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The Behavioral and Neurobiological Response to Sound Stress in Salmon

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Sound pollution · Tubercular nuclei · Neuronal activity · Brain neurochemistry

Abstract
Introduction: Noise associated with human activities in aquatic environments can affect the physiology and behavior of aquatic species which may have consequences at the population and ecosystem levels. Low-frequency sound is particularly stressful for fish since it is an important factor in predator-prey interactions. Even though behavioral and physiological studies have been conducted to assess the effects of sound on fish species, neurobiological studies are still lacking. ***Methods:*** In this study, we exposed farmed salmon to low-frequency sound for 5 min a day for 30 trials and conducted behavioral observations and tissue sampling before sound exposure (timepoint zero; T0) and after 1 (T1), 10 (T2), 20 (T3), and 30 (T4) exposures, to assess markers of stress. These included plasma cortisol, neuronal activity, monoaminergic signaling, and gene expression in 4 areas of the forebrain. ***Results:*** We found that sound exposure induced an activation of the stress response by eliciting an initial startle behavioral response, together with increased plasma cortisol levels and a decrease in neuronal activity in the hypothalamic tubercular nuclei (TN). At T3 and T4 salmon showed a degree of habituation in their behavioral and cortisol response. However, at T4, salmon showed signs of chronic stress with increased serotonergic activity levels in the dorsolateral and dorsomedial pallium, the preoptic area, and the TN, as well as an inhibition of growth and reproduction transcripts in the TN. ***Conclusions:*** Together, our results suggest that prolonged exposure to sound results in chronic stress that leads to neurological changes which suggest a reduction of life fitness traits.

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Introduction

In salmonids, as in other fish species, sound is detected both in the inner ear by the otoliths and by the lateral line which acts as an accessory hearing organ [1]. Fish are able to establish directional responses to oscillations and acceleration, such as those produced by predators and prey as they swim [1, 2]. Importantly, the sound frequency

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caused by a fish swimming through water is below 10 Hz and as such, many fish species have a high sensitivity for low frequencies (i.e., infrasound) [1–5]. In this context, it has been reported that salmonids display strong awareness and avoidance to infrasound, and it has been proposed that this has evolved as an important factor in predator-prey interactions [1–5].

Noise associated with human activities in aquatic environments, such as boat traffic, construction work, hydroelectrical power plants, pumps, seismic exploration, among others, has increased exponentially since the 1990s [6]. This is a problem for the majority of aquatic species since sound travels approximately 1,500 m/s in water compared to approximately 340 m/s in air [6], and travel over large areas, which may have consequences at the population and ecosystem levels [7]. Even though several studies have assessed some of the effects of sound stress on behavior and physiology of several fish species e.g., [8, 9], there is little information regarding how noise stress affects neural systems and their regulation.

Studies conducted on salmonids report that fish respond with a behavioral startle response to low frequencies (above 10 Hz) at intensities of 10–15 dB re 1 μPa, while higher frequencies (e.g., 150 Hz) do not evoke a response [2–5]. Furthermore, a behavioral habituation to sound stress was reported after 20 trials in which individuals did not show a startle response but instead simply avoided the area immediately adjacent to the sound source [4]. However, it is yet unclear if habituation to sound stress implies a reduction in hearing sensitivity accompanied by a lower physiological stress response, such as plasma cortisol or brain monoamine levels or simply a change in behavioral tactics from startle response to avoidance. Importantly, commercial aquaculture activities represent a major source of anthropomorphic noise pollution for farmed and wild fish [10, 11]. Farmed fish are especially vulnerable to potential sound stress since they are confined to tanks or cages where it is not possible to avoid sound sources [10, 11].

The purpose of this study was to assess the immediate behavioral, physiological, and neurobiological response to sound and explore possible habituation responses over 30 trials. We therefore exposed farmed salmon to a sound source below 10 Hz once a day and sampled fish throughout the 30 trials. We assessed their behavioral response, cortisol levels, neuronal activity throughout the whole brain, as well as monoaminergic activity and total transcriptome analysis (i.e., RNAseq) in specific areas in the brain. We hypothesized that salmon would react with whole system startle responses and acute elevations in plasma cortisol and brain monoamine activity to the initial sound exposures, but that behavioral, neurochemical, and physiological habituation to sound would be evident towards the end of the experimental trials.

Materials and Methods

Experimental Fish

Atlantic salmon from the AquaGen strain were hatched, fed at the Institute of Marine Research Matre Research station and reared in tanks according to size up until smoltification. The fish were moved into a 15 m^3 tank (Ø = 5 m, water depth approx. 76 cm) supplied with aerated seawater from 90 m depth at a temperature of 8.6°C, with an approximate flow rate of 220 L/min, a natural photoperiod supplied with lamps (Philips HC-50W) above tanks and >80% O_2 saturation. Surplus feeding was applied in all tanks using Skretting Optiline-feed, size 4 and feed provided by automatic Betten feeders during the light hours. When fish were 20 months from hatching and about 1.9 kg, as 1+ postsmolt, they were tagged and moved to 3 of the described 15 m^3 tanks (n = 100 fish/tank) and used for the experiment maintaining the same conditions as described above. All fish were caught from the rearing tank, sedated, and individually marked with colored floy tags (standard anchor T-bar [TBA], Hallprint.com) with color beads arranged in 100 different combinations melted on the marker for each replicate tank. In addition, a subset of fish were sampled at this point (see sampling details further below). The fish were left undisturbed for 22 days to acclimatize between tagging and the start of the experiment.

Experiment Set-Up

The experimental design consisted of subjecting the fish to a low-frequency sound source (approximately 10 Hz) 5 times per week for 5 min for 42 days between 8:30 a.m. and 9:30 a.m. (in order to provide predictability of the stimuli which in turn may promote control of the stressor and habituation) [12]. Note that in total, the sound exposure was conducted 30 times during the 42-day experimental period since there were no sound exposures during the weekends. For this end, we made six low-frequency underwater sound generators, based on the model by Knudsen et al. [2]. In short, a black plastic tube with a 25-mm-diameter plastic piston in the front was set to 10 Hz by running RS Pro AC 400 V motor at 600 rpm (see schematic online suppl. Fig. S1 for further details; for all online suppl. material, see https://doi.org/10.1159/000539329). Two machines were attached on opposite sides of the tanks with the piston face angled 30° toward the tank center and positioned approximately 20 cm below the water surface. Video recordings were taken 3 times a week (including sampling days) by three Go Pro Hero 10 video cameras which were mounted on a Ø = 20 cm disc positioned in the middle of each tank covering 360°. The cameras were manually started 10 min before and stopped 5 min after sound exposure.

Sampling

A total of 106 fish were sampled throughout the experiment at different timepoints. That is, fish were sampled at the start of the acclimation period (Timepoint 0; T0, n = 22 fish), and after being subjected to 5 min of sound (n = 7 fish per tank for a total of 21) one (T1), 10 (T2), 20 (T3), and 30 (T4) times (online suppl. Fig.

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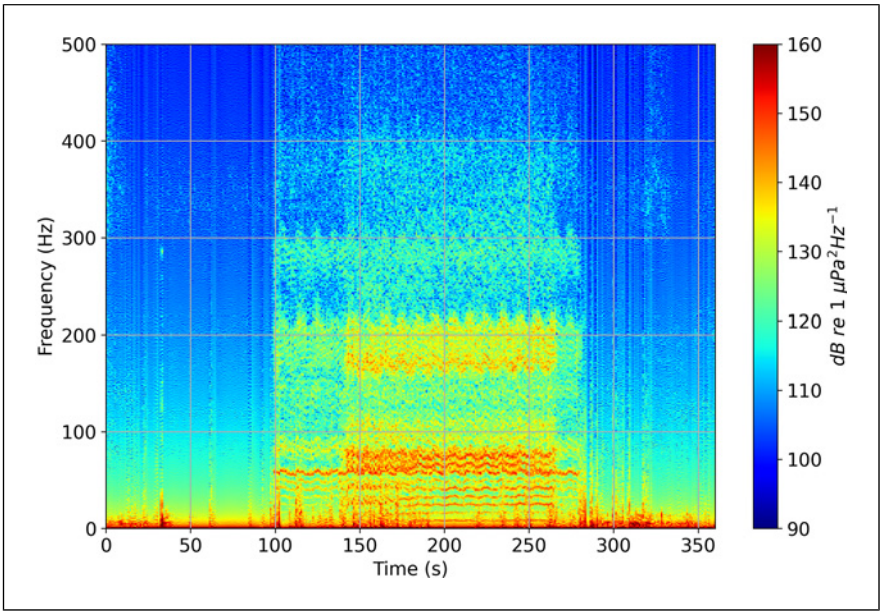

Fig. 1. Power spectral density of the hydrophone (channel *O* of the acoustic vector sensor) of the tank soundscape with and without the sound generators switched on. It was calculated with a segment length of 1 s, Hanning window, and 50% overlap. The period depicted here begins with no sound generator switched on (0–100 s). The first sound generator (250 cm away) is switched on at 100 s and off at 255 s, while the second sound generator (50 cm away) is switched on at 140 s and off at 260 s.

