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The Effects of Continuous Acoustic Stress on ROS Levels and Antioxidant-related Gene Expression in the Black Porgy (*Acanthopagrus schlegelii*)

Hao-Yi Chang[1], Tzu-Hao Lin[2], Kazuhiko Anraku[3], and Yi Ta Shao[1,4,*]

[1]*Institute of Marine Biology, National Taiwan Ocean University, Keelung 202, Taiwan. E-mail: luck77995@gmail.com*
[2]*Department of Marine Biodiversity Research, Japan Agency for Marine-Earth Science and Technology, Kanagawa, 237-0061, Japan. E-mail: schonkopf@gmail.com*
[3]*Fisheries Department, Kagoshima University, Kaogoshima 890-0056, Japan. E-mail: anraku@fish.kagoshima-u.ac.jp*
[4]*Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung 202, Taiwan*

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Hao-Yi Chang, Tzu-Hao Lin, Kazuhiko Anraku, and Yi Ta Shao (2018) Short-term exposure to strong underwater noise is known to seriously impact fish. However, the chronic physiological effects of continuous exposure to weak noise, *i.e.* the operation noise from offshore wind farms (OWF), remain unclear. Since more and more OWF will be built in the near future, their operation noise is an emerging ecological issue. To investigate the long-term physiological effects of such underwater noise on fish, black porgies (*Acanthopagrus schlegelii*) were exposed to two types of simulated wind farm noise—quiet (QC: 109 dB re 1 μPa / 125.4 Hz; approx. 100 m away from the wind turbine) and noisy (NC: 138 dB re 1 μPa / 125.4 Hz; near the turbine)—for up to 2 weeks. Measurement of auditory-evoked potentials showed that black porgies can hear sound stimuli under both NC and QC scenarios. Although no significant difference was found in plasma cortisol levels, the fish under NC conditions exhibited higher plasma reactive oxygen species (ROS) levels than the control group at week 2. Moreover, alterations were found in mRNA levels of hepatic antioxidant-related genes (*sod1*, *cat* and *gpx*), with cat downregulated and *gpx* upregulated after one week of QC exposure. Our results suggest that the black porgy may adapt to QC levels of noise by modulating the antioxidant system to keep ROS levels low. However, such antioxidant response was not observed under NC conditions; instead, ROS accumulated to measurably higher levels. This study suggests that continuous OWF operation noise represents a potential stressor to fish. Furthermore, this is the first study to demonstrate that chronic exposure to noise could induce ROS accumulation in fish plasma.

Key words: Black porgy, Underwater noise, Reactive oxygen species, Antioxidant, Auditory evoked potential.

BACKGROUND

Natural underwater soundscapes are composed of geophonic and biophonic sounds, such as wind, waves and bubble noises, whale calls, fish choruses and sounds of snapping shrimp (Hildebrand 2009). After the industrial revolution, anthropogenic noise became another prominent source of underwater soundscapes, altering the soundscape and increasing the background noise level (*e.g.* Andrew et al. 2002; Hildebrand 2009; McDonald et al. 2006; Slabbekoorn et al. 2010). Offshore wind farms (OWF) are an emerging green energy sources that have been installed

*Correspondence: Tel: +886-2-24622192 ext. 5327. Fax: +886-2-24629381. E-mail: itshao@mail.ntou.edu.tw

over the past decades (Krupp et al. 2009). The OWF's piles can be used to anchor floating cages for offshore aquaculture development and may have artificial reef effects that benefit the local marine ecosystems (Boehlert and Gill 2010). The low frequencies sound of wind turbine operation is a consistent anthropogenic noise in the ocean (Andersson et al. 2011).

Anthropogenic noise may influence marine animals in many ways (*e.g.* Kunc et al. 2016; Peng et al. 2015), and the impacts of artificial noise depend on their intensity and duration. Intense underwater noise, such as that from pile driving, military sonar, seismic exploration and ship traffic, may cause hearing loss, auditory tissue damage, physical injury, behavioral changes and sometimes even death in underwater animals (McCauley et al. 2003; Popper and Hastings 2009a b). Recent studies indicate that continuous noise with lower sound pressure may also induce stress responses. Nevertheless, such physiological responses may be relatively minor. For example, lined seahorses (*Hippocampus erectus*) exposed to noise from air pumps (123.3 ± 1.0 dB) had elevated adrenal cortisol concentrations and weaker immune function compared to the control group (110.6 ± 0.6 dB) during a four-week experimental period (Anderson et al. 2011). Additionally, the conversion rates and clearance of cortisol both increased in milkfish (*Chanos chamos*) exposed to simulated wind farm noise (Wei et al. 2018).

The level of circulating cortisol is a measurable parameter indicative of stress. When animals are stressed, cortisol is released by the adrenal cortex, or head kidney in teleosts, which quickly raises circulating levels. However, the elevated cortisol levels do not last long and may return to resting levels as the animal adapts to the stressors (Barton 2002). High cortisol levels can suppress immunity or other somatic functions, including reproduction and growth. This action might mediate observed effects of chronic exposure to anthropogenic noise on long-term physiological effects. Rolland et al. (2012) reported that right whales (*Eubalaena glacialis*) exposed to low-frequency (20-200 Hz) shipping noise had higher fecal metabolites of stress-related hormones (glucocorticoids) than those not exposed (Rolland et al. 2012). Likewise, the increase in reactive oxygen species (ROS) levels in plasma or inner ear lymph of terrestrial mammals may also be induced by chronic noise exposure (Kight and Swaddle 2011).

ROS are oxidative metabolites mainly derived from mitochondrial respiratory machinery (Le Bras et al. 2005). The term includes oxygen-centered and oxygen-related compounds, such as superoxide radical (O_2^-), hydroxyl radical (OH^-), singlet oxygen (O_2) and hydrogen peroxide (H_2O_2) (Nordberg and Arnér 2001). Under normal physiological conditions, intracellular ROS levels are controlled by antioxidants (Floyd 1999) such as glutathione (GSH), vitamin C, vitamin E, carotenoids and radical-scavenging enzymes (*e.g.* superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST)) (Jayaraj et al. 2007). In teleosts, the major antioxidant defense system operates in the liver and kidney (reviewed in Basha and Rani 2003). In ROS metabolism, intracelluar or extracellular SOD(s) removes O_2^- through dismutation to O_2 and H_2O_2; the H_2O_2 is then sequentially reduced to H_2O and O_2 by CAT or GPx, which counteracts the toxicity (Kashiwagi et al. 1997). However, if the production of ROS exceeds the scavenging capacity in the organisms, accumulation of ROS may damage cells via direct attacks on biological molecules (Floyd 1999). Recently, measurements of ROS levels and antioxidative enzyme activities in fish under ambient stress have been used as indicators of long-term stress (Jin et al. 2010; Tseng et al. 2011). The effects of chronic sound exposure on the ROS regulatory system have not been previously investigated in any fish species.

