Vessel noise pollution as a human threat to fish: assessment of the stress response in gilthead sea bream (*Sparus aurata*, Linnaeus 1758)

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Abstract

This study examined the effects of boat noise pollution on the stress indices of gilthead sea bream (*Sparus aurata*, Linnaeus 1758). To assess the stress response in these fish, biometric values and plasma parameters such as ACTH, cortisol, glucose, lactate, haematocrit, Hsp70, total protein, cholesterol, triglycerides, and osmolarility were analyzed. After habituation of the animals, the experiment was carried out in a tank fitted with underwater speakers where the fish were exposed to sound treatments (in duplicate) consisting of: 10 days of no sound (control treatment; the animals were only exposed to the experimental tank's background noise); and 10 days of noise derived from original recordings of motor boats, including recreational boats, hydrofoil, fishing boat and ferry boat (vessel noise treatment).

The exposure to noise produced significant variations in almost all the plasma parameters assessed, but no differences were observed in weights and fork lengths. A PERMANOVA analysis highlighted significantly increased values ($p<0.05$) of ACTH, cortisol, glucose, lactate, haematocrit, Hsp70, cholesterol, triglycerides, and osmolarility in the fish exposed to vessel noise for 10 days.

This study clearly highlights that: anthropogenic noise negatively affects fish, and they are valuable targets for detailed investigations into the effects of this global pollutant.

Finally, these experimental studies could represent part of the science that is able to improve the quality of the policies related to management plans for maritime spaces (Marine Strategy Framework Directive 56/2008 CE) that are aimed at stemming this pollutant phenomenon.

Keywords: Underwater noise pollution, *Sparus aurata*, Vessel traffic, Stress response, Plasma parameters.

Introduction

Over the past few decades, human activities have produced increasing background sea noise pollution (Ross 2005; Hildebrand 2009), changing the acoustic characteristics of marine ecosystems (coastal, pelagic, deep) globally. In particular, vessel traffic has increased greatly, and noise emissions account for > 90% of the acoustic energy that humans emit into the sea (Green et al. 1994). Vessel traffic does not generally generate such intense noise, but the acoustic pollution it produces is constant over time, may affect large areas and could pose a serious hazard not only to individual animals, but also to entire populations (Weilgart 2007; Panigada et al. 2008; Clark et al. 2009; Slabbekoorn et al. 2010). In view of this, anthropogenic noise is now recognized as a major 21st century pollutant, appearing in international legislation like the Marine Strategy Framework Directive 56/2008 CE.

Low frequency (6 – 3000 Hz) underwater noise from vessels comes from: mechanical vibrations produced by engines, power transmission units and generators; the hull interacting with water whilst underway; and cavitation on rotating propeller blades. The highest intensities usually fall within frequencies ranging from 0.1 to 1 kHz (McDonald et al. 2014). This frequency range has been shown to be a potential threat to fish, because most audiograms of marine fish species indicate that their greatest sensitivity to sound falls within this range (Popper et al. 2003).

Noise pollution generated by vessel traffic is able to affect the behavioural, ecological and biochemical parameters of marine fish, as documented in several studies (Scholik and Yan 2001; Sandström et al. 2005; Wysocki et al. 2006; Sarà et al. 2007; Graham and Cooke 2008; Codarin et al. 2009; Picciulin et al. 2010; Bruintjes and Radford 2013; Holles et al. 2013; Voellmy et al. 2014a; Voellmy et al. 2014b).

Fish that are affected by adverse stress stimuli exhibit biochemical parameter changes (Heath 1990) that could reveal a poor animal welfare status. Physiological responses to stress vary widely between species (Barton 2002). However, it has been observed that noise can affect typical haematological stress biomarkers, including cortisol, glucose, lactate and haematocrit (Smith et al., 2004). In this regard, Buscaino et al. (2010) demonstrated a disturbance effect from noise exposure (0.1–1 kHz linear sweep, 150 dB re 1 μPa rms) on glucose, lactate and haematocrit levels in sea bream and sea bass. Furthermore, Filiciotto et al. (2013) observed higher levels of serum cortisol, glucose, red blood cell counts, haematocrit values and haemoglobin content, and lower levels of white blood cells in fish exposed to onshore aquaculture system noise compared to noise from offshore aquaculture systems.

Meanwhile, Wysocki et al. (2006) have demonstrated that the underwater ship noise can elicit a significant cortisol stress increase in different freshwater fish like *Cyprinus carpio*, *Gobio gobio* and *Perca fluviatilis*.