S2). At each sampling, fish from each tank were netted at the same time and directly transferred into a 200 L container with a lethal concentration of MS222 (Finquel vet, 2 g/L; Western Chemical Inc, Washington, DC, USA). When fish showed no signs of life (approximately within 10 s), the fish were individually recognized by their floy tag, measured, and weighed, before a blood sample was taken from their caudal vein with heparinized syringes (to prevent coagulation). The blood samples were centrifuged for 5 min at 10,000 rcf and 4°C. Plasma samples were placed in individually marked Eppendorf tubes and immediately frozen in dry ice and stored at −80°C. Fish were then decapitated, and their brains quickly excised (within 2 min) and sampled in two different ways: (1) whole brains were placed in 4% paraformaldehyde (PF) in 0.1 mol/L Sørensen's phosphate buffer (PB; 28 mmol/L NaH_2PO_4, 71 mmol/L Na_2HPO_4, pH 7.2) for 62 h at 4°C. The tissue was then washed three times for 20 min in PB, cryopreserved between 24 and 32 h in 25% sucrose in PB at 4°C, embedded in Tissue-Tek O.C.T.-Compound (Sakura Fintek) and stored at −80°C until sectioning for immunohistochemistry (n = 9 per timepoint) and (2) brains were immediately placed into a mold and frozen on dry ice in Tissue-Tek O.C.T.-Compound (Sakura Fintek) and stored at −80°C until sectioning and microdissection for monoamine neurochemistry and gene expression analyses (n = 12 per timepoint).

Sound Measurements

To characterize the soundscape experienced by the salmon with or without sound generators, sound intensity and direction was measured using an acoustic vector sensor (VHS-100, Ocean Applies Acoustic-Tech, China) suspended by rubber bands in midwater at various positions within each of the three tanks without fish. This system consists of three orthogonal accelerometers and one hydrophone, with four output channels: the directional channel X, Y, Z for particle acceleration, and one omnidirectional sound pressure channel (channel O) with a sensitivity of −178 dB re 1 V/µPa (20–4,000 Hz). Measurements were made in at least 8 positions in the central part of the tank between the two sound generators (~50–150 cm from the nearest sound generator at ~50 cm increments). Measurements were also made at one additional midwater position 50 cm from the tank wall, equidistant from the two sound generators (~250 cm), to approximate the quietest conditions available to fish while the sound generators were running. At each position, measurements were made of the ambient tank soundscape with and without the sound generators switched on. At least 10 s of data were collected at each position, with and without the sound generators running, sampled at 10 kHz and decimated to a sample rate of 2 kHz without loss of integrity (Fig. 1).

Mean sound pressure levels associated with the primary signal (5–15 Hz) ranged from 142 to 146 dB re 1 µPa near the sound generators (50–75 cm) and approached ambient levels (~136–137 dB re 1 µPa) at the most distant position (250 cm; Fig. 2). The maximum 1 s mean sound pressure level recorded was 169 dB re 1 µPa over 5–500 Hz at 50 cm from the nearest noise generator, while the median 1 s mean sound level in the same position was 132 dB re 1 µPa with the sound generator switched on.

Behavioral Analysis

Video recordings taken during the sampling days were used to quantify the behavioral response to low-frequency sound. Behavioral observations were made 30 s prior to the sound generators being switched on and again 60 s after the sound generators were switched on. At each of these timepoints, counts were made of individuals who were, or were not, exhibiting typical swimming behavior (i.e., holding their position against the water current). The number of fish scored was dependent on the number residing within the field of view of the camera (mean 8.5 fish, range 0–33 fish).

We also observed the behavior of individuals at the moment when the sound generators were switched on. This involved identifying the first individual within the video field of view to respond to the new sound and scoring the strength of the response exhibited by that

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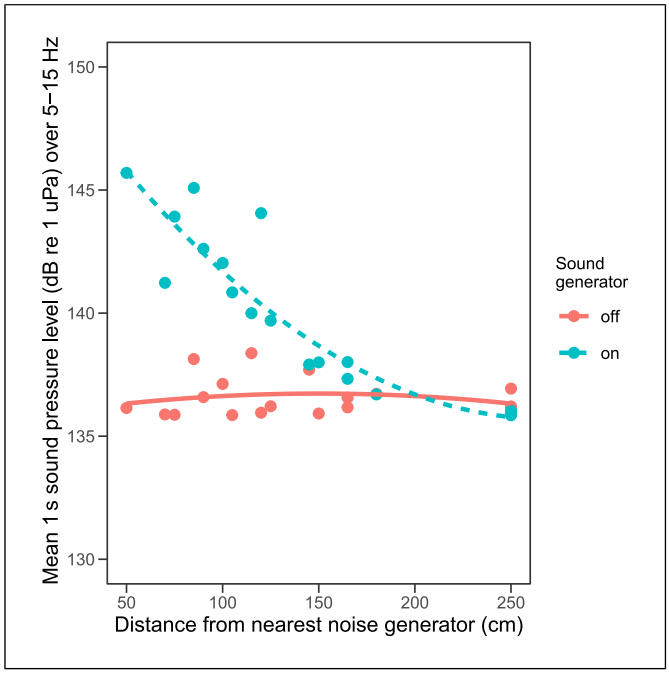

Fig. 2. Mean sound pressure level according to the distance between the acoustic vector sensor and the nearest sound generator. Values are pooled among the three tanks, and color-coded to indicate whether the sound generator was switched on or off. The effect of distance is illustrated by first-order polynomial fits.

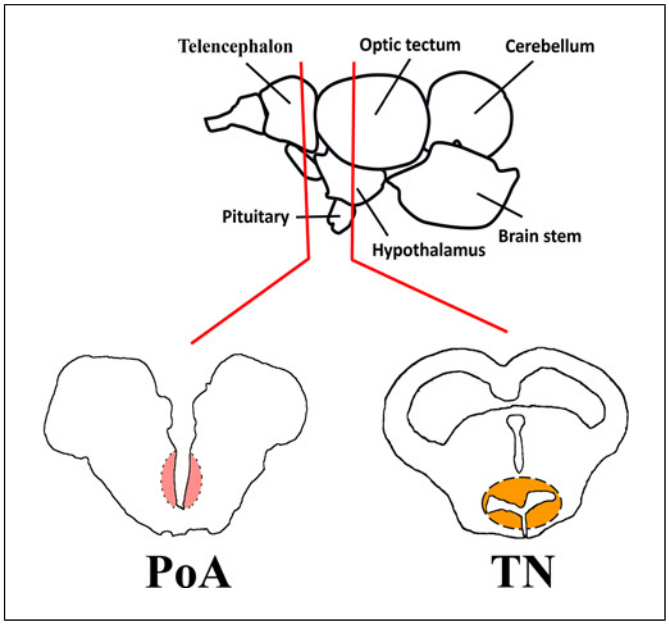

Fig. 3. Atlantic salmon brain. Sagittal view with the regions of interest (ROI) indicated by red lines. Below is the transverse view of ROI examples: on the left the preoptic area (PoA) and on the right the tubercular nuclei (TN).

individual on an ordinal scale: 0 = no change/response; 1 = changed direction and/or moved away from the sound source without increasing swimming speed; 2 = as for 1, but with an increase in swimming speed; 3 = teleost startle response (i.e., vigorous tail flip by which fish rapidly change their direction [13]).

Brain Sectioning and Immunohistochemistry

Brain sections were obtained by using a Leica CM3050 cryostat (Leica, Wezlar, Germany) set at −24°C. The thickness of the slices was set to 14 µm and thaw mounted onto marked glass slides (Fisherbrand™ Tissue Path Superfrost™ Plus Gold Slides). The glass slides were then placed in an oven at 65°C for 10 min and stored at −80°C for further analysis.

Immunohistochemistry for the neuronal activity marker extracellular signal-regulated kinase (pERK) was conducted on 6 fish per time point (T0–T4, for a total of 30 samples). The protocol was adjusted from Randlett et al. [14] and included both positive and negative controls. Notably, this protein is highly conserved in vertebrates [15] and this antibody has been successfully used to stain pERK in several mammalian, amphibian and fish species. The slides were left at room temperature for 1 h before antigen retrieval by incubation in 150 mM Tris-HCL (pH 9) for 10 min at room temperature in a plastic container with a lid, followed by heating at 70°C for 25 min and 5 min cool down at room temperature. Next, the slides were washed 3 × 5 min in PBST (Phosphate buffered saline +0.1% Tween) in Couplin staining jars. A PAP-pen (Abcam, Cambridge, United Kingdom, Cat. ab2601) was used to mark the border of the slides to create a hydrophobic barrier. Subsequently, the tissue was blocked in a blocking solution consisting of PBST +1% Bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) + 2% Normal Goat Serum (Invitrogen, USA) + 1% dimethyl sulfoxide (DMSO; Sigma-Aldrich) in the staining jars for 1 h. Following this, the slides were placed in a humid chamber and the pERK primary antibody (Cell Signaling, # 4370) was added by pipetting. The primary antibody was diluted 1:500 in 200 µL × slide of PBST +1% BSA +1% DMSO. The primary antibody was incubated at 4°C overnight. The following day, the slides were rinsed off by washing three times with 1,000 µL PBST. The slides where then immediately soaked in PBST for 15 min, followed by soaking twice in Phosphate buffered saline (PBS) for 15 min. The slides were then placed in the humid chamber and the secondary antibody (Alexa Fluor 568 Donkey anti-rabbit IgG red; catalog #A10042; Thermo Fisher Scientific, Waltham, MA, USA) was added by pipetting, followed by incubation for 45 min at room temperature in the dark. The secondary antibody was diluted 1:500 in 200 µL per slide in PBS +1% BSA. Following incubation, the slides were placed in the staining jar and washed three times with PBS for 5 min. Vectashield® antifade mounting medium with DAPI (Vector Laboratories, Newark, CA, USA) was added before mounting the cover glass. Finally, the cover glass was sealed to the slides by applying transparent nail polish around the edges and stored at 4°C until photographed.