OWF noise exposure has been shown to result in higher cortisol turnover rate in up to a week in milkfish (Wei et al. 2018). In this study, we investigate the ROS levels and antioxidant-related genes in fish exposed to a longer period (up to 2 weeks) of continuous noise to understand their long-term of physiological responses. Black porgies (*Acanthopagrus schlegelii*) were exposed to either high or low levels of underwater noise. This species is a common in many coastal embayments of west Taiwan, where OWF construction is scheduled. Moreover, it is a potential aquaculture species to be kept in offshore ocean farms that may be affected by OWFs. In addition to plasma cortisol levels, circulation ROS levels and mRNA levels of hepatic Cu/Zn-SOD (*sod1*), CAT (*cat*) and GPx (*gpx*) were measured in fish kept under detectable noise conditions for 24 hr, one week and two weeks. In previous studies, elevated plasma ROS levels revealed possible oxidative stress in animals (reviewed in Adler et al. 1999), and indicated the noise impacts on human (Purnami and Manyakori 2018; Nyilo and Putri

2018). Furthermore, there are three isoforms of SOD in most chordates, which differ in their metal cofactor(s), located in the cytoplasm, mitochondria and extracellular, respectively (Zelko et al. 2002). However, *sod1*, the cytosolic form, is the only one that has been studied in the black porgy (An et al. 2010). The results of the current study describe a novel aspect of chronic physiological stress response in fish, revealing that ROS homeostasis may change under weak but continuous noise.

MATERIALS AND METHODS

Experimental animals

This study used black porgy (*Acanthopagrus schlegelii*) sub adults greater than 1 year of age (15.6 ± 0.3 cm, 118.5 ± 5.0 g, n = 185). The experimental fish were bred in the Aquatic Animal Center of National Taiwan Ocean University and transported to the research field rented from Aquaticlch Biotech Company Ltd., Yilan in 2016 March. Before the experiment, the fish were kept in a concrete pools (3 × 7 × 1.5 m) (c. 200 fish per pool) with flowing fresh seawater for the sound perturbation experiment. Those fish were kept outdoors, but shielded from the sun with black gauze to maintain the temperature below 27°C. For the hearing threshold determination, another set of fish were transported to the laboratory directly and kept in 600L fiber reinforced plastic (FRP) tanks (10 fish per tank) with a filtered system. The fish were fed once a day with commercial pellets *ad libitum* (United Aquaculture Feeds, Taiwan). All animal care and experiments followed protocol (105039) approved by the Institutional Animal Care and Use Committee of National Taiwan Ocean University.

Hearing threshold determination

To ensure that fish could detect the noise in sound perturbation experiments, the hearing thresholds of the back porgies were measured by the auditory evoked potentials (AEP) method (first described by Kenyon et al. (1998); modified by Babaran et al. 2008)). In general, fish in the same size range were immobilized by injecting gallamine triethiodide (Flaxedil; ALX-550-180-M500, Enzo Life Sciences, New York, NY, USA) into the dorsal muscle (c. 0.3 mg per kg of body weight). The immobilized fish were wrapped in nylon mesh and suspended in the experimental tank with the top of their heads kept about 2 mm above the water surface. The experimental tank (0.5 × 0.5 × 0.5 m) was made of flexible plastic supported by netting that was hung from a metal frame. Babaran et al. (2008) indicated that tanks made from this material are the most efficient at transmitting sound that originates in the air and penetrates the water. The experimental apparatus was placed on a vibration-free air table (Vibraplane, Kinetic Systems, Boston, MA, USA) located inside a soundproof room (1.9 × 1.6 × 2.6 m) to minimize disturbances from noise and vibration.

A recording electrode (a Teflon-coated silver wire, 0.6 mm in diameter with a 2-mm exposed tip) was placed on the midline of the skull between the eyes. A reference electrode of a similar specification was placed 0.5 cm in front of the recording electrode. EEG paste (Ten20® conductive EEG paste, D.O. Weaver and Co., Aurora, CO, USA) was used to cover the area of skin attached to the electrodes in order to enhance the conduction of evoked potentials from the brain to the electrodes. During the recordings, a small tube was used to irrigate the gills with oxygenated seawater to keep the fish alive.

AEP were enhanced using a differential amplifier (DP-301, Warner Instruments, Hamden, CT, USA) and input into software (LabChart v8, AD Instruments, Sydney, Australia) through an analog-digital converter (PowerLab 4/35, AD Instruments). The band pass filter on the amplifier was set at 10 Hz-10 kHz while the sampling frequency of the analog-digital converter was fixed at 10 kHz.

One hundred-millisecond tone burst stimuli were generated by a functional synthesizer (DF1906, NF Co., Yokohama, Japan) with waveform editor software (arbitrary waveform editor 0106, NF Co.), and amplified by a 120 W mixer amplifier (BG-2120, TOA Electronics Inc., San Francisco, CA, USA) (frequency response: 50 Hz-20 kHz). The stimuli were produced using a 45-cm diameter woofer (Barcorna, Taiwan) suspended 1.25 m above the test subjects. Eight sound frequencies were administered: 100, 200, 400, 600, 800, 1000, 1200 and 1500 Hz. Each tone burst (100 ms) included a sound wave consisting of 5-cycle waves (including one rise, one fall and three plateau), in which the duration of the sound stimuli were: wave duration (ms) = 5 × 1000 / frequency (Hz), and inter-stimulus intervals (tone burst duration - wave duration) (Fig. S1). Additionally, each AEP represented the average response of 400 burst stimulus presentations (200 averages for each polarity/phase were added together to cancel mechanical noises). The signal

frequency and peak-to-peak voltage level were both controlled by software (arbitrary waveform editor 0106, NF Co.). A hydrophone with an effective sensitivity of -166 dB re 1 V/μPa (BII-7121, Benthowave Instrument Inc., Collingwood, Canada) was placed in the experimental tank near the fish to monitor the testing sound intensity. A charged amplifier (F8, ZOOM Corporation, Tokyo, Japan) was used in the recording system. The sound recordings were fed into a computer, synchronized to the time domain of auditory evoked signals.