Although exposure to acoustic stimuli in marine crustaceans has been shown to have an effect on Heat shock protein 70 (Hsp70) expression and Total Protein Concentration (PC) of haemolymph (Celi et al. 2013, Filiciotto et al. 2014; Celi et al. 2015), until now there has been no data on the modulation of Hsps in fish under acoustic stress conditions. Moreover, although there is no evidence of an acoustic exposure effect on osmolarity in aquatic organisms, many papers report that osmolarity changes under stress conditions in fish (Mugnier et al. 1998; Lowe and Davison 2005).

Proteins, cholesterol and triglycerides play a vital role in the physiology of living organisms, and the literature has shown that the levels of these parameters can be modulated in fish exposed to different stressors (Mc Donald 1980; Hadi 2009; Muazzez 2009; Kori-Siakpere 2011; Parvathi 2011), e.g. handling and confinement cause a steady increase in the plasma Adrenocorticotropic Hormone (ACTH) level in both coho salmon and rainbow trout (Sumpter 1986).

However, the physiological effects caused by vessel noise have not been reported for *S. aurata* in either a field or experimental context.

Given these statements, in the present study we investigated whether boat noise exposure contributes to biometric and haematological indices of stress changes in gilthead sea bream. Studied specimens in control tank-based experiments were exposed to a medium-term random sequence of boat noises, and ACTH, cortisol, glucose, lactate, haematocrit, Hsp70, total protein, cholesterol, triglyceride and osmolarility levels were then analyzed.

Materials and Methods

Study animals and husbandry

Forty gilthead sea bream (*Sparus aurata*, Linnaeus 1758) aged one year with a mean initial weight (± SD) of 139.74 g (± 29.75) and a fork length (± SD) of 20.49 cm (±1.45) were obtained from a commercial fish farm (Sicily) and maintained in the aquaculture experimental plant of the Marine and Coastal Environment Institute of the National Research Council (IAMC-CNR) of Messina, Italy.

The aquaculture experimental plant is authorized by the Ministry of Health with the decree number 105/2014-A to use animals for experimental or other scientific purposes.

Prior to the experiment, gilthead sea bream specimens were stabulated in a 5 m^3 (2.3 m diameter, flat bottom, 1.15 m depth) circular fibre glass tank under a natural photo period. The tank was equipped with a flow-through system (with a complete water renewal each hour) of sea water. The water quality parameters were maintained in a range that was suitable for gilthead sea bream (an ambient water temperature of 20.06 ± 2.01 °C (mean ± SD) and a salinity of 38.31 ± 0.22 ‰ (mean ± SD) were maintained over the course of the husbandry). The fish were fed ad libitum everyday using commercial 4.0 mm marine dry pellets (NaturAlleva, Cologna Veneta, VR, Italy).

Experimental set-up and protocol

The experiments were conducted between October and November 2014. Prior to starting, the 40 sea bream were randomly sorted from the holding tank using a net, individually weighed and measured, assigned to four identical experimental circular fibre glass tanks (in groups of 10 specimens), and acclimated there for 10 days. The experimental tanks (1.3 m^3, 1.4 m diameter and depth of 1.0 m) were equipped with a flow-through system of sea water. Each tank underwent complete water renewal every hour. During the entire study period, the salinity was 38.23 ± 0.26 ‰ (mean ± SD), the temperature 18.05 ± 1.33 °C, and the dissolved oxygen 6-8 mg/L (mean ± SD, 7.41 ± 0.13). The photo and thermo periods were natural. The water quality parameters were maintained in a range suitable for gilthead sea bream. During the experimental period, the animals were fed with commercial pellets (NaturAlleva, Cologna Veneta, VR, Italy) twice a day at a rate of 1.1% of body weight.

A laboratory enclosure was placed 2 m away from the tanks, and the equipment required for audio projection was installed there. Each experimental tank was fitted with an underwater speaker, and sound treatments were duplicated and consisted of: 10 days of no sound (control treatment; the animals were only exposed to the experimental tank's background noise); and nine chosen files of noise derived from original recordings of motor boats (vessel noise treatment).

The same basic experimental set-up was used for all the trials.

Noise recording and playback

Acoustic recordings were obtained in a sea area off Capo Granitola, south-west of Sicily (Italy), when different boats were passing by. Noises from seven recreational boats, hydrofoil, fishing boat, and ferry boat were chosen to be used in the study.

