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Research Article

No trout about it: behavioural and transcriptional effects of long-term noise exposure in brook trout (*Salvelinus fontinalis*)

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Abstract

Exposure to acute noise sources can lead to negative behavioural outcomes and fitness deficits in fishes, but it is unknown whether fish can habituate to chronic exposures. As underwater noise increases globally, understanding how long-term exposures can affect behavioural, morphological, and transcriptional measures of stress in fish is critical. We tested responses in captive brook trout (*Salvelinus fontinalis*) immediately after acute exposure to a noise and after 2 weeks of chronic exposure. Behavioural tests quantified fish movements before, during, and after sound presentation, with morphological and transcriptional changes assessed through ears and whole brains samples, respectively. Pre-control and pre-experimental brook trout exhibited increased swimming distance and velocity, but after the 2-week exposure, post-experimental fish showed no response to noise while the post-control group remained responsive. Post-experimental fish showed significant differences in transcription levels of genes involved in neuroplastic, appetite, and stress responses relative to the other groups. Together these results suggest that while fish may appear unresponsive via behavioural metrics to anthropogenic noises after chronic exposure, they still show significant changes at the transcriptional level with possible long-term effects.

Key words: anthropogenic noise, fish behaviour, fish bioacoustics, transcriptional profiling, habituation

Introduction

Anthropogenic noise is drastically changing the soundscape of aquatic ecosystems (Slabbekoorn et al. 2010; Mickle and Higgs 2018; Duarte et al. 2021), causing potentially detrimental effects on fish (Cox et al. 2018; Mickle and Higgs 2018; Pieniazek et al. 2023). Globally, the most dominant source of underwater anthropogenic noise is vessel noise, which propagates efficiently over long distances (Vasconcelos et al. 2007; Frisk 2012; Possenti et al. 2024). Underwater, vessel noise can overlap with fish communication (Radford et al. 2014), masking important acoustic signals (Popper and Hastings 2009; Casper et al. 2013) that are critical for fish survival and reproduction (Fay and Popper 2000; Rollo et al. 2007; Slabbekoorn et al. 2010; Ladich and Fay 2013). Intense sounds may also lead to direct damage to the sensory receptors and related structures responsible for sound detection (McCauley et al. 2003; Popper 2003; Kunc et al. 2016; Mickle and Higgs 2018). However, since fish can regenerate auditory receptor cells (Popper and Hoxter 1984; Corwin and Oberholtzer 1997) it is unclear how long-term exposure may be ameliorated by these regenerative mechanisms (Smith and Monroe 2016), especially given that some fish have been shown to habituate to sound sources after long-term exposures (Johansson et al. 2016; Nedelec et al. 2016; Magnhagen et al. 2017).

While long-term anthropogenic sound can induce behavioural, and sometimes physiological, changes, the integrated analysis of these behavioural and internal effects is critical to fully understand how sound impacts fish and how these impacts may manifest long-term (Mickle and Higgs 2018). In one of the few examples examining both behavioural and genetic responses to enhanced sound exposure, Barcellos et al. (2018) exposed zebrafish (*Danio rerio*) to classical music and found increased activity in music-exposed fish relative to controls as well as significant changes to mRNA expression of brain-derived neurotrophic factor (*bdnf*) and select pro-inflammatory genes. Butler and Maruska (2021) used a random selection of pure tones ranging from 100 to 2000 Hz and showed differential gene expression in genes related to both feeding and maternal care, and corresponding changes in relevant behaviours, in noise-exposed compared to control African cichlids (*Astatotilapia burtoni*). Exposing Atlantic salmon (*Salmo salar*) juveniles to intense seismic sound results in significant differential expression of genes thought to indicate tissue damage (Andrews et al. 2014), while black porgy (*Acanthopagrus schlegelii*) showed

differential expression of genes related to immune function when exposed to wind farm noise (Chang et al. 2018). Lastly, when Atlantic salmon were exposed to low-frequency sound, they showed both an acute startle response and signs of chronic stress with an inhibition of neural-tissue growth and reproduction transcripts (Oppedal et al. 2024). Although there have been limited studies investigating noise effects on gene expression in fish (Celi et al. 2016; Wei et al. 2018; Butler and Maruska 2021; Sapozhnikova et al. 2021; Faria et al. 2022), there has been extensive research examining transcriptional changes in genes induced by other environmental stressors (Prunet et al. 2008; Logan and Buckley 2015; Akbarzadeh et al. 2018; Connon et al. 2018; Bernos et al. 2020). Given that previous research has shown that anthropogenic noise is a stressor in aquatic environments, it is expected that, in the current study, sound will induce transcriptional changes reflective of that stress like those in the studies mentioned above.

Understanding the long-term consequences noise stressors may impose on fish behaviour could potentially be a direct indicator of their physiological welfare and health status, both in the wild and in captivity. The purpose of the current study was to examine how brook trout (*Salvelinus fontinalis*) respond to long-term noise exposure at behavioural, morphological, and molecular levels to understand the possible interdependent relationship between metrics. Specifically, acoustic startle escape responses and general swimming behaviours were quantified through recorded behavioural trials and morphological changes in hearing-related tissues were analyzed in response to noise exposure. The relative transcription of 28 genes was measured within the dissected brains of brook trout after noise exposure using a custom nanofluidic OpenArray® chip. To date, this is the first study that integrates a molecular approach with behavioural and morphological metrics to analyze the habituation of fish to noise, providing novel evidence of transcriptional changes of markers possibly linked to habituation.

Methodology

Study species

For this study 150 juvenile brook trout were obtained from the Codrington Fish Culture Station (Codrington, ON) via the Great Lakes Institute for Environmental Research (Windsor, ON). All fish used in this experiment were captively reared from eggs and shared genetic ancestry and introgression from stocking. As members of the salmonid family, brook trout are expected to have hearing ranges most sensitive to 100–300 Hz, although this has yet to be measured directly (Kaciem et al. 2020). Care and use of experimental animals complied with Canadian Council of Animal Care animal welfare laws, guidelines, and policies as approved by the University of Windsor Animal Care Committee (AUPP #21-07).

Experimental housing conditions and sound exposure protocol

In the current study, fish were randomly sorted into one of two housing rooms, the control room or the experimental sound exposure room, and held in 379 L tanks with each tank containing ten 19 L buckets (7–8 fish/bucket) to act as separate housing containers. Each bucket had additional holes drilled into the sides as well as an air stone to ensure adequate water exchange and oxygen supply between the buckets and the tank in which they were being held. All fish were maintained at a constant 14 °C water temperature, a pH of 6.5–7, and a 12:12 light:dark cycle to mimic natural conditions. Water conditions and temperature were monitored daily, and the fish were acclimatized to their respective tanks for 7 days prior to start of the experimental period.