Following previous studies on AEP, each experiment started at the highest sound pressure level (SPL) to induce suprathreshold responses; the SPL was then reduced in ~5 dB steps until traceable and repeatable waveforms were no longer detectable (Yan and Curtsinger 2000; Akamatsu et al. 2003). For each sound stimulus, recordings were performed twice to compare the similarities of the first and second averaged ABRs by calculating the correlation coefficient (*r*) (Fig. S2). The AEPs were deemed to have responded to the sound stimulus only when $r > 0.3$ (Babaran et al. 2008). The black porgies' audiograms are shown in figure 1; each frequency includes data from 5 fish.

Experimental setup and sound perturbation

One week before noise perturbation experiments, the black porgies were moved from the FRP tank and kept separately in mesh nylon floating cages (1.5 × 1.8 × 1.5 m, 10 fish per cage), which were then placed inside a concrete pool (3 × 7 × 1.5 m). Two concrete pools (control and treatment) were used in the experiment. The pools were approximately 25 m apart, and between the pools was a 2 m deep ditch and other empty pools. In the treatment pool, four floating cages were lined up in two rows in front of the submerged loudspeaker that administered the noise treatments (Fig. S3). To equalize the fish number per pool with the treatment group, another four cages with fish were placed in the corresponding positions of the control pool. During the experiment, the fish were fed with 5-10% of body weight of pellets every day, and the tanks were supplied with approximately 20 L/min of fresh seawater.

The noise exposure experiment was done following the protocol from Wei et al. (2018) with identical devices and setup (including pools and water level). Briefly, Dr. Mathias H. Andersson provided us with the noise recording used in an earlier sound exposure experiment (Wei et al. 2018). The noise treatment file was broadcast by a mixer amplifier (BG-2120, TOA Electronics, Inc.) connected to an underwater loudspeaker (AQ339 Clark Synthesis Diluvio Ltd. frequency response: 20 Hz-17 kHz), which was suspended in the water, 0.7-0.8 m deep, and facing the floating cages. The sound pressure levels in the experimental pools were calibrated with an MR-1000 portable sound recorder (frequency response: 20 Hz-40 kHz ± 1 dB, Korg Inc., Tokyo, Japan) and a hydrophone (effective sensitivity: -165 dB re 1 V/μPa, frequency response: 16 Hz-44 kHz + 2/-3 dB) (C54XRS, Cetacean Research Technology, Seattle, WA, U.S.A.). During the recording, the hydrophone was suspended on a fixed wooden bar at a depth of 1 m below the surface near the center of the cage.

Two noise levels were used for the perturbation. In the noise perturbation pools, the distances from the rows of cages to the loudspeaker were adjusted to correspond to noisy conditions (NC) or quiet conditions (QC). In NC, the distance between the loudspeaker and the floating cage was approximately 1 m, where the spectral level in the cage was 138/115 dB re 1 μPa at 125.4/315.5 Hz. This spectral level is similar to that measured near the wind turbine (Andersson et al. 2011), and the sound pressure level was far above the black porgy hearing threshold (Fig. 1). The sound spectral level measured in QC (109/99 dB re 1 μPa at 125.4/315.5 Hz), which corresponded to the sound spectral level 100 m from the wind turbine (Andersson et al. 2011), was just high enough for the fish to hear (Fig 1). The floating cages in the control condition (CC) were placed in another concrete pool. The ambient noise in CC was 80 dB re 1 μPa at 125.4 Hz. (Fig. 1).

Three independent sets of experiments were performed between June 2016 and October 2016 for replicates. The pool used for control or treatment were swapped in different sets of experiments, and the side that the loudspeaker stood (right or left of the pool) was alternated to avoid possible confounding effects of position. In each set of experiments, 5 fish were taken from the NC, QC and CC groups to be dissected at each time point (24 hr, 1 week and 2 weeks). No significant differences in survival rate were found among the NC, QC and control groups. Similarly, feeding behavior was not different among groups.

Dissection

Before dissection, fish were immediately transferred into 0.01% buffered MS-222 (Ethyl 3-aminobenzoate, methanesulfonic acid salt, Sigma-Aldrich, St. Louis, MO, USA) solution for anaesthetization. After this, the caudal peduncle was severed, and blood was collected from the caudal artery in heparinized micro hematocrit tubes (Na-hep. lot no. 1605445; Assistent, Sondheim, Bavaria, Germany). In the experiments, the fish were anaesthetized within 1 min, and the entire sampling process was finished in about 5 min. After centrifugation for 2 min at 13,000 rpm (hematocrit rotor, 185 mm in diameter)—approx. 35017 relative centrifugal force (RCF, G-force)—plasma samples were transferred into another set of heparinized 1.5 ml screw-cap tubes and chilled in liquid nitrogen. Before measurement, plasma samples were stored at -80°C. Liver tissue was removed immediately after sacrifice and stored in RNAlater® solution (P/N: AM7021, Ambion Inc, Carlsbad, CA, USA) at 4°C for 12 hr following the manufacturer's instructions. After this brief incubation, samples were transferred to -80°C for storage. Ten of fifteen liver and plasma samples (3 replicates * 5 fish) were taken randomly from each group for the following study.