IHC Quantification

All the slides from the pERK immunostaining were sent to the Norbrain Slidescanning Facility (Institute of Basic Medical Sciences, University of Oslo) and high-resolution images of the histological sections were acquired using an automated slide

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scanning system (Axio Scan Z1, Carl Zeiss Microscopy, Munich, Germany). The images were inspected using the Zeiss ZEN Lite Blue software (Carl Zeiss Microscopy) and saved in a CZI format. Following this, the Zen Lite Blue software was used to adjust brightness and contrast to obtain individual TIFF format pictures per slice for further analysis.

The Fiji platform [16] in ImageJ2 [17] were used to quantify the pERK-labeled cells. An initial qualitative analysis of two whole brain samples per timepoints (T0 and T1) was used to identify regions of interest (ROIs) and these areas were identified using salmonid stereotaxic atlases [18–22]. Consequently, cropped images for each ROI were used to quantify the immunoreactive cells for six samples at T0, T1, T2, and T4 for the preoptic area (PoA) and the whole tubercular nuclei (TN; which includes the NAT, nucleus anterior tuberis; the NPT, nucleus posterior tuberis; and the NLT, nucleus lateralis tuberis) of the hypothalamus (Fig. 3). The IHC toolbox [23] plugin for ImageJ was used to quantify the immune stained cells. A model was first created on representative pictures for all areas and was then used for analysis of the immune-stained cells for all areas. The IHC toolbox plugging model was then used to obtain binary pictures of all recognized pERK immunoreactive cells. All labeled cells were then counted using the "analyze particles" function in imageJ. The number of immunoreactive cells was counted within the ROI for both lobes in each section and pooled together (since there were no lateralization differences). All sections for each fish brain sample were pooled together into an average number of labeled cells per fish which was further used in the statistical analysis (online suppl. Fig. S3). Notably, this represents the relative neuronal activity since it is not possible for us to claim that we have counted every single possible activated cell within the neuronal population for each ROI and each fish.

Brain Sectioning and Microdissections

Brain sections were obtained by using a Leica CM3050 cryostat (Leica, Wezlar, Germany) set at −24°C. The thickness of the slices was adjusted to 150 μm and thaw mounted onto marked glass slides (Fisherbrand™ Tissue Path Superfrost™ Plus Gold Slides) and kept cold inside the cryostat before being stored at −80°C until further processing.

A total of 12 brain samples per timepoint were processed for microdissections. The glass slides were kept on an RNase-cleaned cold stage set at −14°C. Using a stereo microscope, four areas were microdissected using a 23-gauge needle: the forebrain dorsolateral (Dl) and dorsomedial (Dm) pallium, the preoptic area (PoA) and the tubercular nuclei (TN). The number of punches (i.e., microdissections from each area) was on average 17 for the Dl, 11 for the Dm and 10 for the PoA and TN (the average represents all slices for each ROI for each sample for all timepoints). Microdissected tissue for each area was collected in a 2 mL homogenizing RNase-free tube containing 4 beads and 100 μL of sodium acetate buffer containing an internal standard (3-4-dihydroxybenzyl amine hydrobromide; DHBA) for monoamine analysis. Subsequently, the samples were placed on dry ice before storage at −80°C.

Monoaminergic Neurochemistry

The frozen samples were thawed and homogenized on a Precellys evolution touch homogenizer (Bertin Technologies, France) at 5,500 rpm for 20 s. Following this, samples were transferred to 1.5 mL Eppendorf tubes and centrifuged for 10 min at 20,000 rcf and 4°C. The supernatant was separated for analyzing monoamine neurochemistry by high-performance liquid chromatography (HPLC), while the remaining pellet was dissolved in 350 μL RLT buffer (QIAGEN RNeasy® Plus Micro Kit) before being refrozen at −80°C for further RNA extraction and protein concentration analysis (see next subsections for further details).

The HPLC system consisted of a mobile phase with 86.27 mM sodium dihydrogen phosphate (NaH_2PO_4), 3.7 μL ethylenediaminetetraacetic acid (EDTA), and 0.81 mM sodium octyl sulfate ($C_8H_{17}NaO_4S$) in deionized water (resistance 18.2 MW) with 7% acetonitrile at pH 3.1. The system was composed of a solvent delivery system (Shimadzu, LC-10AD), an autoinjector (Famos, Spark), a reverse-phase column (4.6 × 100 mm, Hichrom, C18, 3.5 mm), and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at −40 and +320 mV. For oxidizing possible contaminants before analysis, a conditioning electrode with a potential of +40 mV was used. Brain serotonin (5-HT), dopamine (DA) and their main catabolites 5-hydroxyindoleacetic acid (5-HIAA) and 3,4-dihydroxyphenylacetic acid (DOPAC), respectively, were quantified by comparison with standard solutions and corrected for recovery of the internal standard using the Clarity HPLC software (CSW, DataApex Ltd, the Czech Republic). Final concentrations were corrected for the amount of protein recovered for each sample (obtained by Bradford protein analysis as explained below). In addition, the ratio between catabolite and neurotransmitter was calculated as a proxy for monoaminergic activity (e.g., [24]).

RNA Extraction

The RNA extraction of the TN samples was conducted using the RNeasy® Plus Micro Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Concentration of the samples was measured using an Epoch microplate spectrometer (Biotek Instruments, Winooski, VT, USA) and was calculated with the Gen 5 3.00 software (BioTek® Instruments, Inc). RNA integrity (RIN score) was quantified using the Agilent RNA 6000 Pico Kit according to manufacturer's protocol (Agilent, Santa Clara California, USA), with scores ≥8, indicative of excellent RNA quality. RNA samples were kept at −80°C until further analysis.

Transcriptome Sequencing

Sequencing of total RNA in the TN was completed by Novogene (England). After additional quality testing at Novogene, total RNA samples were enriched with oligo (dT) magnetic beads for extraction of mRNA. First-strand cDNA was synthesized by randomly fragmenting the mRNA in a fragmentation buffer, combining it with random hexamers and assembling it with M-MuLV reverse transcriptase. Complementary strands were then synthesized by nick translation using a custom (Illumina) synthesis buffer containing dNTPs, RNase H and *Escherichia coli* polymerase I. The resultant cDNA library underwent adapter ligation, terminal repair, poly A-tailing, size selection and PCR enrichment, before a final quality assessment—concentration by Qubit 2.0 fluorometer (Life Technologies), insert size by Agilent 2100 Bioanalyzer and quantification by qPCR. Libraries were sequenced as 150bp, paired-end reads on an Illumina Hiseq 2500 instrument. For sequencing analysis, the samples for each group were compared to each other to identify common regulated genes.

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Read Mapping and Quantification

All analyses in this section were completed on a Linux server using command-line operations. Quality metrics, such as phred quality score, duplicate reads, and adapter contamination, were generated for each sample using Fastqc v0.11.8 [25] and the sample qc reports were collated using multiqc v1.9 [26]. As the sequence data had already been cleaned at the Novogene sequencing facility, quality across all bases was high (>30 phred score) and no adapters were present, so a quality trim was not required. Reads were aligned to the Atlantic salmon Ssal_v3.1. (April 21, 2021) reference genome using HISAT2 v2.2.1 [27]. Reads that aligned to gene regions (as per genome feature definitions in ICSASG_v2 annotation) were counted using featurecounts v2.0.1 [28]. This count table formed the basis for gene expression analysis, which was performed using various R Statistical Software (v4.3.2: R Core Team 2023) packages.

Differential Expression Gene Analysis and Functional Annotation

In order to examine for batch effects and outliers, sample variance was estimated based on read count density, principal component analysis (PCA) and Euclidean distance, using base R functions "density" and "prcomp," and visualized using ggplot2 [29] and pheatmap [30]. Differential expression of genes (DEGs) between treatments was estimated with the DESeq2 package [31], which uses a negative binomial generalized linear model and shrinkage estimation of expression differences, designed to detect even small expression changes. Significantly ($p < 0.05$) different DEGs were estimated via a Wald test and then p values were adjusted for false discovery using Benjamini-Hochberg [32].