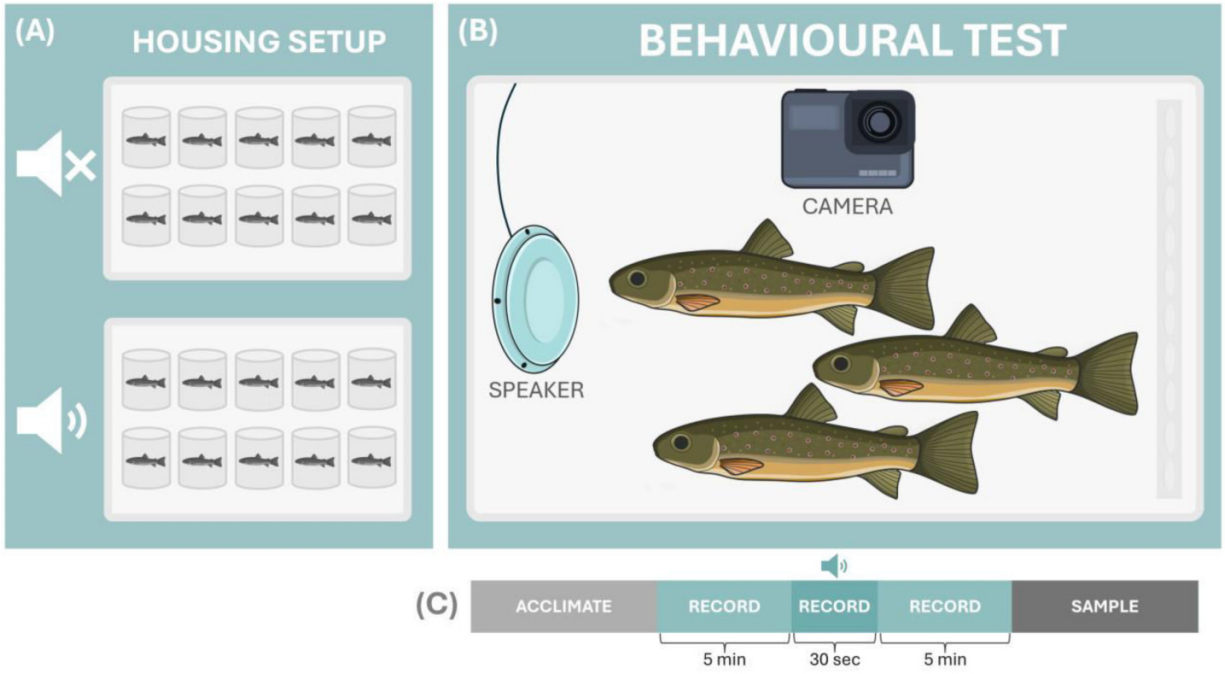
In the control room, no additional noise was played beyond the ambient noise. In the sound-exposed room, playback of boat noise (a broad-band sound file spanning from 10 to 5000 Hz, with predominant energy from 10 to 1000 Hz) was presented through an underwater speaker (Clark Synthesis Diluvio AQ339; Lubell Labs) at 135 dB re 1 μPa. The speaker was positioned at one end of the housing tank and connected to an amplifier (Scosche SA300). The sound was played continuously 24 h a day for the 2-week duration of the experimental period. Noise levels were measured in the housing tanks using a hydrophone system (InterOcean Systems, Acoustic Calibration and System Model 902) and, where possible, using a waterproof accelerometer (model 4524 cubic triaxial deltatron, Bruel & Kjær) to estimate noise levels as pressure and particle motion. It is recognized that there are inherent problems with complex acoustics in small tanks (Parvulescu 1964; Rogers et al. 1995), but the current set of experiments would not be feasible in a field setting and can still provide useful information about possible noise effects. The ambient noise in both the housing tanks was below 120 dB re 1 μPa root mean square (RMS) (equivalent to -27.62 re 1 m·s^{-2}) prior to the addition of fish. In the sound-exposed room, the boat noise recording was played at 135 dB re 1 μPa RMS (equivalent to -28.42 re 1 m·s^{-2}). The speaker was placed at one end of the tank and noise was quantified using the hydrophone at the speaker, positioned in the middle of the tank and at the far end of the tank, and sound reliably ranged from 132 dB re 1 μPa RMS (equivalent to -28.26 re 1 m·s^{-2}) to 135 dB re 1 μPa RMS (equivalent to -28.42 re 1 m·s^{-2}). Treatment groups will be referred to as follows for the duration of this study: pre-control (control fish before the 2-week sound exposure period that *will not* be receiving experimental treatment), pre-experimental (fish that *will be* receiving experimental treatment during the 2-week sound exposure period), post-control (control fish after the 2-week sound exposure period), and post-experimental (fish that received experimental treatment after the 2-week sound exposure period).

Behavioural testing

Behavioural tests were conducted before and after the 2-week sound exposure period (Fig. 1) to assess changes in behavioural responses of the brook trout to acute sound stimuli following prolonged exposure. Three fish at a time were transferred from one of the housing tanks into an experimental tank (757 L) that was equipped with a camera (GoPro Hero, US), a Plexiglas barrier perforated with 1 cm holes to reduce acoustic reflection, and a speaker set-up identically to

Fig. 1. Visual representation of exposure timeline for (A) control and (B) experimental fish. Behavioural tests are conducted before and after a 2-week period of either silence or boat noise exposure.

Fig. 2. Visual representation of experimental setup depicting (A) separate rooms for control and experimental sound exposure brook trout with ten 19 L buckets, (B) behavioural testing setup equipped with a GoPro camera, underwater speaker, and perforated Plexiglass barrier, and (C) behavioural testing timeline. Behavioural tests involved a 5 min acclimation period, followed by video recording during sound exposure, and tissue sampling.



the noise-exposed housing tank. Water conditions in the experimental tank were identical to those of the housing tanks (14 °C and a pH of 7).

The brook trout were acclimated to the behavioural tank for a minimum of 5 min before the start of the behavioural trials (Fig. 2). Following the acclimation period, the same boat noise that was used for the pre-exposed housing tank was played for 30 s. The camera recorded the behaviour of the brook trout during the 5 min of acclimation time, the 30 s of sound exposure, and 5 min after the presentation of sound to observe any differential responses in the chosen behavioural metrics: distance moved, swimming velocity, distance from speaker, turn angle, and startle responses. The same behavioural trials were repeated for the pre-control ($n = 10$), pre-experimental ($n = 10$), post-control ($n = 10$), and post-experimental ($n = 10$) treatment groups (Fig. 2).

After the completion of behavioural testing, fish were humanely euthanized using 2-phenoxyethanol (Sigma–Aldrich: 1 mL of 2-phenoxy ethanol per 2 L of water). The total length of each brook trout was measured, and brook trout heads were preserved in RNAlater at −20 °C for future analysis.

Otolith morphology and ciliary bundle counts

A subset of 10 random brook trout from both the sound-exposed room and the control room were sampled following the end of the 2-week exposure period ($n_{control} = 10$, $n_{experimental} = 10$) to analyze effects of sound on otolith morphology and hair cell counts. Heads from these fish were fully submerged in 4% paraformaldehyde (PFA) and stored at 4 °C before further dissection of ears.

Auditory hair cell counts

Saccules were removed from each brook trout using a Leica S6D Stereo zoom microscope (Microscope Central, USA) and stored in a 4% PFA solution at 4 °C. Images of the saccule were taken using Eclipse Imaging software (Empix Imaging Inc., Mississauga, ON) at 8X magnification, exposure at 349 ms,

Table 1. List of gene functional classes, symbol, and names for the 19 genes successfully amplified and used in the current experiments.

Gene function	Gene symbol	Gene name
Neural	*bdnf*	Brain-derived neurotrophic factor
Neural	*sox2*	SRY-box 2
Neural	*neurod-1*	Neurogenic differentiation factor 1
Neural	*neurog-1*	Neurogenin 1
Neural	*pcna*	Proliferating cell nuclear antigen
Stress	*cirbpa*	Cold inducible RNA binding protein a
Stress	*hsd-11b2*	11β-hydroxysteroid dehydrogenase 2
Stress	*crfb-1*	Corticotropin-releasing factor b1
Stress	*gr2*	Glucocorticoid receptor 2
Stress	*mr*	Mineralocorticoid receptor
Stress	*hsp70a*	Heat shock protein 70a
Stress	*avt*	Arginine vasotocin
Appetite	*npy*	Neuropeptide-y
Appetite	*agrp-1*	Agouti-related neuropeptide 1
Appetite	*cck-1*	Cholecystokinin isoform l
Appetite	*lepr*	Leptin receptor
Reference	*rpl13a*	Ribosomal protein 13a
Reference	*rp17*	60s ribosomal protein L7
Reference	*rps9*	40S ribosomal protein S9

Note: Gene functional classes were determined based on the known function of each gene in fish, with relevant references given in the text.

and an image gain of 49.44% from the Leica S6D Stereo zoom microscope. Measurements of the otolith's length, perimeter, and area were obtained from the images.