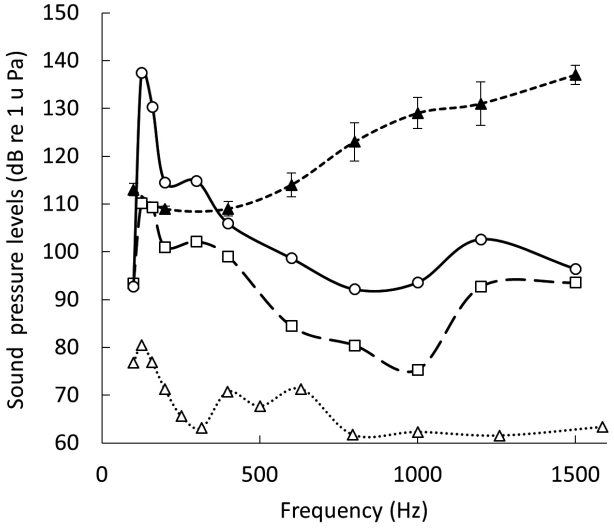

Fig. 1. Acoustic characteristics of the noise perturbation experiment. The 1/3-octave band spectral characteristics of the noisy condition (NC; solid line with open circles), quiet condition (QC, dashed line with open squares), ambient noise (control, CC, dotted line with open triangles) and the hearing curve of the black porgy (dashed line with solid triangles) (Means ± SEM are shown). N = 5 at each frequency in the audiogram.

Plasma cortisol and reactive oxygen species (ROS) level measurements

Plasma cortisol and ROS levels were determined by commercial enzyme-linked immunosorbent assay kits (cortisol: DNOV001, Dietzenbach, Germany; ROS: MBS021826, MyBioSource, San Diego, CA, USA). The cortisol kit has been used in previous studies to test many teleost species, *e.g.* Nile tilapia, *Oreochromis niloticus* (Antache et al. 2014); zebrafish, *Danio rerio* (Lin et al. 2016); European eel, *Anguilla anguilla* (Sbaihi et al. 2009; Renault et al. 2011; Sancho et al. 2017); and orange spotted grouper, *Epinephelus coioides* (Lee et al. 2017). Moreover, the ROS kit was designed to react with fish plasma according to the manufacturer's instructions.

Cortisol standards, ROS standards and test solutions were prepared according to the manufacturer's instructions. For cortisol, a standard curve was generated from six cortisol standards: 0, 31.25, 62.50, 125.00, 250.00, and 500.00 ng/mL; seven standards were used for ROS: 15.62, 31.25, 62.50, 125.00, 250.00, 500.00, 1000.00 IU/mL. Plasma samples were measured directly, without dilution, and absorbance of both was measured at 450 nm with a multi-detection microplate reader for both assays (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA).

RNA extraction, reverse transcription, cloning and q-PCR

Total RNA was extracted from 100 mg liver tissue using RNeasy® Plus Universal Tissue Mini Kit (cat. 73404, Qiagen, Hilden, North Rhine-Westphalia, Germany) according to the manufacturer's protocols.

Total RNA contents and quality were measured and checked by NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). For all samples, 4 μg of total RNA were reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (cat. 4368814, Applied Biosystems, Foster City, CA, USA) and RNAse Inhibitor (Y9240L, Enzymatics, Beverly, MA, USA).

The specific primers designed for q-PCR were based on the published sequences from NCBI (accession numbers are given in Table 1). Before the measurement, the amplification efficiency and melting curve of each q-PCR primer pair was tested by 10-fold serial dilutions of the templates (1/1 to 1/1000) with three replicates for each gene. The efficiency of each set of

primers ranged from 95.1 to 97.0% (Table 1). The R-squared values of all standard curves were above 0.995 in all rounds of the experiment. Expression of target gene mRNA was determined by q-PCR with the Roche LightCycler® 480 System (Roche Applied Science, Mannheim, Baden-Württemberg, Germany). PCR reactions contained 40 ng (for target genes measurements) or 4 ng (for reference gene measurements) cDNA and 50 nM of each specific primers (Table 1) (An et al. 2010) and the LightCycler® 480 SYBR Green I Mastermix (cat no. 4887352001, Roche Applied Science, Mannheim, Baden-Württemberg, Germany) in a final volume of 10 μl. Reactions were performed in a white LightCycler® 480 Multiwell Plate 384 (cat no. 04729749001, Roche Applied Science, Mannheim, Baden-Württemberg, Germany) with sealing foil (Roche, cat. no. 04729757001). All q-PCR reactions were performed as follows: 1 cycle of 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. PCR products were subjected to a melting-curve analysis, and representative samples were electrophoresed to verify that only a single product was present. Control reactions were conducted with RNA free water to determine background content. Three replicates were performed for each gene/sample. Expression levels of target genes were normalized against the reference gene, *actb* (β-actin). The efficiency-corrected relative expression method was used to quantify the relative expression levels (Weltzien et al. 2005).

Statistics

We used SPSS v. 20 to compare data through Mann-Whitney non-parametrical *U*-tests. Multiple group comparisons were performed by the one-way Kruskal-Wallis test, followed by post hoc multiple comparisons with Dunn's test (Olsvik et al. 2005).

RESULTS

Audiogram

Our audiograms of the black porgies show that the frequency with the highest sensitivity (lowest threshold) was 400 Hz, for which the threshold was 109 dB re 1 μPa. The hearing sensitivity decreased gradually at higher and lower frequencies. Furthermore, the NC sound spectrum was well above the detectable threshold at 125.4 Hz, but only a small portion of the QC spectrum was above the detectable limit (Fig. 1).

Plasma cortisol and ROS levels

The fish exposed to NC showed a trend toward increased plasma cortisol levels after 24 hr of exposure, but the change was not statistically different from the control (Mann-Whitney *U*-tests $p = 0.229$, $U = 66$, $W = 111$). After noise exposure for 1 or 2 weeks, the cortisol level of the NC group had returned to the resting level. No significant differences were found between the control and QC group during the experimental period (Fig. 2A).

In addition, under QC noise exposure, there was no significant change in ROS levels along the experimental period, and there was no difference between QC and controls at any examined time point. On the other hand, the ROS levels in the fish under NC conditions were found to increase gradually after 24 hr of exposure so that the ROS levels measured at 2 weeks were higher than the levels measured at 24 hr (Mann-Whitney *U*-tests $p < 0.05$, $U = 78$, $W = 133$). The NC-exposed fish also exhibited higher ROS levels in the second week than the control group (Mann-Whitney *U*-tests

Table 1. The primer sequences used for q-PCR. The reference gene was *actb*

Gene	Primer sequence	Product size (bp)	Acc. No.
sod1 *Ei* = 0.97	F 5'-GTTGCCAAGATAGACATCAC-3' R 5'-TTAGACTCTCCTCGTTGC-3'	89	AJ000249.1
gpx *Ei* = 0.96	F 5'-CAGGAGAACTGCAAGAAT-3' R 5'-TTCCATTCACATCCACCTT-3'	72	GU799605.1
cat *Ei* = 0.97	F 5'-GCAACTACCAGCGTGATG -3' R 5'-CAGACACCTTGAACTTGGA-3'	92	GU370345.1
actb *Ei* = 0.95	F 5'-GCAAGAGAGGTATCCTGACC-3' R 5'-CTCAGCTCGTTGTAGAAGG-3'	67	AY491380.1

Ei indicates the efficiency for each q-PCR primer.