The R package clusterProfiler 4.10.0 [33] was used to test for enrichment of DEGs in KEGG (Kyoto Encyclopedia of genes) pathways and GO (Gene Ontology) terms. Primary gene identifiers (Entrez ID) were annotated to gene symbols and other identifiers within the Ssal_v3.1 reference genome database using the AnnotationHub 3.10.0 package [34].

Bradford Protein Analysis

The flow-through following RNA extraction was treated with 1,400 µL ice-cold acetone and incubated for 30 min at −20°C. After centrifuging for 10 min at 20,000 rcf, the pellets were washed with 100 µL ice-cold ethanol before resuspension in 20 µL 0.4 NaOH buffer. The resuspended pellets were used for Bradford protein assay analysis, as described by Vindas et al. [35] and used in the final calculation of monoamine concentrations.

Analysis of Plasma Cortisol

Cortisol in plasma from EDTA-treated blood was analyzed using a commercially available DetectX® cortisol enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, USA) previously validated for Atlantic salmon (see manufacturer's website for further details: https://www.arborassays.com/product/k003-h-cortisol-eia-kit/#publications), following the manufacturers' protocol. The absorbance of the prepared ELISA plate was read in a plate reader at 450 nm and the concentrations were calculated using the four-parameter logistics curve.

Statistics

All statistical analyses were done using RStudio software v. 4.3.2 (R Development Core Team, http://www.rproject.org). To compare swimming behavior with or without the sound generators operating, and also test for a hypothesized effect of acclimation over time, the binomial response variable was fitted with a generalized linear model (quasibinomial family, logit link function) using R [36]. The model contained fixed effects for the trial day (range 1–42), treatment (before or during sound exposure), and tank identity, including full interactions. Repeated observations within each tank (and potentially of the same fish) over time are not truly independent, although the tank effect was not significant and was not involved in any significant interaction effects. Model fit was assessed using simulated residual plots generated using the DHARMa package [37], and the model was interpreted using an analysis of the deviance table (car package [38]), followed by extraction of condition predictions (i.e., marginal means) using the ggeffects package [39]. To analyze the immediate response to sound, we tested for a change in response strength over the duration of the trial by treating response strength scores as integers and fitting a linear model using the lm function in R. Trial day and tank identity were used as fixed effects, including an interaction effect. Exploratory plotting provided no evidence of a non-linear effect of trial day on response strength. The model was assessed and interpreted similarly to the previous model.

The cortisol, pERK neuronal activity, and monoamine neurochemistry were analyzed with a linear mixed effect (LME) model with timepoint as a fixed effect and tank as a random effect. Interactive effects between groups were assessed using Tukey-Kramer honestly significant difference post hoc test. Visual inspection of the qqnorm and residual plots to check the assumptions of normality and homoscedasticity confirmed that these models conformed to these assumptions. Note that the cortisol, the Dm 5-HT, the Dm 5-HIAA/5-HT, the Dm DA, the PoA DA, the TN 5-HT, the TN 5-HIAA, the TN 5-HIAA/5-HT, and the TN DOPAC/DA were log-transformed to achieve homoscedasticity. Data outliers for both cortisol and monoamine neurochemistry data were determined by the Rosner test (setting the K value at 4). Significance was assigned at $p \leq 0.05$ and data is presented as mean ± SEM. All data can be found in the online supplementary data file 1.

Results

Behavioral Response to Sound Stress

There was a significant interaction between treatment and day ($\chi^2_{(1)} = 6.63$, $p < 0.01$) which indicates that the prevalence of fish displaying normal behavior in response to sound increased over time, while there was no change in behavior over time while the sound sources were turned off. In other words, fish initially changed their swimming behavior in response to the sound generators, but this response decreased over time (Fig. 4).

To analyze the immediate response to the sound generators being started, we tested for a change in response strength over the duration of the trial. We found no significant effect of day ($F_{(1)} = 2.34$, $p = 0.13$), tank ($F_{(2)} = 1.25$, $p = 0.29$), or their interaction ($F_{(2)} = 2.22$, $p = 0.11$).

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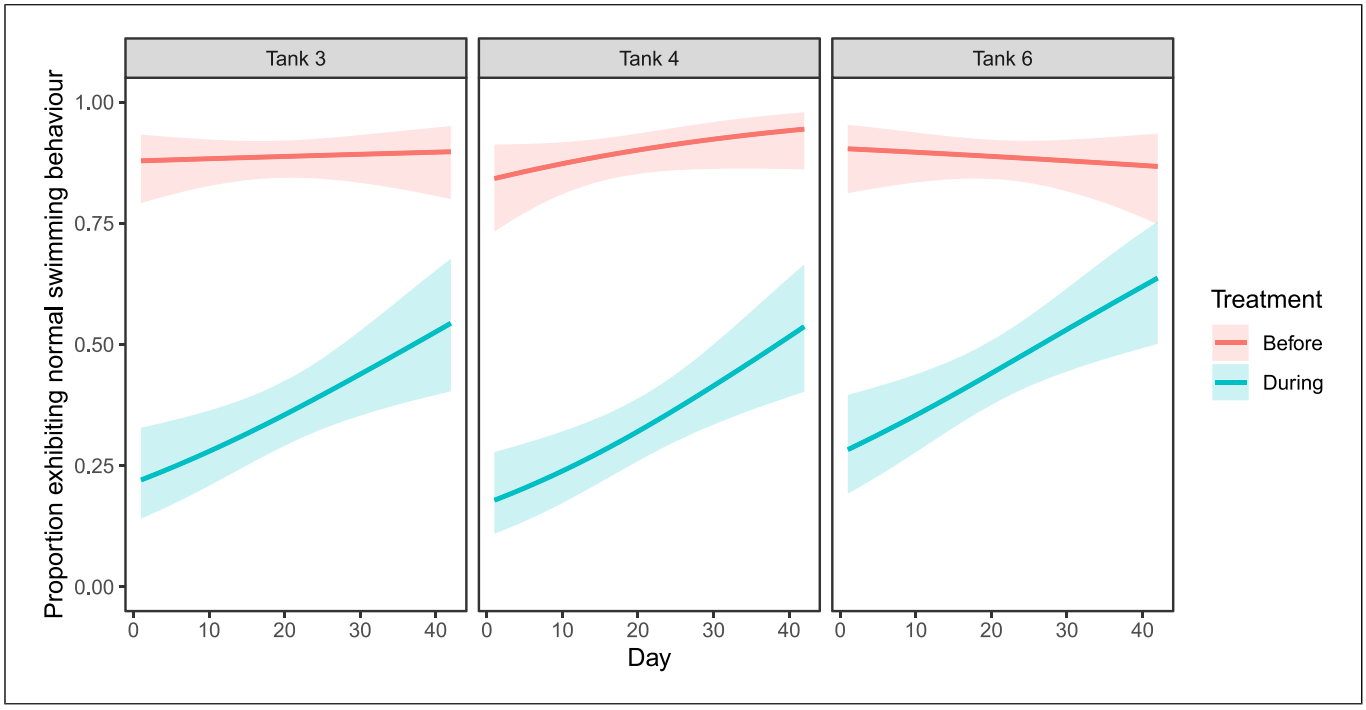

Fig. 4. Predicted proportion of salmon exhibiting typical behavior (i.e., maintaining their position in the water column swimming against the current) before and during exposure to the sound stress. The increasing proportion exhibiting typical behavior during sound exposure between Day 1 and Day 42 (i.e., after 30 days of sound stress) indicates an increasing number of fish showing normal swimming behavior.

That is, each tank population showed approximately the same startle response when the sound generators were turned on and this did not change over time (Fig. 5).

Plasma Cortisol

The blood plasma cortisol levels for a subset of fish were analyzed at timepoint 0 (no sound exposure; T0), at T1 (after the fish had experienced sound once), at T2 (after experiencing 10 rounds of sound), at T3 (after experiencing 20 rounds of sound), and at T4 (after experiencing 30 rounds of sound). There was a significant effect of time ($\chi^2_{(4)} = 80.6$, $p < 0.001$) in that the T1 values were significantly higher than all other groups: T0 ($p < 0.001$), T2 ($p = 0.003$), T3 ($p < 0.001$), and T4 ($p < 0.001$). Furthermore, levels at T2 were higher than T0 ($p = 0.01$), T3 ($p = 0.005$), and T4 ($p = 0.003$). The remaining timepoints were not significantly different from one another (Fig. 6).

Neuronal Activity

The quantification of the neuronal marker pERK in the PoA showed no significant effect of time ($\chi^2_{(3)} = 5.14$, $p = 0.16$). The quantification of pERK in the TN showed a significant effect of time ($\chi^2_{(3)} = 15.9$, $p = 0.001$) with T0 showing a higher number of stained cells compared to T1 ($p = 0.04$), T2 ($p = 0.03$), and a tendency compared to T4 ($p = 0.09$). The remaining timepoints were not significantly different from one another (Fig. 7a). For example pictures of pERK neuronal activity in the TN at T0 and T1, see Figure 7b, and for a full overview of quantified neuronal activity for all timepoints, see the online supplementary data file 1.