The saccular sensory epithelia were rinsed in phosphate buffer, and then stained with a solution of 12.5 μL of Oregon Green 488 Phalloidin (Life Technologies Corporation, Eugene, OR) and 200 μL of phosphate buffer (Higgs et al. 2002; Schuck and Smith 2009). The sample was then stored in a dark storage container for 20 min in the phalloidin solution (Higgs et al. 2002). A small volume of phosphate buffer was placed on a microscope slide (Fisher Scientific, US), and the sensory epithelia were then transferred onto microscope slides and a glass coverslip (Fisher Scientific, US) was placed on top of the microscope slide. Once saccules were stained, ciliary bundles of hair cells were visualized through images collected from a Leica microscope (Leica DM IRB inverted fluorescence microscope, using the software Las A.F. 4.5). Ciliary bundles were counted in three regions along the anterior, middle, and posterior saccule using a magnified view of the epithelium. Images were imported into Adobe Photoshop CC 2019 to create three identical boxes of 200 μm^2 in size (in magnified view) representing 19% of the total saccular area (Higgs et al. 2002). Ciliary bundles within the box were then counted using Image J software (National Institutes of Health). Hair cell damage was characterized as a difference in absolute number of ciliary bundles between fish exposed to the sound and control fish. Damage caused from dissection was notably different from ciliary bundle loss, as dissection damage often appeared as a tear while ciliary loss appeared as dark spots (Hastings et al. 1996). Comparisons in ciliary bundles of hair cell number were made between the pre-experimental and post-experimental brook trout.

Quantifying gene transcription

RNA extraction and cDNA synthesis

Whole brain tissue samples were obtained from the pre-control (n = 30), pre-experimental (n = 30), post-control (n = 30), and post-experimental (n = 30) treatment groups by aseptically dissecting the fish heads and storing them at −20 °C prior to RNA extraction. Brain tissue was mechanically homogenized in 2 mL microcentrifuge tubes using 400 mg of 1 mm diameter glass beads and a cell disrupter. Total RNA was isolated using an adapted version of Invitrogen Trizol Reagent (ThermoFisher Scientific, Cat #15596026) method supplied by the manufacturer. Following isolation, all RNA samples were tested for concentration ($ng \cdot \mu L^{-1}$) and quality (28S:18S bands ∼ 2.0) using a Tecan Spark® Multimode Microplate Reader. Isolated RNA was stored at −80 °C. cDNA was synthesized from the RNA samples using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Cat # 4368814) as per manufacturer instructions and stored at −20 °C until further processing.

Nanofluid OpenArray® plate

To simultaneously measure differential gene transcription of 19 candidate genes (including three endogenous control genes; Table 1), nanofluidic TaqMan® assays custom designed in the OpenArray® platform (gene expression platform, ThermoFisher Scientific) were used. The concentration of all cDNA samples was diluted to 100 $ng \cdot \mu L^{-1}$ and 2.5μL of each sample was mixed with 2.5μL TaqMan® OpenArray® Real-Time PCR Mastermix (Applied Biosystems™) for loading on the

OpenArray® instrument (ThermoFisher Scientific Inc., Mississauga). The mixture was loaded using the QuantStudio 12K Flex Accufill System (Applied Biosystems™), and the OpenArray® plate was run on the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). After the initial denaturation at 95 °C for 3 min, 40 cycles of the following program were used for amplification: denaturation at 95 °C for 10 s and annealing at 60 °C for 10 s.

Statistical analyses

All statistical analyses with graphs were performed using R Statistical Software (v4.3.2; R Core Team 2024) with an alpha level of $p < 0.05$. In cases where the data met the assumptions of normality, parametric testing was used.

Otolith morphology and auditory hair cell analysis

One-tailed independent samples *t* tests were conducted to evaluate the effect of boat noise treatment on otolith area and auditory hair cells counts.

Behavioural analysis

Video analysis was conducted using behavioural analysis software, Ethovision XT (Noldus, VA, USA), which quantified the total distance moved, average swimming velocity, distance from speaker, and average turn angle for all three fish in the experimental tank. Only the data for 30 s before, during, and after the noise was played were used for statistical purposes. Startle responses were quantified by direct observation of the trial videos and counted only when all three stages of a C-start (the fish turning 180 degrees, the body moving in the opposite direction to the head, and a quick burst of swimming in a new direction) occurred within the critical 15 s window following acoustic stimulus (Liu and Hale 2014).

To examine the impact of the boat noise treatment on acoustic startle responses, a χ^2 test for independence was utilized. Specifically, the frequency of acoustic startle responses before and after experimental treatment was assessed, classified into four categories representing the combinations of treatment group (control/experimental) and time point (before/after the 2-week period).

To quantify more subtle changes in behaviour, video recordings of fish before, during, and after sound presentation were analyzed using Ethovision XT video tracking software (Noldus, VA, USA). The total distance moved, swimming velocity, distance of the fish from the speaker, and the turn angle of fish were quantified as response variables and treatment (pre-control, pre-experimental, post-control, and post-experimental) and sound status (before, during, and after 30 s sound exposure) were used as main effects in a Multivariate Analysis of variance (MANOVA) model including both main effects and their interaction. If a significant multivariate effect was found, univariate ANOVAs were conducted on each swimming behaviour to understand which specific variables contributed to the observed multivariate effect with Tukey HSD testing of any significant univariate main effects. To avoid pseudo-replication of behavioural data, a "fish" identifier was added to the statistical model. However, there was no effect of "fish" ($p = 0.565$) allowing each fish to be perceived as an independent data point.

Gene transcription analysis

Differences in transcription between treatments were analyzed using a calculation of relative transcription derived from Ct values normalized to PCR efficiencies and endogenous control genes (Tuomi et al. 2010). The assay was classified as a true positive when two replicates were positive. The standard curve for each marker was generated by linear regression analysis of the Ct value versus the amounts of the template DNA using log copies/through-holes for TaqMan® assays.

Out of the four reference genes initially included, only three (*rpl13a*, *rpl7*, and *rps9*) were used for data analysis as they did not show treatment effects. The normalized quantity of the target gene was corrected for by using the calculated amplification efficiency and divided by the geometric mean of the three reference genes to obtain the relative transcription of each sample for each gene of interest. The 19 genes were categorized based on their functional class (Table 1) and genes were only included in the analysis if they were able to amplify >90% of the brook the trout samples.

Relative transcription levels were first tested in two-way ANOVA with main effects of treatment and gene, where "treatment" represented the pre-control/pre-experimental/post-control/post-experimental treatment groups and "gene" represented the 19 genes being analyzed for transcriptional changes, with relative transcription as the dependent variable. There was a significant treatment × gene interaction ($F_{12,714} = 4.88$, $p \ll 0.001$) so genes were grouped by function (neural, stress, or appetite genes) and individual ANOVAs of treatment effects were run for each group. To avoid Type I error inflation, a sequential Bonferroni was applied within each group.