$p < 0.05$, $U = 82$, $W = 137$) (Fig. 2B).

Expression of genes involved in the antioxidant pathway

Relative to the reference gene (*actb*), the mRNA levels of hepatic *sod1* showed no difference between groups at any examined time point (Fig. 3A). However, hepatic cat mRNA levels tended to decrease after 24 hr of noise perturbation; this trend was found in the control as well. After 1 week of exposure, the fish under QC had lower hepatic *cat* mRNA levels compared to both CC and NC fish (one-way Kruskal-Wallis $p < 0.05$, *d.f.* = 2, $H = 17.11$) (Fig. 3B). Furthermore, there was no significant change across time for hepatic *gpx* mRNA levels. Nevertheless, *gpx* mRNA levels were higher in the QC fish than CC and NC after 1 week

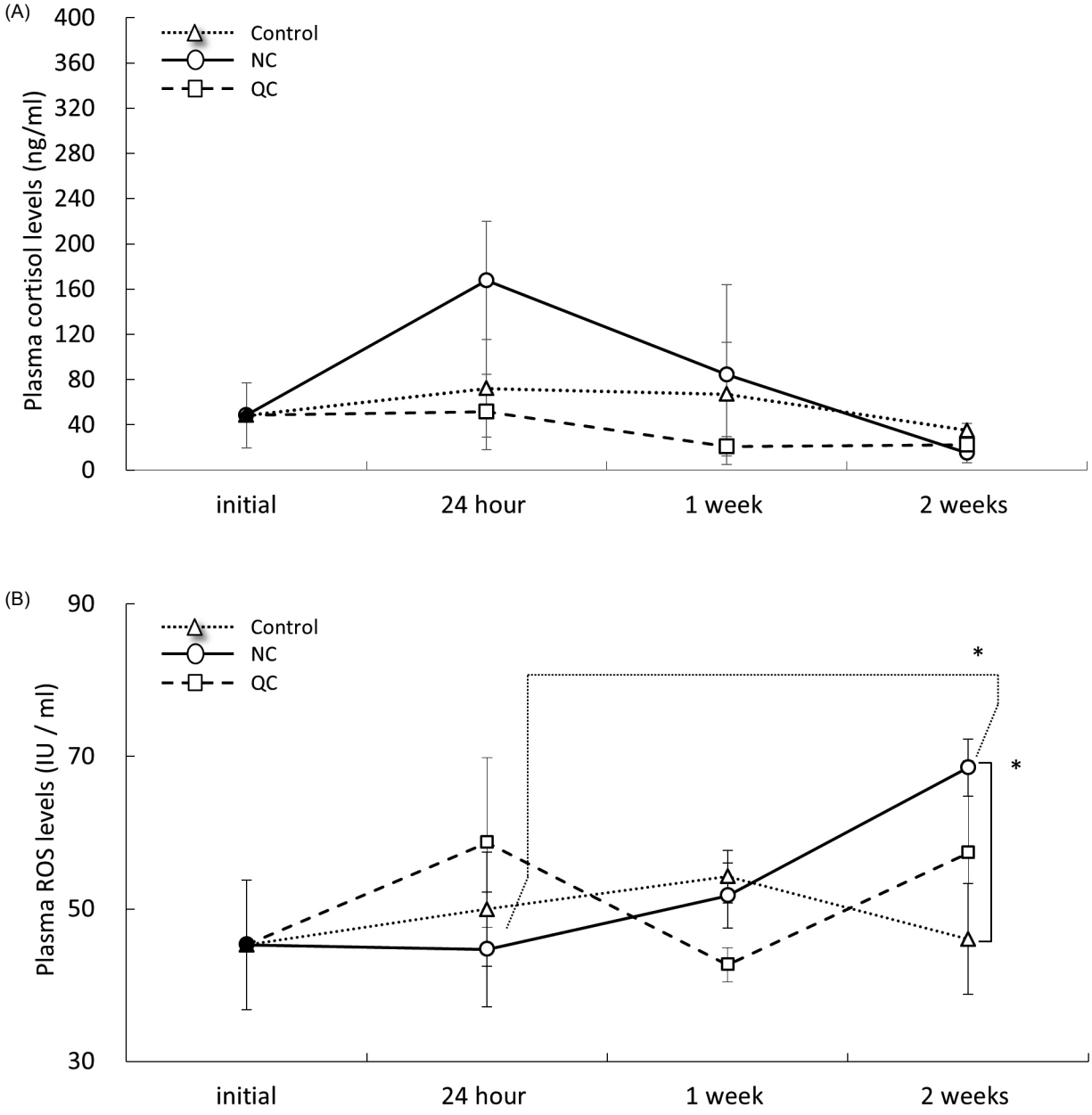

Fig. 2. The effects of exposure to the underwater noise on plasma (A) cortisol and (B) ROS levels during the 2-week treatment period. NC (solid line), QC (dashed line) and CC (dotted line). Means ± SEM are shown. N = 5 for initial control and N = 10 for all other groups. The measured samples were randomly selected from each treatment pool. *Mann-Whitney *U*-tests $p < 0.05$. Solid bracket indicates comparisons between two groups at the same time point; dotted bracket indicate the comparison of the same group between different time points.