Monoamine Neurochemistry

We quantified monoamine neurochemistry in 4 areas of the forebrain. These areas were chosen due to either their differential activity levels observed from the qualitative analysis of neuronal activity in response to sound over the course of the experiment (i.e., the PoA and the TN), or both due to high neuronal activity levels for all timepoints, as well as their well-known function in the processing of stress and emotional stimuli (i.e., the Dl and the Dm) [40, 41].

Serotonin

There was a significant effect of time on the 5-HT concentrations in the Dl ($\chi^2_{(2)} = 8.81$, $p = 0.01$), with a tendency for lower 5-HT levels at T0, although there were

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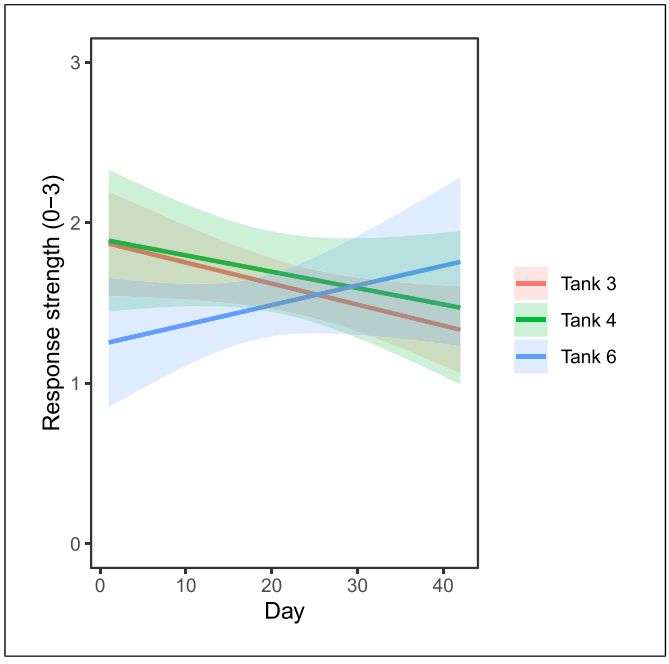

Fig. 5. Predicted response strength of salmon upon exposure to a noise generating machine according to a linear model fitted to integer-coded response strengths over time. Responses are shown separately for each tank population. The mean response strength did not significantly change over time, nor did it differ between tanks. Scale: 0 = no change; 1 = changed direction and/or moved away from the sound source without increasing swimming speed; 2 = as for 1, but with an increase in swimming speed; 3 = teleost startle response.

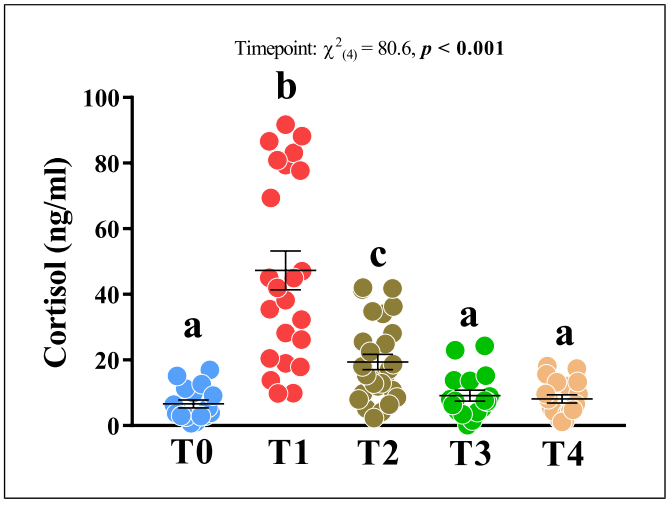

Fig. 6. Mean (±SEM) blood plasma cortisol levels of Atlantic salmon before being subjected to sound stress (T0) and after experiencing sound stress once (T1), 10 (T2), 20 (T3), and 30 (T4) times. Linear mixed effect model statistics are given in each panel. Small letters indicate posthoc significant ($p < 0.05$) differences between the groups.

no significant differences between the timepoints when using a post hoc analysis. There were no significant differences of 5-HT concentrations in the Dm ($\chi^2_{(2)} = 0.14$, $p = 0.93$), the PoA ($\chi^2_{(2)} = 1.71$, $p = 0.42$), or the TN ($\chi^2_{(2)} = 2.1$, $p = 0.35$) (Fig. 8a).

The Catabolite 5-HIAA

There was a significant effect of time on the 5-HIAA concentrations in: the Dl ($\chi^2_{(2)} = 30.1$, $p < 0.001$), with higher concentrations at T4 compared to T1 ($p = 0.02$) and T0 ($p < 0.001$), but no significant differences between T0 and T1 ($p = 0.12$); the PoA ($\chi^2_{(2)} = 11.7$, $p = 0.003$), with T4 showing higher concentrations than T0 ($p = 0.008$), but not T1 ($p = 0.18$) and no differences between T0 and T1 ($p = 0.33$); and the TN ($\chi^2_{(2)} = 10.5$, $p = 0.005$), with T0 showing the lowest concentrations compared to T1 ($p = 0.03$) and T4 ($p = 0.02$), but no differences between T1 and T4 ($p = 0.84$). There were no significant effects of time ($\chi^2_{(2)} = 3.95$, $p = 0.14$) on the Dm 5-HIAA concentrations (Fig. 8b).

The Serotonergic Activity (5-HIAA/5-HT)

There was a significant effect of time on the serotonergic activity in: the Dl ($\chi^2_{(2)} = 30.6$, $p < 0.001$), with higher activity at T4 compared to T1 ($p = 0.001$) and T0 ($p < 0.001$), but no significant differences between T0 and T1 ($p = 0.78$); the Dm ($\chi^2_{(2)} = 18$, $p < 0.001$), with T4 showing higher activity compared to both T1 ($p = 0.01$) and T0 ($p = 0.002$), but no differences between T0 and T1 ($p = 0.91$); the PoA ($\chi^2_{(2)} = 20.1$, $p < 0.001$), with T4 showing the highest activity concentrations compared to T1 ($p = 0.02$) and T0 ($p < 0.001$), but no differences between T0 and T1 ($p = 0.56$). There were no significant effects of time ($\chi^2_{(2)} = 4.47$, $p = 0.11$) on the TN (Fig. 8c).

Dopamine

There was no significant effect of time on DA concentrations in all studied areas (online suppl. Fig. S4a).

The Catabolite 3,4-DOPAC

There was no significant effect of time on DA concentrations in all studied areas (online suppl. Fig. S4b).

The Dopaminergic Activity (DOPAC/DA)

There was a significant effect of time on the dopaminergic activity in the Dm ($\chi^2_{(2)} = 11.5$, $p = 0.003$), with higher activity at T0 compared to T1 ($p = 0.02$) and T4 ($p = 0.04$) but no significant differences between T1 and T4 ($p = 0.93$). There were no other significant effects in dopaminergic activity in the remaining areas (online suppl. Fig. S4c).

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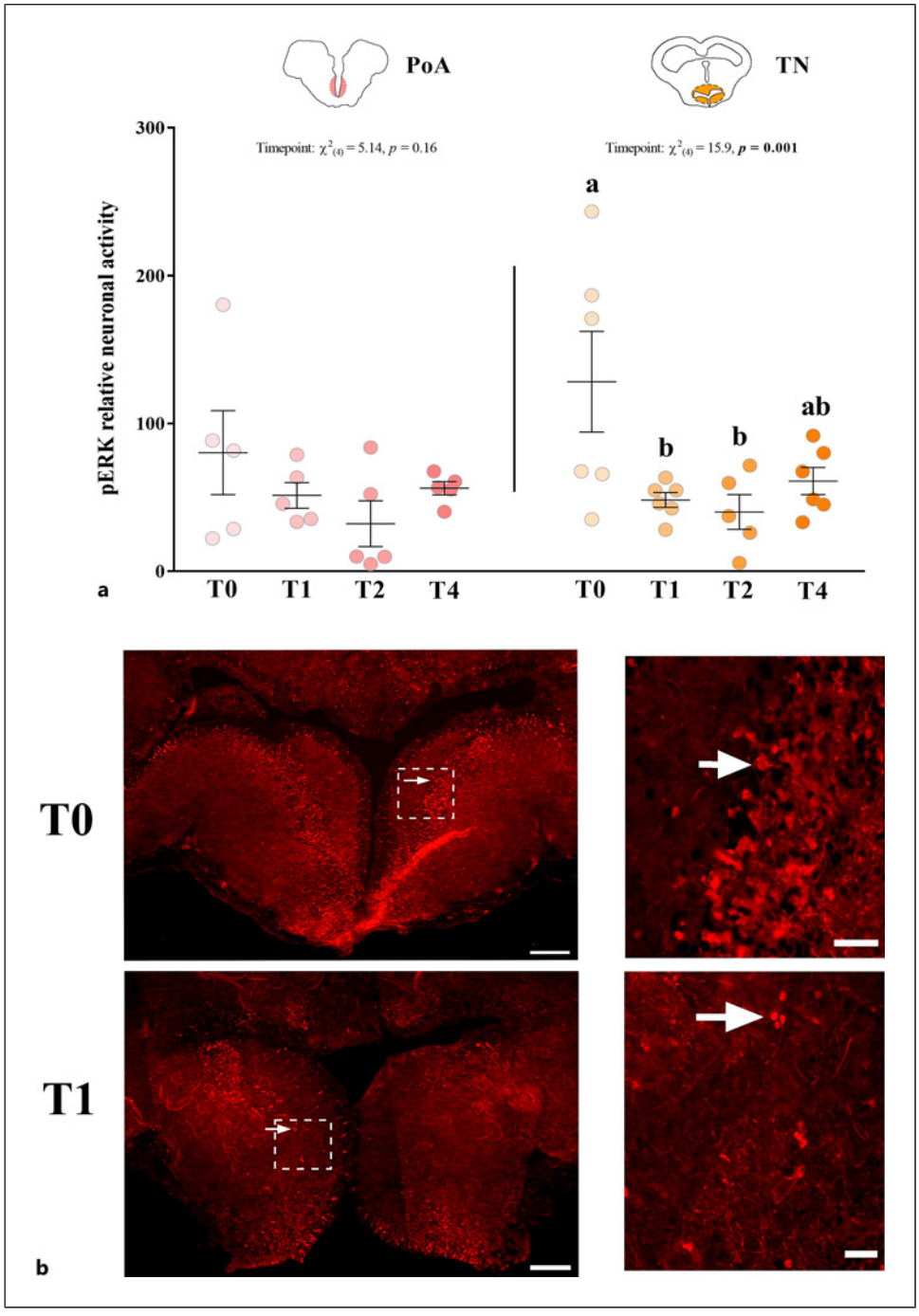