Results

Auditory morphology

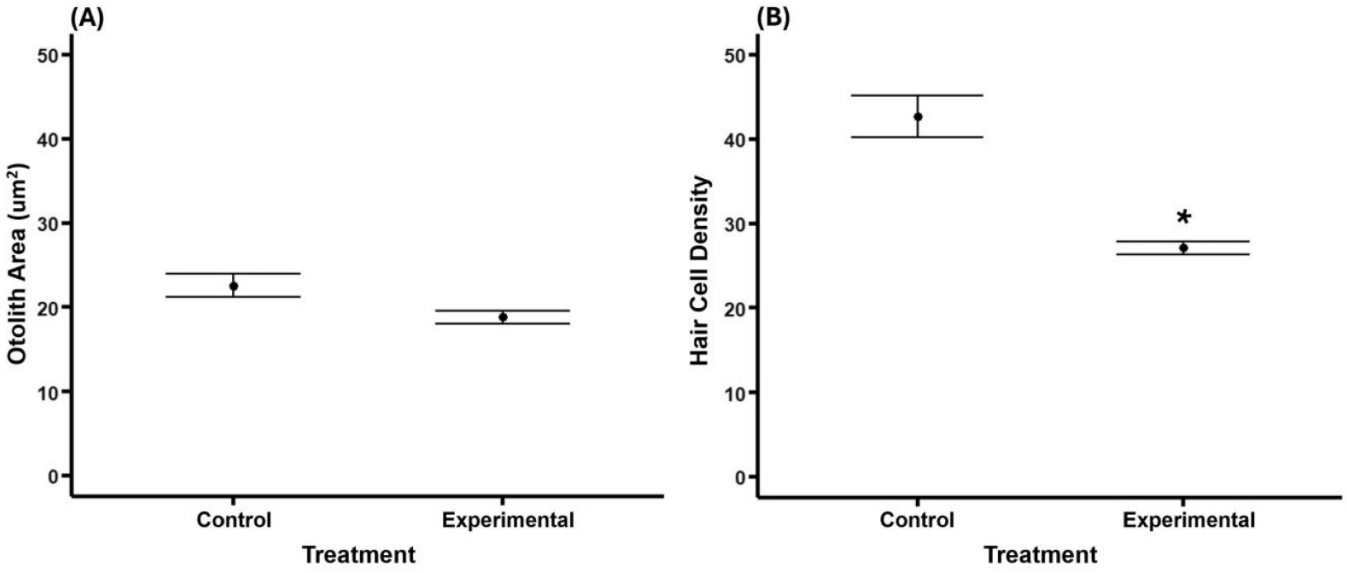
There was no significant effect of the sound exposure after 2 weeks on otolith area ($t_{15} = 0.89$, $p = 0.39$), with fish from control tanks having a mean otolith area of 21.0 μm² (±2.6) and those from the sound-exposed tanks having a mean area of 18.2 μm² (±1.9; Fig. 3A). There was a significant effect of sound exposure on hair cell density ($t_6 = 6.47$, $p = 0.047$), with control animals having a mean hair cell density of 42.7 (±6.0) hair cells per 200 μm² and sound-exposed animals having a mean hair cell density of 27.0 μm² (±2.3; Fig. 3B).

Swimming behaviour

Startle responses

For startle behaviour, 28 control fish and 30 sound-exposed fish had C-starts in response to the 30 s sound exposure before the 2-week sound exposure while 27 control fish and 14 sound-exposed fish showing C-starts after the 2-week sound exposure. While there were fewer startles in the exposed fish

Fig. 3. (A) Otolith area and (B) hair cell density in number of hair cells per 200 μm^2 box for post-control and post-experimental brook trout. Error bars represent ± 1 S.E with $*$ representing significant differences between control and experimental brook trout at a p-value of <0.05.



after the 2 weeks, it was not quite statistically significant ($\chi 2$ calc = 3.00, $\chi 21$, 0.05 = 3.84, $p = 0.08$).

There was no significant interaction between treatment and sound status ($F_{3,330} = 1.04$, $p = 0.40$, Pillai Trace = 0.074) in the overall MANOVA, so the interaction term was ignored for subsequent model approximations. There was a significant main effect of both treatment ($F_{12,687} = 11.35$, p « 0.001, Pillai Trace = 0.37) and sound status ($F_{8,656} = 5.41$, p « 0.001, Pillai Trace = 0.12) on the multivariate behavioural responses; individual post hoc analyses were done on each behavioural metric.

Total distance moved

There were significant main effects of treatment ($F_{3,330} = 19.92$, p « 0.001) and sound status ($F_{2,330} = 17.49$, p « 0.001) on total distance moved in the trials. Across three of the four treatment groups (pre- and post-control fish and pre-experimental fish), fish moved significantly more during sound playback than before or after sound playback (Fig. 4A). Post-experimental fish showed no significant differences between treatments; however, they did move significantly less than the other treatment groups (Fig. 4A), especially while the sound was playing (Table 2).

Swimming velocity

There were also significant main effects of treatment ($F_{3,330} = 27.54$, p « 0.001) and sound status ($F_{2,330} = 19.628$, p « 0.001) on the swimming velocity of fish in the trials. All fish swam faster when sound was played than before or after sound presentation (Fig. 4B). Post-experimental fish moved significantly slower than fish in the other three treatment groups regardless of sound exposure (Fig. 4B; Table 2).

Distance to speaker

There was no effect of sound status ($F_{2,330} = 0.14$, $p = 0.87$) on the distance fish kept from the speaker but there was a significant main effect of treatment ($F_{3,330} = 18.04$, p « 0.001) on this metric. There was no significant effect of pre-control versus post-control fish ($p = 0.73$), but all other comparisons showed a significant (p « 0.001) effect of treatment on the distance from the speaker, with post-experimental fish staying further from the speaker than the other treatments (Fig. 4C; Table 2).

Average turn angle

There were once again significant main effects of treatment ($F_{3,330} = 24.58$, p « 0.001) and sound status ($F_{2,330} = 16.51$, p « 0.001) on the average turn angle fish exhibited in the experiments. Control fish turned less than experimental fish, both before and after sound exposure, and post-experimental fish showed the highest degree of active turning across all sound exposures (Fig. 4D; Table 2).