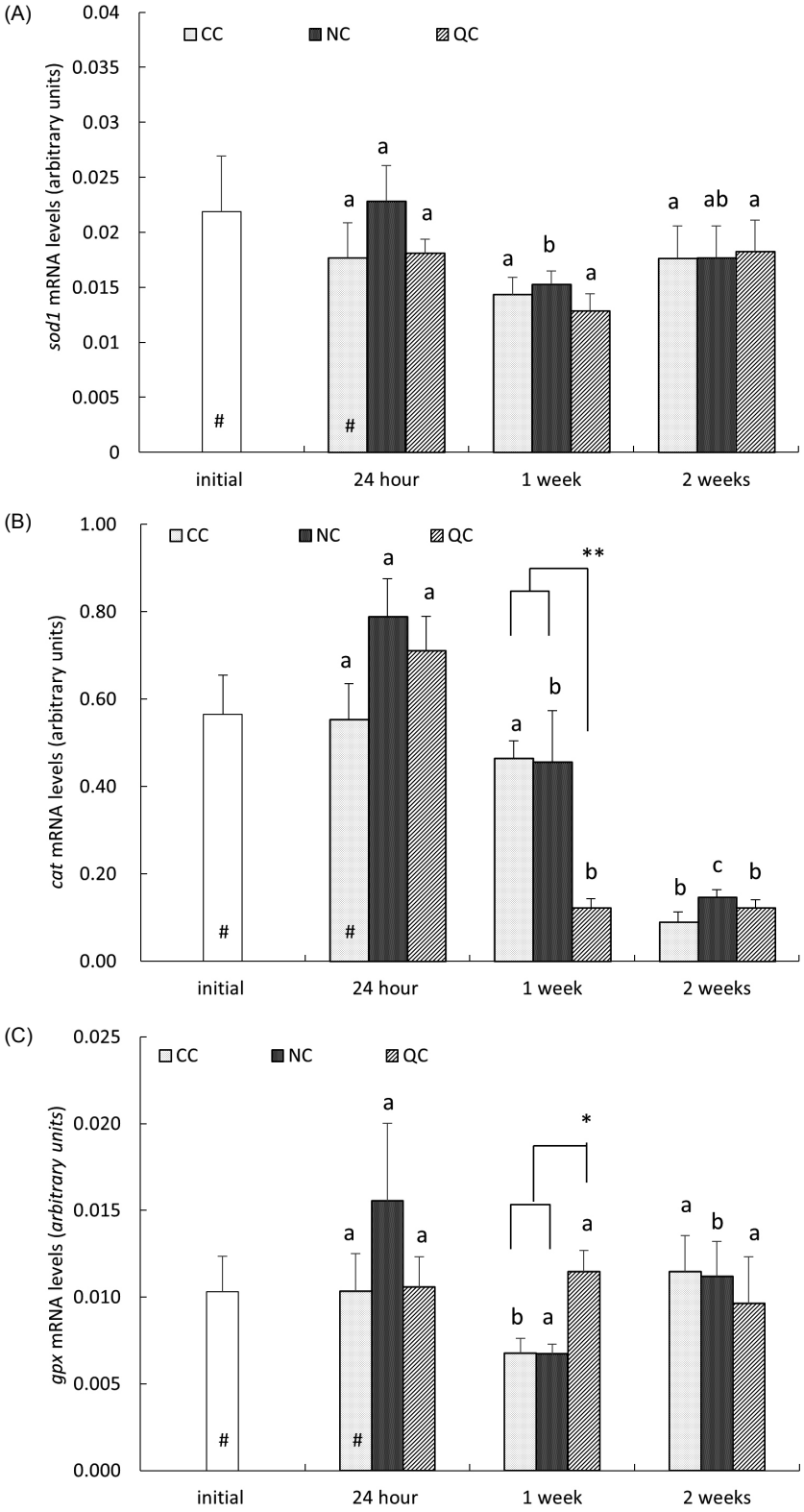

Fig. 3. Relative expression levels of the indicated genes in fish exposed to wind turbine noise. (A) hepatic *sod1* mRNA levels; (B) hepatic *cat* mRNA levels; (C) hepatic *gpx* mRNA levels. Means ± SEM are shown. # indicated the group N = 8, in all other groups N = 10. The measured samples were randomly selected from each treatment pool. One-way Kruskal-Wallis ** $p < 0.005$ or * $p < 0.05$. Solid bracket indicates comparisons between two groups at the same time point. Values with different letters differ from each other in the same group (CC, NC or QC) (one-way Kruskal-Wallis $p < 0.05$).

of exposure (one-way Kruskal-Wallis $p < 0.05$, *d.f.* = 2, $H = 13.67$), whereas the difference was not observed at 2 weeks (one-way Kruskal-Wallis $p = 0.779$, *d.f.* = 2, $H = 0.65$) (Fig. 3C).

DISCUSSION

Richardson et al. (1995) found that the impacts of anthropogenic underwater noise on animals can be divided into four zones based on the distances from the sound source and its intensity: zone of injury, zone of masking, responsiveness and zone of audibility. OWF is a possible anthropogenic noise source, and the sounds could be different between when it is being constructed and when it is in operation. During the wind farm construction stage, the extreme pile-driving noise and vibration have been known to have strong effects on marine mammals (Bailey et al. 2010) and fishes (*e.g.* Thomsen et al. 2006; Hammar et al. 2014) in zones nearby. Nevertheless, the construction stage may last only a few months or weeks. Once the OWF begins to operate, turbine noises become a continuous acoustical noise source (Nedwell and Howell 2004). Compared to the pile-driving noise, the operation noise has smaller impact zones (Nedwell and Howell 2004) and the effects may depend more on sensory abilities of the animals (reviewed in Slabbekoorn et al. 2010). Audiograms from black porgies have been reported in earlier studies using either behavioral tests (Ye 1992) or AEP (Wu et al. 2009). However, the previous data either do not include the dominant frequency (125.4 Hz and 315.5 Hz) of the sound treatment used in the present study (Wu et al. 2009) or the absolute scale of sound pressure levels is not reported (Ye 1992). AEP results in this study suggest that the black porgy can definitely hear the OWF operation noise when it is near the pile (NC, c. 1 m), and may be able to detect that noise within 100 m distance (QC). Thus, the results of the present experiment suggest that the impact zones of the OWF operation noise on black porgy should be very small, and the species may not able to detect the OWF sound from more than 100 m from the wind turbine.