Fig. 7. a Mean (±SEM) relative number of labeled cells of the neuronal activity marker pERK in the preoptic area (PoA) and the tubercular nuclei (TN) of Atlantic salmon before being subjected to sound stress (T0) and after experiencing sound stress once (T1), 10 (T2), and 30 (T4) times. Linear mixed effect model statistics are given in each panel. Small letters indicate posthoc significant differences between the groups. **b** Example of pERK immunohistochemistry in the TN of Atlantic salmon at the start of the experiment (timepoint 0; T0) and after being subjected to 5 min of low-frequency sound (timepoint 1; T1). Arrows indicate stained cells. The square areas indicate magnified pictures to the right for each timepoint showing labeled cells. The scale bare represents 200 µm (pictures to the left) and 50 µm (pictures to the right).

Total Transcriptome Gene Expression Analysis (RNAseq)

Differential expression analysis was conducted on three different contrast groups, T0, T1, and T4. There were in total 64 genes differentially regulated between T0 and T1, 72 between T0 and T4 and 12 between T1 and T4 (Fig. 9). See online supplementary data file 1 and 2 for a complete list of all DEG's.

Over-representation analysis identified one significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway between T0 vs. T4. Enriched pathways are grouping of genes participating in same cellular biological systems, containing an over-represented number of significantly DEGs in the analyzed samples ($p < 0.05$) compared to the

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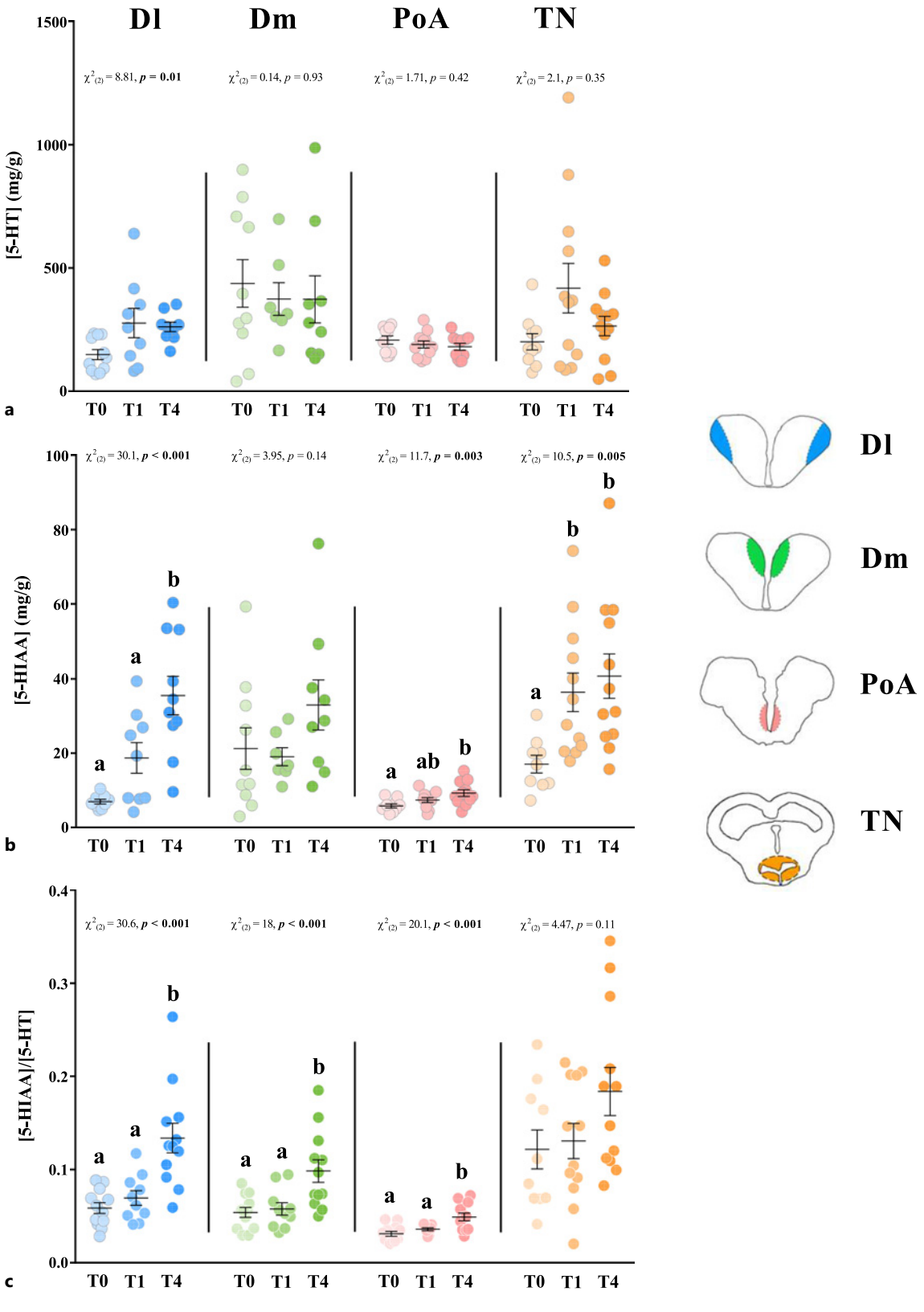

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background number of genes in each pathway. The pathway is endocrine system and is overrepresented by the downregulation of genes related to growth, see Table 1.

To identify biological processes affected by sound stress over time, a Gene Ontology (GO) overrepresentation analysis was performed by functionally annotating all DEGs with their molecular function. We found the same four GO terms to be overrepresented between T0 versus T1 and T0 versus T4 (Table 2). These genes are associated with hormone, ligand, and receptor activity and includes *prolactin* (FC −14.76 at T4), *somatotropin-2* (FC −14.33 at T1 and −10.79 at T4), *glycoprotein hormones β5* (FC −10.98 at T1 and −11.91 at T4), *α chain 2* (FC −14.3 at T4), and *α chain 1* (FC −10.27 at T1 and −9.42 at T4), *somatolactin* (FC −9.37 at T1 and −9.35 at T4), *propiomelanocortin a* (FC −3.06 at T4), *a2* (FC −3.77 at T4), and *b* (FC −8.82 at T1), see online supplementary data file 2 for a complete list.

Discussion

We found that farmed salmon respond with a startle response to a low-frequency sound source in which they changed their swimming behavior pattern. However, fish showed an increasingly normal swimming behavior towards the end of the trial suggesting either habituation or hearing loss. Notably, while the cortisol response shows signs of habituation, with peak levels during the first (timepoint 1; T1) and 10th trials (T2), serotonergic activity was found to be significantly increased at T4, suggesting an increased stress reactivity of these systems to long-term sound stress exposure. Furthermore, we found that the tubercular nuclei (TN) in the hypothalamus showed decreased neuronal activity to sound stress exposure and that this is associated with a downregulation of genes associated with growth and reproduction. Altogether, we show that exposure to 30 rounds of sound leads to altered monoaminergic reactivity levels and the downregulation of life fitness traits in Atlantic salmon, which are indicative of chronic stress.