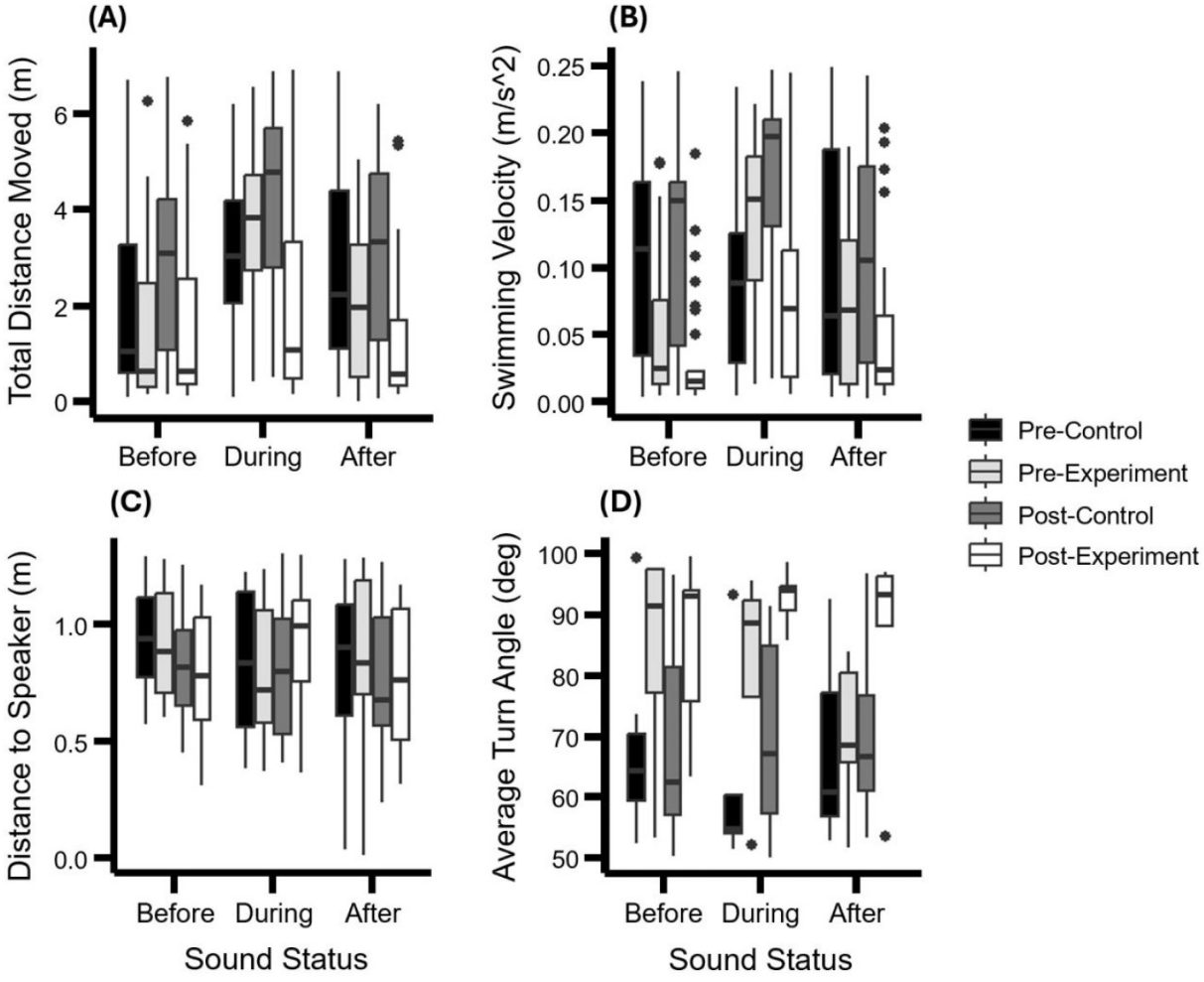
Relative transcription

Neural genes

There was a significant interaction between treatment and gene ($F_{12,714} = 4.88$, p « 0.001), so all gene effects were run as individual ANOVA analyses with treatment as the main effect and relative transcription level as the response variable.

There was a significant effect of experimental treatment on the relative transcription levels of *bdnf* in brook trout ($F_{3,131} = 7.886$, p « 0.001). Post hoc analysis showed there was no difference in relative levels between control and pre-experimental animals before sound exposure ($p = 0.97$); how-

Fig. 4. Behavioural responses of brook trout before, during, and after sound presentation for four experimental categories: (A) total distance moved, (B) swimming velocity, (C) distance to speaker, and (D) average turn angle. The whiskers on the box plot represent 1.5∗interquartile range.



ever, there was a significant upregulation of transcription in post-experimental fish ($p = 0.007$, Fig. 5A).

There was also a significant effect of treatment on the relative transcription levels of *sox2* ($F_{3,174} = 8.577$, $p \ll 0.001$). In contrast to the results seen for *bdnf*, *sox2* transcription in post-experimental fish was significantly downregulated relative to the three other treatment groups ($F_{3,174} = 8.577$, $p = 0.007$) with no difference in transcription levels in the other treatments ($p = 0.95$, Fig. 5B).

There was a significant treatment effect on relative transcription of *NeuroD1* ($F_{3,180} = 6.54$, $p = 0.003$) but here the post hoc effects were different than in the other neural genes. There was no difference in relative transcription between the control and sound-exposed fish after 2 weeks ($p = 0.99$) but there was a significant difference in transcription level in the pre- and post-control animals ($p = 0.012$; Fig. 5C).

There were no significant treatment effects on relative transcription of *NeuroG* ($F_{3,729} = 3.114$, $p = 0.0257$) or *PCNA* ($F_{3,155} = 2.834$, $p = 0.0402$) after sequential Bonferroni correction for multiple tests.

Stress genes

There was not a significant interaction between treatment and gene ($F_{24,1320} = 1.47$, $p = 0.09$) for stress genes; however, all gene effects were run as individual ANOVA analyses with treatment as the main effect and relative transcription level as the response variable to maintain between functional groups.

There was a significant effect of treatment on the relative transcription of *cirbpa* ($F_{3,181} = 19.39$, $p < 0.001$) overall. Post hoc analysis showed that the significance was mainly driven by the downregulated transcription in post-experimental fish compared to the post-control fish ($p < 0.001$). There was no statistical difference in *cirbpa* transcription in the other two treatment groups (Fig. 6A).

There was an overall significant treatment effect on the transcription of *hsd11b2* ($F_{3,106} = 5.55$, $p = 0.001$) and post hoc analysis revealed that there was a significant increase in relative transcription in post-experimental fish compared to the post-control fish ($p = 0.011$) and compared to the pre-exposed fish ($p = 0.007$, Fig. 6B). There was no significant difference between any of the other treatment groups.

Table 2. Mean and standard deviation (SD) values for the four behaviours measured for all experimental treatments.

Experimental group	Before/during/after acute sound presentation		Distance moved	Velocity	Distance from speaker	Average turn angle
Pre-experimental	Before	Mean	1.5	0.1	1.2	89.4
		SD	1.7	0.1	0.4	36.9
	During	Mean	4.6	0.2	1.2	53.4
		SD	2.8	0.1	0.5	34.3
	After	Mean	2.5	0.1	1.2	78.0
		SD	2.4	0.1	0.6	36.5
Pre-control	Before	Mean	3.3	0.1	1.1	73.8
		SD	4.1	0.1	0.3	34.5
	During	Mean	5.5	0.2	1.1	56.0
		SD	3.6	0.2	0.4	34.4
	After	Mean	4.7	0.2	0.9	70.7
		SD	4.6	0.2	0.5	35.0
Post-experimental	Before	Mean	1.7	0.0	1.5	106.3
		SD	1.8	0.0	0.5	25.5
	During	Mean	2.1	0.1	1.5	93.7
		SD	3.4	0.1	0.5	37.6
	After	Mean	1.2	0.1	1.5	105.0
		SD	1.4	0.1	0.5	25.6
Post-control	Before	Mean	3.5	0.2	1.0	80.2
		SD	2.5	0.1	0.4	33.5
	During	Mean	6.3	0.3	1.1	48.7
		SD	3.4	0.2	0.5	24.6
	After	Mean	3.3	0.2	1.1	64.4
		SD	2.1	0.1	0.5	35.1

Note: Distance moved represents the total distance moved during each presentation, velocity is the averaged swimming velocity, distance from speaker represents how far each fish was from the speaker, and average turn angle represents the turn angle of each fish in the tank.

There was a significant effect of treatment on relative transcription values of *crf-b1* overall ($F_{3,87} = 4.81$, $p = 0.0365$) with, again, the largest increase in transcription seen in fish after 2 weeks of sound exposure (Fig. 6C). All other post hoc tested revealed no significant differences between groups.

For the remainder of the stress response genes (*gr2*, *mr*, *hsp70a*, and *avt*) there was no effect of treatment on their relative transcription ($p > 0.05$).

Appetite genes

There was a significant interaction between treatment and gene ($F_{12,574} = 22.68$, $p \ll 0.001$) for appetite genes so all gene effects were run as individual ANOVA analyses with treatment as the main effect and relative transcription level as the response variable.

All appetite related genes explored in the current study showed significant overall treatment effects on their relative transcription (*agrp* $F_{3,69} = 14.49$ $p = 0.007$, *cart* $F_{3,144} = 4.23$, $p = 0.007$, *cck* $F_{3,119} = 6.70$, $p < 0.001$, *npy* $F_{3,169} = 15.18$, $p < 0.001$, *lepr* $F_{3,73} = 4.81$, $p = 0.004$). Of those five genes, *agrp*, *cck*, *npy*, and *lepr*, all showed the same pattern of being significantly upregulated in response to 2 weeks of sound exposure, with no differences between the other three treatments (Fig. 7). For the fifth gene, *cart*, there was a significant increase in transcription in the control animals after 2 weeks but not in the other three treatments.

