavobacteriosis, a recurrent and widespread disease causing high mortality and which is often the gateway for serious skin lesions and fin damages, further studies are needed to investigate this cognitive enrichment strategy under real farm conditions and on other farmed fish species. If the effects are confirmed, this cognitive enrichment may have a significant positive economic and fish welfare impact for the aquaculture industry.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2024.101081.

Ethics approval

The study design was approved by the Ethics Committee (CEFEA-74) of INRAE 'Comité d'éthique Finistérien en Expérimentation Animale' and has received authorisation from the French Ministry of Education, Research, and Innovation under the agreement number 2020-C2EA74-VC-01-V2 (18th December 2020). This study was carried out in compliance with the ARRIVE 2.0 guidelines, and with the Guidelines of the National Legislation on Animal Care of the French Ministry of Research (Decree No 2013-118, 1 February 2013) and following EU legal frameworks relating to the protection of animals used for scientific purposes (i.e., Directive 2010/63/EU). The scientists in charge of the experiments received training and personal authorisation.

Data and model availability statement

The data/models were not deposited in an official repository. The data are available on request from the corresponding author.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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Declaration of interest

None.

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