The behavioral results from our study are in agreement with previous studies conducted on salmonid species [2–5]. That is, the immediate response of salmon to the low-frequency sound source was a startle response, in which individuals change their swimming direction to avoid the sound generator. Furthermore, we found that 1 min into the sound cue a higher number of individuals maintained their normal swimming activity in response to sound stress through the course of the experiment. This means that fewer individuals continued to respond to the sound stress. This is partly in agreement with observations conducted on Chinook salmon and rainbow trout which changed their behavioral response from startle to avoidance over the course of 20 sound exposure trials [4]. It is thus possible that the majority of salmon in our experiment started avoiding the sound source over the course of the sound exposure trials by avoiding the areas in the immediate vicinity of the sound source generators. However, the individuals who were captured on the video footage in the proximity of the sound source continue reacting with a startle response to sound exposure onset, which suggests that even after 30 trials these fish do not show a habituation to immediate sound exposure. Alternatively, it is possible that fish experienced a reduction in hearing sensitivity (i.e., temporary threshold shift) and that this was the cause of the apparent "habituation." We did not test for hearing loss in this study. However, the sound intensities recorded during sound exposure treatments were generally below levels reported to cause a temporary threshold shift in fish with swim bladders (reviewed by Popper and Hawkins [42]). This includes the maximum 1s mean sound pressure level of 169 dB re 1 μPa over 5–500 Hz at 50 cm from the nearest noise generator, and a median 1s level in the same position of 132 dB re 1 μPa with the sound generator switched on.

In response to stress, fish increase their blood cortisol levels over a short period of time (the peak in salmonids is between 30 and 60 min) before levels decrease back to basal levels. More precisely, in salmonids, plasma post-stress cortisol levels after 30 min are between 80 and 100 ng/mL, while basal levels are normally ≤10 ng/mL (e.g., [43–47]). While an increase

Fig. 8. Mean (±SEM) serotonin (5-HT; **a**) and its main catabolite 5-hydroxy-indoleacetic acid (5-HIAA; **b**) concentrations, as well as the 5-HIAA/5-HT ratio (**c**) in the dorsolateral pallium (Dl), the dorsomedial pallium (Dm), the preoptic area (PoA), and the tubercular nuclei (TN) of Atlantic salmon before being subjected to sound stress (T0) and after experiencing sound stress once (T1), and 30 (T4) times. Linear mixed effect model statistics are given in each panel. Small letters indicate post hoc significant differences between the groups.

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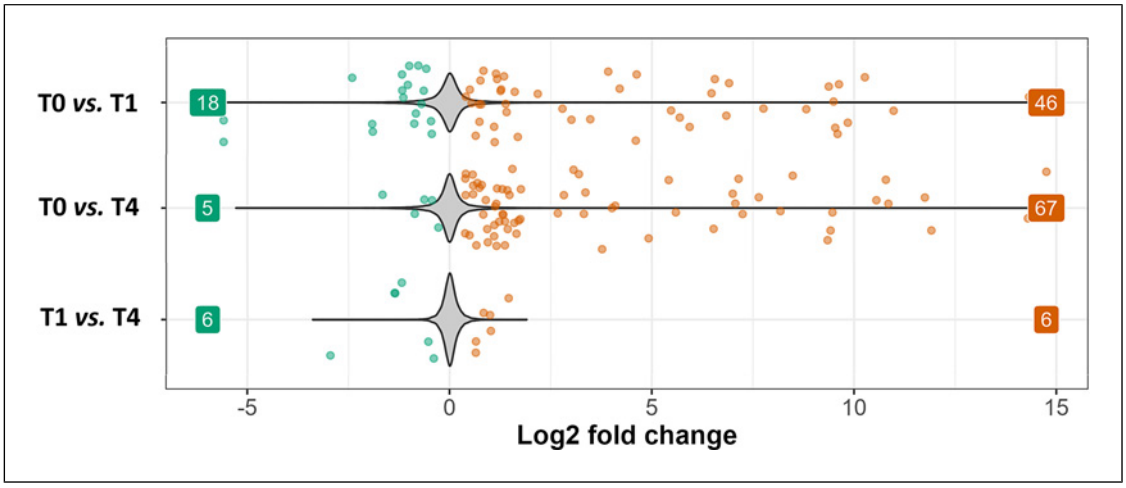

Fig. 9. Violin plot showing Log2 fold change scores of significantly differentially expressed genes (DEGs) in the tuberculi nuclei (TN) of Atlantic salmon before being subjected to sound stress (T0) and after experiencing sound stress once (T1) and 30 (T4) times. Upregulated DEGs are shown in green and downregulated in orange. Statistical analysis is based on the DESeq2 package [31].

Table 1. Enriched KEGG pathway in the tubercular nuclei (TN) of Atlantic salmon before being subjected to sound stress (T0) versus after experiencing sound stress 30 times (T4)

Pathway	Log2FC	Symbol	Entrez ID	Description
sasa04912	−14.3	LOC106610036	106610036	*glycoprotein hormones α chain 2-like*
sasa04912	−9.42	glha1	100286517	*glycoprotein hormones α chain 1*
sasa04912	−1.16	LOC106603906	106603906	*early growth response protein 1*
sasa04912	−0.83	LOC106611930	106611930	*early growth response protein 1*
sasa04912	−0.57	LOC106604926	106604926	*early growth response protein 1*

The pathway was found based on significantly differentially expressed genes ($p < 0.05$).

Table 2. Gene Ontology (GO) over-representation test of molecular function processes in the tubercular nuclei (TN) of Atlantic salmon before being subjected to sound stress (T0) versus after experiencing sound stress 1 (T1) or 30 times (T4)

Comparison	GO term	Description	Adjusted *p* value	Gene count
T0 versus T1	GO:0005179	hormone activity	0.000029	6
	GO:0030546	signaling receptor activator activity	0.00026	7
	GO:0048018	receptor ligand activity	0.00026	7
	GO:0030545	signaling receptor regulator activity	0.00026	7
T0 versus T4	GO:0005179	hormone activity	6.6E−09	9
	GO:0030546	signaling receptor activator activity	0.000000084	11
	GO:0048018	receptor ligand activity	0.000000084	11
	GO:0030545	signaling receptor regulator activity	0.000000084	11

The GO terms were found based on significantly differentially expressed genes ($p < 0.05$).

in cortisol response to acute stress is adaptive, problems may arise when stress persists over a long period of time as this can lead to chronically elevated cortisol levels which are associated with reductions in fitness and increased susceptibility to illness [48, 49]. We found that in response to 5 min of aversive sound (T1),

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salmon showed a significant increase in cortisol, which was also maintained at T2 (after 10 trials of sound stress). Even though we sampled individuals shortly after sound exposure, the cortisol response increased approximately 10 times compared to undisturbed individuals. This suggests that if we had waited at least 30 min following sound exposure, we would have obtained values ≥100 ng/mL indicative of an acute stress response [47]. Interestingly, cortisol levels did not respond to sound exposure at T3 and T4, which suggests that fish were no longer surmounting a cortisol response, at least in the same magnitude, to the sound exposure. An increase in cortisol levels in response to acute sound stress exposure has also been reported in sea bass (*Dicentrarchus labrax*) [50], gold fish (*Carassius auratus*) [51], common carp (*Cyprinus carpio*), the gudgeon (*Gobio gobio*), and the European perch (*Perca fluviatilis*) [52]. Furthermore, similarly to our results, long-term exposure to sound stress did not result in chronic elevation of cortisol levels in goldfish [51]. These results suggest that any possible sound stress that fish may be experiencing from long-term exposure to a low-frequency sound generator does not result in the production of an increase in cortisol levels. In other words, these results would be indicative of physiological habituation to sound stress, which along with our behavioral data, would indicate that exposure to low-frequency sound over 30 trials results in at least partial habituation. However, our results on neuronal activity, monoaminergic signaling, and gene expression, suggest otherwise.

Neuronal activity in the brain is important for regulating neuronal plasticity and gene transcription, which is important for the development of the neuronal circuitry [53]. In this way, brain circuitry is shaped to respond and adapt to environmental stimuli [54]. In order to quantify the relative neuronal activity in salmon exposed to repeated low-frequency sound bursts, we used the phosphorylated extracellular signal-regulated kinase (pERK) marker, which is associated with activity-dependent regulation of neuronal functions [55]. Importantly, pERK has relatively low baseline activity that rapidly increases after stimulation within 1–3 min, peaks at 10 min, and then returns to baseline within 2 h [56]. We found that in response to daily 5 min of sound stress, there was a significant decrease of neuronal activity (i.e., pERK) in the hypothalamic TN. Our results are indicative that salmon are responding with a region-specific down-regulation of neuronal activity in the TN to sound stress exposure throughout the course of the experiment (from T1 to T4). This decrease in neuronal activity may be indicative of an inhibition or an activation of specific neuronal networks in response to stress. However, more studies are needed to elucidate specific neurons that have been activated and inhibited within this neural area in response to sound stress. Interestingly, areas within the TN have been associated with processing of mechanosensory signaling, such as low-frequency sound, via the lateral line. Moreover, the nucleus anterior tuberis (NAT), which is part of the TN, has been described as an essential auditory nucleus [57, 58]. Therefore, we believe that our results on neuronal activity in the TN are directly in response to sound stress. Furthermore, we also observed (but did not quantify) high neuronal activity in the dorsomedial (Dm) and dorsolateral (Dl) pallium for all timepoints (i.e., there were no observable differences between timepoints), which have been proposed as functionally equivalent to the mammalian amygdala and hippocampus [41, 59, 60]. Even though there were no observable differences between timepoints, we still decided to include these areas along with the PoA (which is associated with the regulation of the hypothalamic-pituitary interrenal stress axis [61]) and the TN for quantification of monoamine neurochemistry.