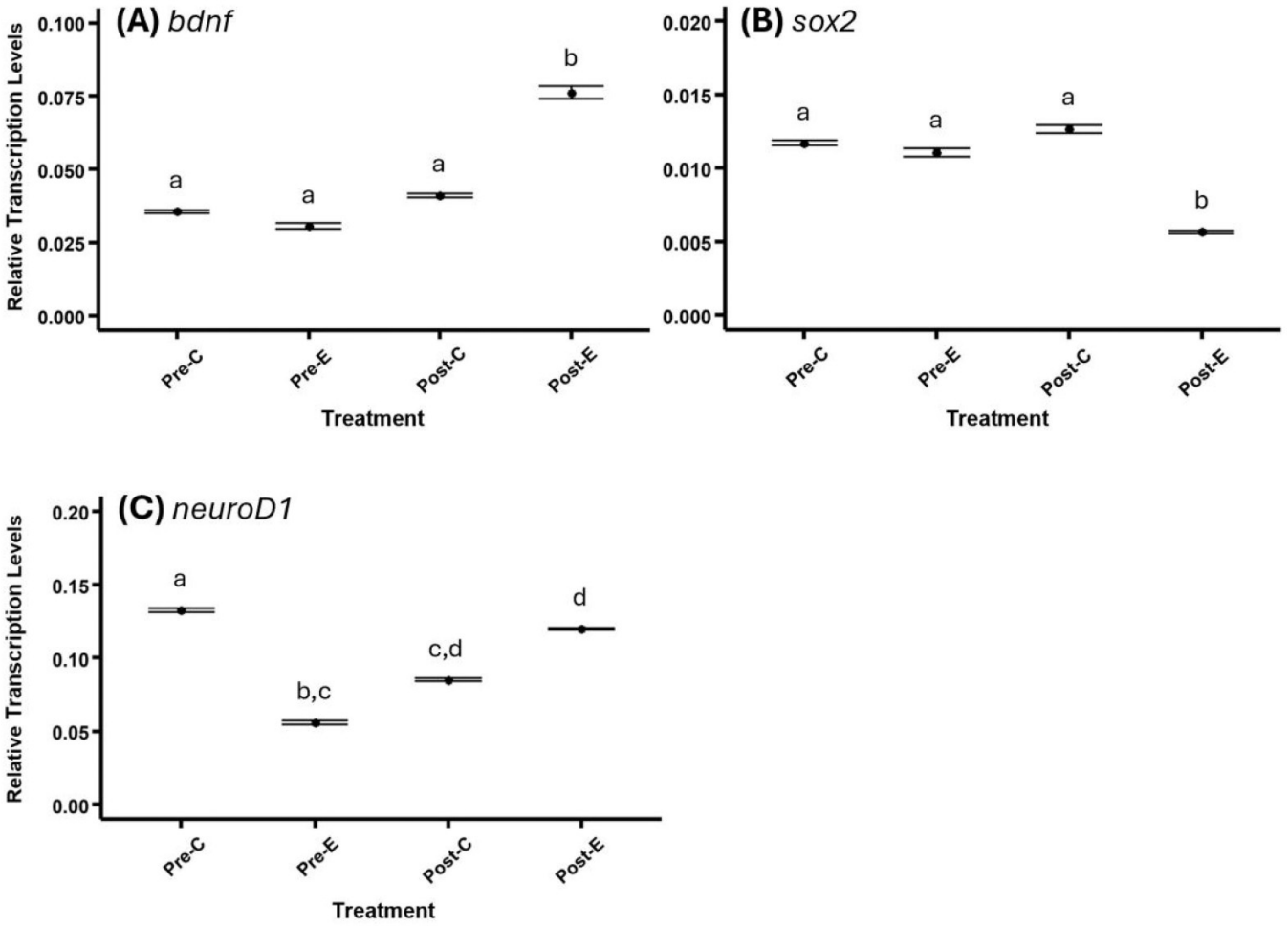
Discussion

The current study found that 2 weeks of constant boat noise exposure had some, albeit limited, effects on auditory morphology and behavioural responses to short-term boat noise suggesting that fish exposed to boat noises over long time periods may have the ability to habituate to acute exposures, therefore lessening the concerns of anthropogenic noise in natural environments. This apparent ability to habituate, however, is belied by the significant changes in gene transcription seen in brain tissues of these fish, suggesting that prolonged exposure may act as a significant stressor at the molecular level. Investigating responses at these different levels of organization allows for the effects of noise to be seen and the true impact of this anthropogenic stressor be fully realized.

Behavioural responses to continued sound exposure

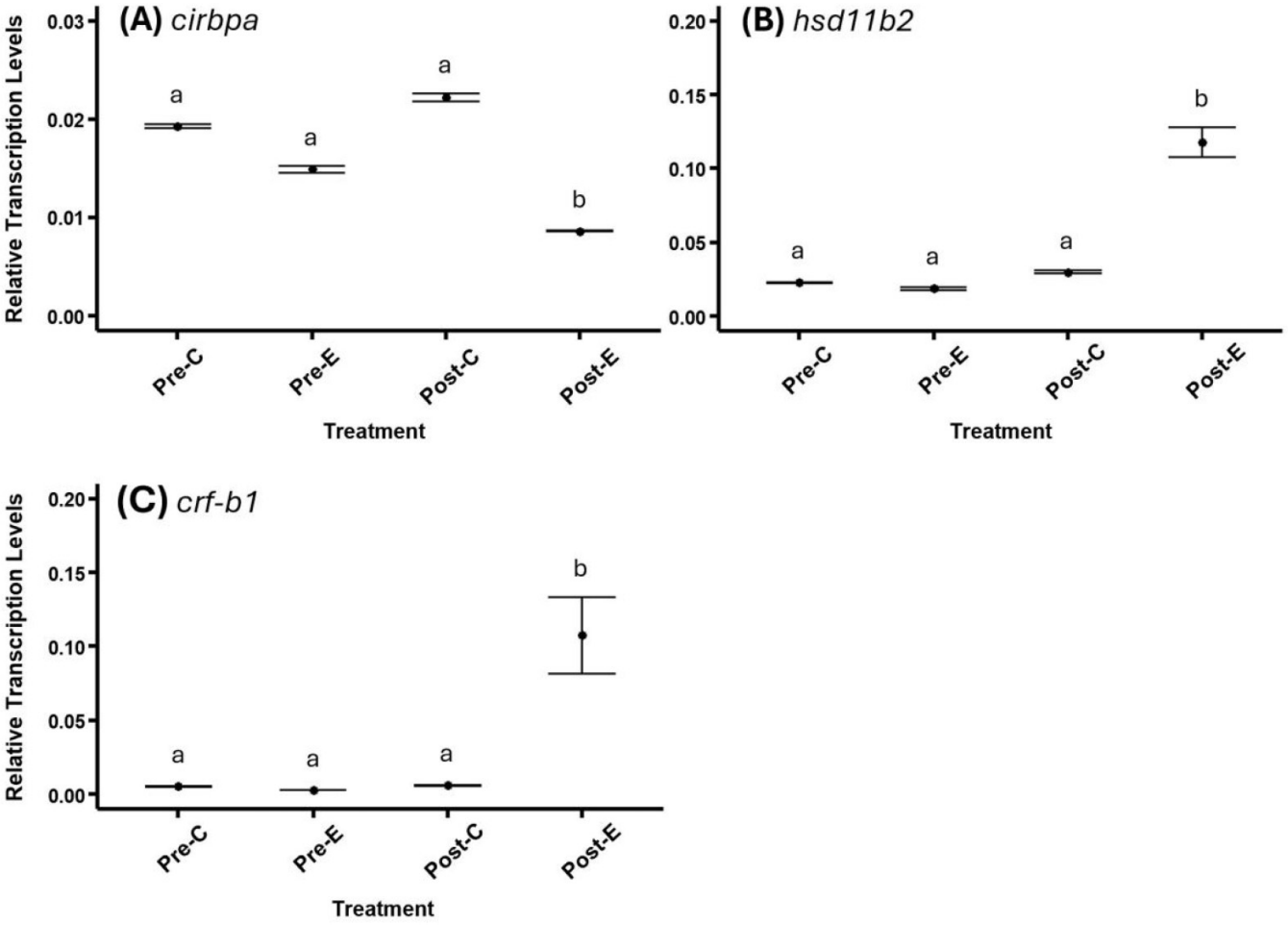
Fish that were exposed to sound for 2 weeks ignored subsequent bursts of boat noise as measured by total distance moved and swimming velocity when compared to unexposed fish, suggesting an ability to habituate to and