There are many physical factors that may influence sound transmission and attenuation in open ocean, meaning that the OWF operation noise range that the black porgy detects could be different in the field. In addition, noise has been shown to cause hearing impairment in fish (Scholik and Yan 2001; Smith et al. 2004; Codarin et al. 2007), which would temporarily shift their hearing threshold and potentially reduce noise-induced physiological responses. A temporary threshold shift (TTS) that results from sound perturbation ought to depend on the level of stimulus and the species' audio sensitivity. As such, TTS is more easily observed when the fish are exposed to louder noises. Additionally, hearing impairment is much more frequently found in hearing specialists compared to hearing generalists, even upon exposure to the same ambient sound. Amoser and Ladich (2005) indicated that the hearing ability of common carp (*Cyprinus carpio*) is heavily affected by loud stream and river noises (c. 100 dB re 1 μPa 2k-10k Hz) but is only moderately affected by quiet habitat noise (c. 80 dB re 1 μPa 2k-3k Hz). On the other hand, the European perch (*Perca fluviatilis*) hearing thresholds were only slightly influenced by the loudest administered noise levels (Amoser and Ladich 2005). Likewise, another comparative study indicated that noise exposure may cause different levels of TTS in different species. White noise exposure (0.3-2.0 kHz, 142 dB re: 1 μPa) results in only a mild elevation in the auditory threshold of bluegill sunfish (*Lepomis macrochirus*) (Scholik and Yan 2002), but similar levels of white noise result in significant TTS in fathead minnows (*Pimephales promelas*) (Scholik and Yan 2001). Although TTS may occur, continuous noise exposure with lower sound pressure, *e.g.* air pumps or wind turbines, causes stress responses for weeks or a month in some species, indicating that the fishes are indeed influenced by the sounds, even if they have been exposed for a long period of time (Anderson et al. 2011; Wei et al. 2018).

Noise has been known to induce stress responses in fishes (reviewed in Slabbekoorn et al. 2010), including both acute and chronic responses. Most studies indicate that noise exposure can increase plasma cortisol levels, but the effect may only last for a very short period. A significant elevation in whole body cortisol concentration in juvenile red drums (*Sciaenops ocellatus*) and spotted sea trout (*Cynoscion nebulosus*) was shown at 15 min to 30 min after noise exposure, but the level decreased within the next 60 min (Spiga et al. 2012). The upregulation of genes related to cortisol synthesis (head kidney *star* (steroidogenic acute regulatory protein) and *hsd11b2* (hydroxysteroid 11-β dehydrogenase 2)) suggests that the milkfish has higher cortisol turnover rate after 1 week of noise exposure, but

circulation cortisol levels do not stay elevated for more than 24 hr (Wei et al. 2018). On the other hand, Anderson et al. (2011) found that lined seahorses maintained elevations in plasma cortisol level in noisy conditions (123.3 ± 1.0 dB SPL re: 1 μPa, mean ± SE) for 3 months, exhibiting 1.5-fold higher plasma cortisol levels than the fish under quiet conditions (110.6 ± 0.6 dB SPL re: 1 μPa, mean ± SE). In the present study, plasma cortisol levels in black porgies were only slightly elevated at 24 hr after continuous exposure to the loud noise condition; we suspect that the cortisol levels measured at 24 hr may not be the peak level but may instead be in the decline phase back to the resting levels.

Although the plasma cortisol levels shown in this study did not change significantly under the noise treatments, the changes in both ROS levels and antioxidant-related gene expression were found after 1 or 2 weeks of noise exposure. Previous studies suggest that oxidative stress and antioxidant responses may be linked. Kassahn et al. (2009) concluded that the environmental stressors, *e.g.* heat, cold hypoxia or hyposomotic exposure, can cause oxidative stress and/or redox shift in animals which induce antioxidant responses. Meanwhile, the increase in oxidative stress is hypothesized to stimulate the hypothalamus to release corticotropin-releasing hormone (CRH) and could result a higher cortisol level via the hypothalamic-pituitary-head kidney axis (Kassahn et al. 2009). In addition, two weeks after an exogenous cortisol intracoelomic injection, GSH, an antioxidant, levels in juvenile brown trout (*Salmo trutta*) were found to be significantly higher than the levels of the juveniles that received a placebo injection. However, the exogenous cortisol manipulation does not change the ratio of glutathione disulfide (GSSG) / GSH in brown trout, in which that ratio indicates the oxidative stress (Birnie-Gauvin et al. 2017). Therefore, the physiological linkage of cortisol to the ROS / antioxidant responses may not always be correlated. For example, higher cortisol levels were detected in juveniles of rainbow trout (*Oncorhynchus mykiss*) than control fish after the exposure of sodium selenite (Na_2SeO_3) at the pollutant level 2.52 mg/L for 96 h; nevertheless, lipid peroxidation levels, as well as hepatic GSH and GPx concentrations, showed a reduction pattern (Miller et al. 2007). Under continuous acoustical stress, the elevated ROS levels and changes in antioxidant-related gene expression were not dependent on cortisol levels. There was no clear connection between the two stress parameters found in the present study.

Persistent impacts of chronic noise exposure have been reported in some other species, including humans. For instance, long-term exposure to urban noise or aircraft noise has been associated with elevated resting systolic blood pressure or increased levels of epinephrine and norepinephrine (Evans et al. 1998 2001). The mechanisms underlying these concerning effects of noise vary and are not fully described, but the excessive accumulation of ROS molecules and oxidative damage has been suggested to be part of the process (Nawaz and Hasnain 2013). It is believed that neural activation is required to process environmental noise and leads to free radical accumulation based on metabolic demands. Therefore, cellular DNA, protein and lipid damage should be observed under noise perturbation (reviewed in Kight and Swaddle 2011). Recently, 8-oxo-2'-deoxyguanosine (8-OHdG) was used as a biomarker for detecting oxidative DNA damage in a study, indicating that the people exposed to low noise (81-94 dB(A)) and loud noise (> 95 dB(A)) had significantly more oxidative DNA damage and higher plasma cortisol levels than controls (< 80 dB(A)) (Nawaz and Hasnain 2013).