The neurotransmitter serotonin (5-HT) has a crucial role in energy regulation, neural plasticity, behavioral and emotional control, as well as neuroendocrine responses to stress [62, 63]. There is now ample evidence that serotonergic activity in fish, just like in mammals, increases in response to stress [45, 64, 65]. In this context, 5-HT has been proposed to be important in the reallocation of energy resources from processes such as growth and reproduction. Importantly, 5-HT is rapidly replaced intracellularly following its release at the synapse [24]. Furthermore, the formation of 5-HT's main catabolite, 5-HIAA, occurs after 5-HT reuptake from the synapse. Therefore, it is more appropriate to use 5-HIAA levels and the 5-HIAA/5-HT ratio as a proxy for serotonergic activity (i.e., release and uptake of 5-HT) [24, 66]. In this experiment, we found that exposure to sound stress resulted in an increase at T4 compared to T0 of 5-HIAA in the Dl, the PoA, and the TN, and the 5-HIAA/5-HT ratio in the Dl, Dm, and PoA. These results show increased serotonergic activity in all studied brain areas after 30 trials of sound stress, which indicates that individuals experience sound as being stressful even after 30 trials with no signs of habituation at this level. Furthermore, serotonergic

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activity is also significantly increased in the TN at T1 compared to T0, which suggest that this area is particularly affected by sound stress exposure. While none of the other areas show a significant increase in serotonergic activity at T1, it is important to point out that serotonergic activity in salmonids peaks at about 30 min post-stimuli exposure [65] and it is possible that sampling individuals at a later point would have resulted in higher levels within other areas as well, particularly since there appears to be a numerical tendency for increased levels in the Dl and the PoA. However, further experiments allowing for longer exposure times to sound stress before sampling are necessary to shed light on this possibility. Notably, while the cortisol and behavioral data suggest partial habituation to sound stress, our results on serotonergic activity suggest the contrary. The 5-HT system is highly associated with HPI axis reactivity, with both cortisol and 5-HT levels increasing in response to stress and co-regulate each other [64, 67, 68]. However, the brain is a very heterogenous organ and not all neuronal populations respond in the same manner to stimuli. For example, in response to acute stress, 5-HT signaling is normally increased in the raphe nuclei, but not in the Vv or in the Dm [69, 70]. In this context, differences in 5-HT signaling show a higher neuronal region-specific resolution than cortisol levels and may therefore show a different dynamic and sensitivity to stimuli [71]. Furthermore, serotonergic signaling is associated with behavioral inhibition, particularly over longer time periods [72–74], we therefore speculate that increased 5-HT signaling at T4 is associated with the diminished behavioral response to sound that we observed at this timepoint. Alternatively, it is possible that the increase in serotonergic activity was due to samplings T1 and T4 having taken place several weeks after T0 and that this was an effect of time (i.e., the fish were slightly older at the end of the experiment). However, it is important to note that even though serotonergic neurochemistry follows a dynamic regulation in response to different physiological states. It is mainly up- or downregulated to specific events, such as stress [65, 75], smoltification [76], vaccination [77], and aggression [78]. In our experiment, fish were not undergoing any major life stage changes (the few that had matured were not included in our analysis) and were only challenged by the exposure to aversive sound. Therefore, it is unlikely that the increase in serotonergic activity is due to a difference in time/age. Interestingly, dopaminergic activity was found to be downregulated in the Dm in response to sound stress. The dopaminergic system is associated with behavioral flexibility through stimuli salience regulation [79, 80]. In response to stress, dopaminergic activity may increase or decrease [46, 81–84]. This indicates a strong context-, time-, and/or region-specific influence of stress on DA signaling. In this experiment, we found that sound stress results in a downregulation of DA signaling in an area that is associated with emotional processing [41], which suggests a negative salience experience [79].

Importantly, the results on increased serotonergic activity in the TN after T1 and T4 compared to T0, together with results on neuronal activity, highlight its importance either for processing sound stress stimuli or as an area particularly affected during this type of challenge. We, therefore, conducted a whole transcriptome analysis (RNAseq) of this area at T0, T1, and T4 to further understand its role during sound stress. The whole transcriptome profile of the TN at T1 and T4 shows mainly a downregulation of genes associated with the endocrine system (according to KEGG analysis) which has functions related to hormones, receptors, and ligand activity (according to GO molecular function terms). Out of all the genes, the one that showed the highest downregulation was prolactin. Prolactin has been mainly associated with reproduction, osmoregulation and brood care behavior [85]. However, it is also associated with stress regulation and suggested to have an inhibitory role on the HPI axis [86, 87]. Specifically, in rainbow trout, chronic stress caused by a 24-h confinement and a decline in water quality induced a decrease of up to 60% in plasma prolactin levels [86]. Notably, several of the downregulated genes are associated with growth and their downregulation is associated with reduced growth rates. That is, *somatotropin 2*, also called growth hormone (GH), is essential for normal growth development (Cavari et al. [88]) and is inhibited by glucocorticoids in fish (for review see [89]). For example, in salmonids, acute and chronic stress inhibits secretion of GH [90, 91]. Furthermore, the glycoprotein hormones α chain 1 and 2 are neuropeptides associated with the control of reproduction and metabolism in mammals [92] and fish [93]. Moreover, free glycoprotein α has been proposed to be involved in the secretion of prolactin by stimulating the differentiation of prolactin cells in the pituitary [94]. Similarly, somatolactin is a peptide hormone related to GH that also has a role in reproduction [95]. Furthermore, GH, somatolactin, and prolactin are all transactivated by the *pou class 1 homeobox 1* gene [96], which was also one of the significant DEG's

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showing a FC downregulation of −8.48 at T4, compared to T0. In addition, *trypsin I-p1*, which induces increased growth rate by influencing the efficiency of protein digestion [97] was also significantly downregulated showing a FC of −5.42 at T4, compared to T0. Therefore, a downregulation of all these genes would further decrease growth investment (in terms of regulation of growth-associated genes). Meanwhile, the role of the glycoprotein hormones β5 is a bit more diffuse, but it has been associated with the control of reproduction, with decreased expression affecting fecundity [98]. Finally, *proopiomelanocortin* (*pomc*) and its different transcripts (*a*, *a2*, and *b*) are important genes involved in the stress response of the HPI axis and energy homeostasis. It is a precursor of several peptide hormones including adrenocorticotropic hormone, melanocyte-stimulating hormones, and β-endorphins. In rainbow trout, food deprivation and temperature stress result in downregulation of *pomc* expression with tissue-specific differences between the different variants, which is proposed to serve different functions, particularly in energy budget regulation [99]. Taken together, the results on RNAseq suggest that sound stress over 30 trials leads to a downregulation of genes associated with growth and reproduction. Interestingly, the TN has also been associated with food intake regulation through anorexigenic and orexigenic effects depending on the context, and it has been found to be downregulated in fish showing decreased growth rates, compared to their fast growing conspecifics [100], which may explain why growth-associated genes are downregulated in this area. Moreover, in fish, stress has been found to alter food intake regulation via the activation of the HPI axis, which results in modified expression of peptides related to stress and food intake regulation, e.g., [101].

In conclusion, we found that exposing farmed salmon to sound stress over 30 times once a day still resulted in an initial startle behavioral response, together with increased plasma cortisol levels and a decrease in neuronal activity in the TN. Furthermore, at T4 (after 30 times of sound stress) salmon showed signs of chronic stress with increased serotonergic activity levels in the Dl, Dm, PoA, and TN and an inhibition of growth and reproduction transcripts in the TN. Our results suggest that while fish may show a degree of habituation to daily repeated sound stress in behavior and cortisol release, they still show signs of stress while also showing a dramatic decrease in the expression of genes associated with growth and reproduction. These findings are important to consider for the mitigation of anthropogenic sound pollution in bodies of water for both wild and farmed fish species. It is also important to investigate how unpredictable chronic sound stress, such as boats and construction work, may be affecting fish since it has been proposed that this type of sound stress may be more hazardous and difficult to cope with than constant sound stress [52].

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Statement of Ethics

The experiments were performed in accordance with the current Norwegian law for experimentation and procedures on live animals and were approved by the Norwegian Food Safety Authority (Mattilsynet) through FOTS application ID 29693.

Conflict of Interest Statement

The authors declare they have no competing interests.

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Author Contributions

Frode Oppedal: conceptualization, project administration, data analyses, and review of draft; Luke T. Barret: data collection, data analysis, and review of draft. Thomas W.K. Fraser and Tone Vågseth: data collection and review of draft. Guosong Zhang: data processing and review of draft. Lea Jacson and Marie-Aida Dieng: sample and data analysis and review of draft. Oliver G. Andersen: sample analysis and review of draft. Marco A. Vindas: conceptualization, project administration, sample and data analyses, and writing of original draft.

Data Availability Statement

All relevant data are within the paper or published as online supplementary material. Further inquiries can be directed to the corresponding author.

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