Fig. 5. Relative transcription of neural-related genes in pre-control, post-control, pre-experimental, and post-experimental brook trout for (A) *bdnf*, (B) *sox2*, and (C) *neuroD1*. Error bars represent ±1 S.E with lower case letters indicating statistical similarities and differences between individual treatments at a *p*-value of <0.05.



therefore ignore sound inputs. As the impacts of anthropogenic sounds are of increasing concern to survival of fish stocks (Duarte et al. 2021; Ferrier-Pagès et al. 2021; te Velde et al. 2024), an ability of fish to habituate to these sound sources might lessen concern for exposure guidelines. Previous work has shown that fish may be able to habituate to sound sources: three-spot dascyllus (*Dascyllus trimaculatus*) show diminished responses to sound exposures over repeated presentation of boat noises (Nedelec et al. 2016), and European seabass (*Dicentrarchus labrax*) also show reduced response in at least some behavioural measures in response to long-term impulsive sounds (Neo et al. 2015, 2016, 2018; Radford et al. 2016). In Atlantic cod (*Gadus morhua*) larvae, the type and duration of noise exposure did impact growth and development initially, but after 16 days the growth effects largely disappeared (Nedelec et al. 2015). Lake Malawi cichlids (*Cynotilapia zebroides*) collected from sites regularly exposed to boating activity also are less affected by short-term presentations of boat noise than the same species from unexposed sites (Harding et al. 2018), suggesting a potential for fish to habituate to noise sources at a behavioural level, which in turn could lessen the concerns for underwater noise exposures. While this behavioural habituation might be interpreted as a benefit, as it suggests daily movements of fish are no longer affected by anthropogenic noise sources, it is important to recognize that failure to move away from noise sources does not lessen the likelihood of fish exhibiting other signs of physiological stress (Anderson et al. 2011). Previous work has suggested that overreliance on behavioural metrics of responses to prolonged noise exposure can greatly lessen the regulatory import of mitigating these sound exposures (Bejder et al. 2009). In the current study we saw a decrease in hair cell density and previous work has shown that loss of hair cells can cause a temporary threshold shift (Smith et al. 2006; Uribe et al. 2013; Badlowski and Boyle 2024), implying that the reduced behavioural response in our noise-exposed fish is not due to true habituation but rather due to a reduced ability to detect the noise source. If previous reports of behavioural habituation to noise-sources are in fact due to loss of hair cells, then fish are not truly adapting to noise sources but instead are becoming at least partially deaf to these sources, increasing the need for enhanced mitigation measures in noisy habitats.

Fig. 6. Relative transcription of stress-related genes in pre-control, post-control, pre-experimental, and post-experimental brook trout for (A) *cirbpa*, (B) *hsd11b2*, and (C) *crf-b1*. Error bars represent ±1 S.E with lower case letters indicating statistical similarities and differences between individual treatments at a *p*-value of <0.05.



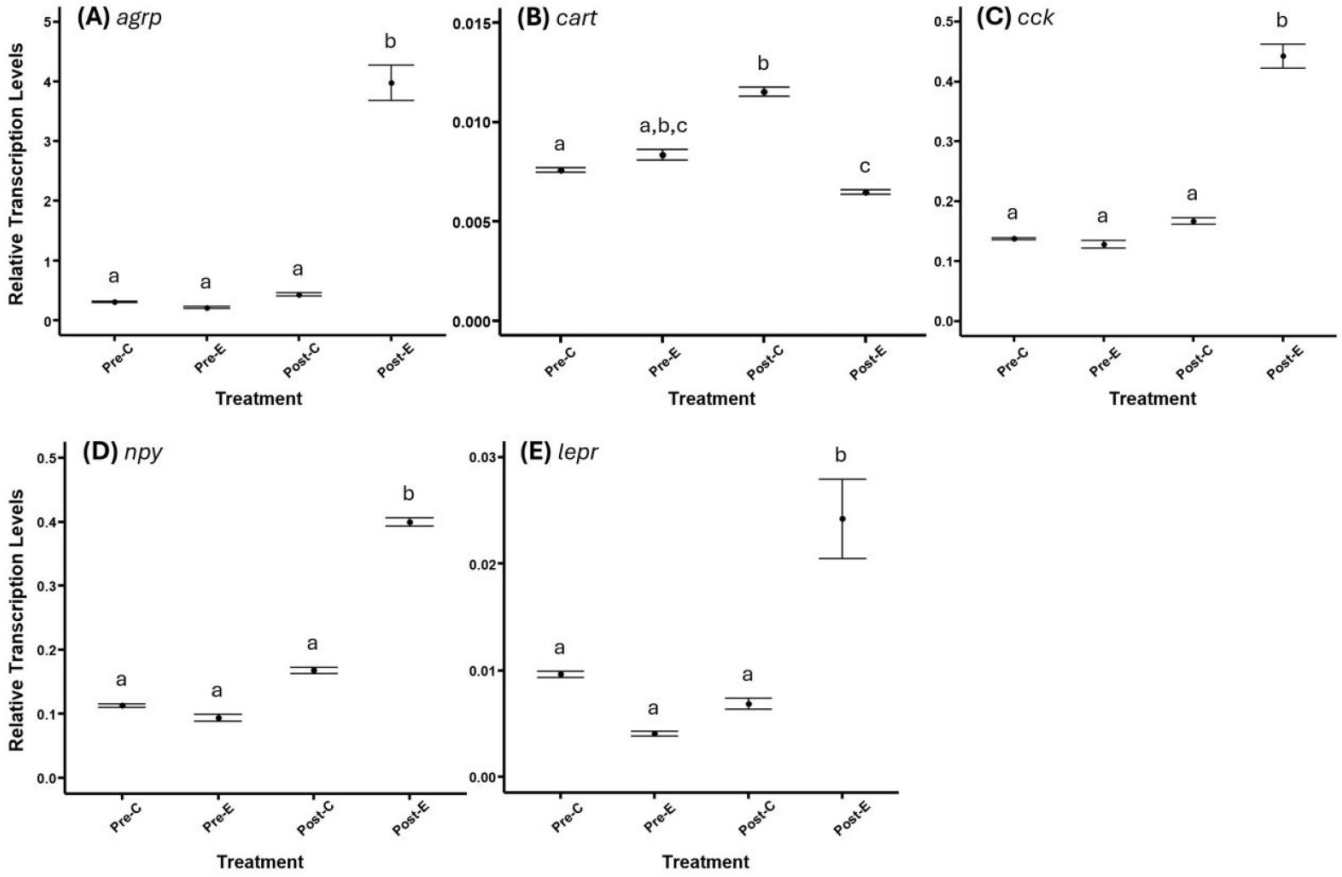
Transcriptional responses to continued sound exposure

In contrast to the behavioural results, measures of gene transcription did show effects of continued noise exposure at neural, stress, and appetitive axes. Along the neural axis both *sox2* and *bdnf* had transcriptional effects to sound exposure, but in different directions.

In the current study, the relative transcription of the *sox2* gene decreased in sound-exposed animals. In fish, *sox2* is part of the *soxB* subgroup of genes, all of which are involved in neural stem cell differentiation and proliferation (Hu et al. 2021). *Sox2* is strongly expressed in developing neuromasts and hair cells (Hernández et al. 2007) and downregulation of *sox2* blocks transdifferentiation of support cells into hair cells (Millimaki et al. 2010). *Sox2* is widely expressed in neurons throughout the adult brain (Taboada et al. 2018; Hu et al. 2021) and is used as a marker of hair cell neurogenesis in the fish central nervous system (Germanà et al. 2011). Interestingly, *sox2* is only downregulated after hair cells begin to die (Baek et al. 2022) so it can act as a marker of hair cell death, and deletion of transcriptional regulatory elements of *sox2* results in a loss of regeneration ability in fish (Jimenez et al. 2022). After hair cell loss, support cells can divide to provide a new source of regenerating hair cells (Beaulieu et al. 2024), but the reduction in *sox2* seen in the current study may block that regeneration potential. Since we did see a significant reduction in hair cell density in the current study, it is possible that the ongoing noise was acting as a stressor, blocking regenerative pathways in the brook trout CNS, and preventing hair cells from regenerating explaining the decrease in the relative transcription of *sox2*. While we cannot say how long the downregulation of *sox2* would last after noise cessation, the critical role of *sox2* in the regeneration process would suggest that the adaptive capacity of fish to regenerate in response to auditory damage might be blocked by continued noise exposure, as expected in heavy shipping areas, and therefore block the ability of fish repair damage and truly habituate to ongoing noise sources.