Thermal shock, hypoosmotic challenges and pollutant exposure have all been reported to raise ROS levels in teleosts (reviewed in Lushchak 2011, Tseng et al. 2011). However, the accumulation of ROS may either be caused by overproduction or deficient clearance. For example, the low capacity antioxidant defenses may be insufficient to balance the generation of ROS, and the subsequent accumulation of ROS may result in oxidative damage (reviewed in Mates 2000). In the black porgy, rapid shifts in ambient temperature and salinity were shown to cause increases in plasma H_2O_2, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) levels within 24 hr, which were accompanied by an elevation in the expression of antioxidant genes, *i.e. sod1*, *cat* and *gpx* (An et al. 2010). The same response was not found in the present study, regarding noise challenge. In this study, although the intensity of the noise used in the perturbation experiment did not exceed the maximum range that could occur in the field, *e.g.* nearby the wind power pile, the level of simulation noise was still far above the fish hearing curve. The ROS levels increased gradually and were consistently elevated during the 2-week NC treatment, but the mRNA levels of antioxidant genes did not parallel the change. The disconnect

between oxidative stress and antioxidant gene expression has been observed in other teleosts as well, where the mRNA levels of antioxidant genes are not highly expressed under strong stressors (Jin et al. 2010). In a study on female zebrafish (*Danio rerio*), low and medium dosages of pesticide exposure (atrazine 10-100 μg/L) could increase hepatic *sod1*, *sod2*, *cat* and *gpx* mRNA levels, but those genes were suppressed under a high dosage pesticide exposure (1000 μg/L) (Jin et al. 2010). Nevertheless, mRNA measurements did not reflect antioxidant enzymes activities. In that study, hepatic SOD and CAT enzyme activities were maintained at high levels under the high doses, despite the low mRNA levels (Jin et al. 2010).

Antioxidant defense network responses involve complex interactions among the enzymes. When animals are under oxidative stress, those antioxidant enzymes function together and remove intracellular ROS, which maintain the balance of the oxidant/antioxidant system (Vertuani et al. 2004). Our results show that hepatic *cat* was downregulated and *gpx* was upregulated in black porgies after 1 week of QC exposure; surprisingly, the ROS levels were slightly lower than other groups at this time point (not significant, one-way Kruskal-Wallis $p = 0.063$ $d.f. = 2$, $H = 1.832$). Apart from that, fish density could be a possible factor that reduced *cat* mRNA levels in all groups during the experiment (Sahin et al. 2014). As of now, the antioxidant response to noise-induced cellular stress is still an open question. The SOD-CAT system provides a first defense against oxidative toxicity, and the activities of those two enzymes in common carp were found to be induced simultaneously as soon as the fish were exposed to pollutants (Dimitrova et al. 1994). However, the SOD and CAT levels may be controlled differentially. For example, wallago catfish (*Wallago attu*) that were collected in highly polluted water exhibited high levels of hepatic SOD activity but low levels of hepatic CAT activity (Pandey et al. 2003). Moreover, CAT has been reported to be suppressed by excess superoxide anion radicals and nitrites (Kono and Fridovich, 1982; Arrillo and Melodia, 1991). Both CAT and GPx enzymes are capable of converting H_2O_2 to H_2O and O_2 (Kashiwagi et al. 1997). In this study, the fact that *cat* and *gpx* levels changed differentially does not indicate that they have different functions or are regulated independently, but alterations to *cat* or *gpx* genes were apparently not fully synchronous. We hypothesized that the mechanisms that regulate *cat* or *gpx* gene may have different sensitivity to oxidative stress or response speed. For example, *cat* mRNA levels may be downregulated as a result of decreased ROS levels in the first week, but *gpx* mRNA levels were kept high, possibly helping to control ROS content in the plasma. After 1 week of noise exposure, the *cat* and *gpx* mRNA profiles in the three groups were opposite, which suggests that the regulation of those two antioxidant-related genes may complement the antioxidant responses. In rock bream (*Oplegnathus fasciatus*), both hepatic *sod1* and *cat* mRNA levels increased after 4-10 days of starvation treatment, but no difference was found in hepatic *gpx* mRNA levels during the 10-day observation period (Nam et al. 2005). Furthermore, the mRNA levels of *cat* and *gpx* under thermal perturbation may fluctuate with time in the bald notothen (*Pagothenia borchgrevinki*) (Almroth et al. 2015). Because of the wide-ranging possibilities for compensatory regulation among different antioxidants, mRNA expression of many antioxidant gene transcripts may not be responsive to ROS levels. Thus, careful examination of ROS contents and diverse antioxidant expression along treatment courses are essential to evaluating the effects of ambient perturbation on cellular oxidative stress in fish.

Overall, we hypothesize that fish can adapt to QC ambient noise, for which significant antioxidant responses were generated in the first week to suppress the possible increase of the plasma ROS levels. By contrast, the comparatively chronic treatment (2 weeks) with the louder noise exposure (NC) induced ROS accumulated with time to reach significantly elevated levels. As far as we know, this is the first study to demonstrate that chronic exposure to noise can induce the accumulation of ROS in fish plasma. Moreover, this study suggests that wind turbine noise could be a potential stressor to fish, but the impacts of continuous noise on fish depend on the exposure, especially the sound levels (distances) and the time. Importantly, the chronic stress found in black porgies under noise conditions was in a very extreme condition (NC) that was continuous for long period, *i.e.* 2 weeks.

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Supplementary Materials

Fig S1. Tone burst stimuli used in the AEP experiment. Only one phase (90°) is shown. Each tone burst (100 ms) includes a sound wave consisting of 5-cycle waves (including one rise, one fall and three plateaus). The duration of the sound stimuli were: wave duration (ms) = 5 × 1000 / frequency (Hz). Interstimulus intervals = 100 ms - wave duration (ms). The amplitude indicates the output levels of the function synthesizer rather than the actual sound pressure. (download)

Fig S2. AEP waveforms from the black porgy (*Acanthopagrus schlegelii*), obtained in response to tone bursts (400 Hz) (A) of opposite polarities, 90° (solid line) and 270° (dotted line). Each experiment was initiated at the highest sound pressure level (SPL) to induce suprathreshold responses (B); the SPL was then reduced in steps of ~5 dB (B-D) until traceable and repeatable waveforms were no longer detectable (E). Recordings were performed twice for each sound stimulus (blue line and red line). The first and second ABRs were compared by calculating a correlation coefficient (r). AEPs were deemed to respond to the sound stimulus only when $r > 0.3$ (D, threshold: 0 dB). A recording from a dead back porgy is shown (F) under the highest sound pressure level. (download)

Fig S3. Diagram of the cage arrangements for the control and treatment pools. (download)