In contrast to *sox2*, the relative transcription of *bdnf* increased. In fish, *bdnf* is a known marker of neuroplastic responses and is often up- and down-regulated in response to acute and chronic environmental stressors, respectively, at the whole brain level (Tognoli et al. 2010; Pavlidis et al. 2015; Mes et al. 2018). In the current study, we saw an increase

Fig. 7. Relative transcription of appetite-related genes in pre-control, post-control, pre-experimental, and post-experimental brook trout for (A) *agrp*, (B) *cart*, (C) *cck*, (D) *npy*, and (E) *lepr*. Error bars represent ±1 S.E with lower case letters indicating statistical similarities and differences between individual treatments at a *p*-value of <0.05.



in the relative transcription of *bdnf* in post-experimental fish when compared to the other treatments, which suggests that post-experimental fish experienced the most severe change in response to the acute stressor. This aligns with previous work in which rainbow trout (*Oncorhynchus mykiss*) showed increased levels of *bdnf* in response to short term confinement stress (Johansen et al. 2012) and temperature stress (Topal et al. 2021). Additionally, *bdnf* has also been shown to be expressed in the lateral line system and inner ear of zebrafish at various life stages, a function that may be more relevant to the current study. This pattern of expression suggests a potential role of *bdnf* in hair cell development, maintenance, and regeneration (Germanà et al. 2020). While we cannot be certain of the cause of an increase in the relative transcription of *bdnf* following noise exposure, it is possible that *bdnf* is a more generalized indicator of neuronal stress (Johansen et al. 2012; Topal et al. 2021) and may activate multiple pathways in neural growth and synaptogenesis (Anand and Mondal 2020; Germanà et al. 2020).

Of the genes known to be expressed along the stress axis, three showed differential transcription after noise exposure with the two corticotropin-related genes (*crf-b1* and *hsd11b2*) showing upregulation indicative of a stress response. The *crf* family of genes are prime regulators of the hypothalamic-pituitary-interrenal axis in fish (Bernier et al. 2009). Expression of *crf* is quickly upregulated in zebrafish in response to a physical stressor and this upregulation is matched by a corresponding increase in whole-body cortisol levels (Fuzzen et al. 2010). Expression of *crf* is widespread throughout the brain of fishes (Alderman and Bernier 2007) so it is a promising overall stress marker to assess the acoustic stress in the current experiment. Atlantic salmon have also shown increases in hypothalamic levels of corticotropin releasing hormone (*crh*; also known as *crf*) following exposure to chronic stress (Madaro et al. 2015; Opinion et al. 2023) in addition to rainbow trout that showed increased levels in response to acute stressors (Doyon et al. 2005).

Similarly, *hsd11b2* is also a marker of cortisol activation in fishes as it catalyses the transformation of cortisol into cortisone during the stress response (Tokarz et al. 2013), although previous studies have shown somewhat contradictory results complicating the interpretation of *hsd11b2* expression, especially when studied in tandem with *crf* (Madaro et al. 2015; Opinion et al. 2023). Atlantic salmon exposed to unpredictable chronic stress showed a down-regulation of *hsd11b2* in the brain when compared to control fish in one study (Madaro et al. 2015) and no difference in *hsd11b2* between treatments in another (Opinion et al. 2023), with both showing an increase in *crf*. Comparatively, both *crf-b1* and *hsd11b2* are increased in the brain of rainbow trout (*Oncorhynchus mykiss*) during periods of social stress corresponding to increased cortisol levels (Best et al. 2023).

Unlike *crf-b1* and *hsd11b2*, *cirbpa* mRNA decreased after noise exposure. Expression levels of *cirbpa* are downregulated in salmonids in response to thermal stress (Jeffries et al. 2012, 2014; Verleih et al. 2015; Beemelmanns et al. 2021). Additionally, these genes appear to be upregulated in zebrafish gills with decreased temperature (Chou et al. 2008). As all three of these genes have been previously shown to have important responses to generalized stress responses in fish, it seems likely that the noise exposures in the current experiment also induced a physiological response indicative of a stressed state. These results are in opposition to the behavioural results, suggesting a more subtle response that would be missed by only observing behaviours.

Of the genes thought to be involved in the appetitive axis, four showed an increase in relative transcription after the 2-week noise exposure. While it may be expected that an acoustic stressor would lead to decreased appetite, and therefore reductions in expression of appetitive genes, the appetitive response to stressors continues to be poorly understood in fish at both the behavioural and molecular level (Conde-Sieira et al. 2018; Pawlak et al. 2023). Depending on species, *agrp* may increase during fasting stress (Kalananthan et al. 2020, 2023) or be unaffected (Jørgensen et al. 2016; Striberny and Jørgensen 2017) and is likely mediated by serotonergic and dopaminergic pathways (He et al. 2018). Similarly, *npy* transcription shows interspecific differences to fasting stress (Narnaware et al. 2000; Murashita et al. 2009) and intraspecific differences in diet supplementation (Frank et al. 2024), but is affected by growth hormone expression while also showing differential effects in different brain regions (Kim et al. 2015) and at different cortisol concentrations in unpredictable ways (Bernier et al. 2004). Orexigenic genes, especially *npy* and *agrp*, may be upregulated during handling stress making it unlikely that they are directly involved in short-term changes in feeding activity (Cortés et al. 2018). In Atlantic salmon, fasting upregulates hypothalamic *lepr* expression (Angotzi et al. 2016) but rainbow trout show no differences in *lepr* expression even after 3 weeks of fasting (Gong and Björnsson 2014). Finally, *cck*, a gene thought to be anoxerigenic (Pawlak et al. 2023) changes expression during fasting in winter flounder (*Pleuronectes americanus*; MacDonald and Volkoff 2009), yellowtail (*Seriola quinqueradiata*; Murashita et al. 2006), and zebrafish (Koven and Schulte 2012) but is unaffected in cavefish (Wall and Volkoff 2013). In the current study we were only able to examine transcription of these genes in whole brain samples; however, it is evident that there are region-specific transcription patterns in many of the genes under investigation in the current project (Pawlak et al. 2023). Despite having only whole brain samples, it is clear that auditory exposure can change transcriptional patterns in the brain and is likely to have extended effects at the transcriptional level.

In conclusion, while long-term exposure to boat noise had limited effects on auditory morphology and behaviour, it showed a more significant effect on levels of transcription in genes important for brain function, physiological stress, and appetite regulation. To date, the current study is one of the first to integrate anatomical and behavioural metrics with transcriptional profiles to determine the effects of anthropogenic noise on fish habituation. While there is increasing concern over the issue of underwater noise on both marine (Duarte et al. 2021; Ferrier-Pagès et al. 2021) and freshwater (te Velde et al. 2024) environments and a call for increased regulation of noise sources (Directive 2008; Nolet 2017), the degree to which fish can habituate to underwater noise remains a key area of investigation (Johansson et al. 2016; Holmes et al. 2017; Rojas et al. 2021). If fish are perceived at a behavioural level to habituate—and therefore ignore—noise sources, it may reduce the push for noise mitigation, which highlights that a comprehensive approach (Mickle and Higgs 2018;Mills et al. 2020) is critical to fully understand both short-term and long-term impacts.

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Formal analysis: RKB, DMH
Funding acquisition: CADS, DDH, DMH
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Resources: AW, CADS, DDH
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Competing interests

The authors declare there are no competing interests.

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