The recordings were made using a calibrated hydrophone (model 8104, Bruel & Kjer, Nærum, Denmark) with a sensitivity of −205.6 dB re 1 V/μPa ± 4.0 dB in the 0.1-Hz to 80-kHz frequency band. The hydrophone was used with a preamplifier (VP1000, Reson, Slangerup, Denmark) with a 1-MHz bandwidth single-ended voltage that had a high-pass filter set at 10 Hz with a 32-dB gain. The equipment was connected to a digital acquisition card (USGH416HB, Avisoft Bioacoustics, set with no gain) managed by the Avisoft Recorder USGH software (Avisoft Bioacoustics, Berlin, Germany). The signals were acquired at 300 kilo-samples s^{-1} at 16 bits and analyzed by the Avisoft-SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany). The format of the files was .wav. The acoustic equipment was powered with the internal battery of a laptop to prevent the intrusion of noise from the AC power supply.

All passing boats were recorded 30–50 m from the hydrophone. The spectrograms from the different vessel noise stimuli are presented in Fig. 1.

The sound of the experimental tank was also recorded to characterize the baseline noise of the study environment. During the entire experimental period, the sea water recirculating flow was directly deployed beyond the tank water's surface to prevent any bubbles, and no air pumps were used. The experimental tank background noise had a lower intensity than the mean of the noise of the boats. The maximum Sound Pressure Level (SPL) (dB re 1 μPa rms) in the recorded frequency band of 0.1–3 kHz was 128 (Fig. 2).

A UW30 underwater speaker (Lubell Labs Inc., Columbus, OH, USA) was placed on one side of the tank and isolated by a neoprene disc to reduce the potential transmission of vibrations from the speaker. The speaker was connected to a Channel Low Impedance Amplifier (model QD-4240 – Inter M, Seoul, Korea) that was in turn connected to the stereo output of a PC running the Avisoft-SASLab recorder software (Avisoft Bioacoustics, Berlin, Germany). Files of the vessel noise treatment were included in a playlist and randomly projected in the "loop mode" for the entire experimental period. The acoustic characteristics of the playback files (vessel noise treatment) are set out in Fig. 2.

Biometric assay and bleeding procedure

Before the start of noise playback (0 days) and at the end of the experimental phase (10 days), all the animals from both the vessel noise and control treatments were captured with a net for body measurement and blood collection purposes.

The specimens were anaesthetized by placing them in a 60-L aquarium with 2-phenoxyethanol (1:300 v/v). The deeply-stunned sea bream were weighed to the nearest 0.1 g, and then measured in terms of fork length to the nearest millimetre before the bleeding procedure. Blood samples were collected in 2.5 ml disposable heparinized syringes within 30-40 seconds through the caudal vein of each fish. To prepare the plasma, the blood samples were centrifuged at 800×g for 10 min at 4 °C, and supernatants were used for the plasma analysis.

The blood was always collected between 9.00 a.m. and 10.00 a.m., with feeding stopped 24 h beforehand.

Animal handling and the use of the specimens complied with the European Community Guidelines for Animal Care (DL 26/2014, application of the European Directive 2010/63/UE) regarding the treatment of animals used for scientific purposes. The anaesthesia was administered and the blood collected by trained researchers.

Plasma analysis

The total cortisol, ACTH and Hsp70 levels were assayed by an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Cusabio Biotech Co., Ltd.) according to the manufacturer's instructions.

The glucose, lactate, cholesterol and triglyceride plasma levels were determined using the Accutrend Plus-instrument (Roche) according to the manufacturer's instructions.

The osmolarity of the plasma samples was measured using a freezing-point depression osmometer (Roebling, Germany). According to Filiciotto et al. (2014), the PC was estimated using a Quibit 2.0 Fluorometer (Invitrogen). The data were quantified with standards.

Data analysis

A Univariate Permutational Multivariate Analysis of Variance (PERMANOVA) was used on standardized biometric and haematological variables to test if the differences observed among the various treated groups were significant. All the PERMANOVA analyses used 999 permutations. *P*-values <0.05 were considered to be statistically significant.

A Random Forest (RF) analysis was performed to classify the sampling units based on ACTH, cortisol, glucose, lactate, haematocrit, Hsp70, total proteins, cholesterol, triglycerides, and osmolarility (Liaw and Wiener 2002). The RF analysis was based on the mean decrease accuracy, which is the normalized difference of the classification accuracy for out-of-bag (OOB) data when the data for that variable is included as observed (Liaw and Wiener 2002). The most important variables for the best classifications were selected by backwards elimination using the OOB error as a minimization criterion (Diaz-Uriarte and Alvarez de Andrès 2006). The selected variables were used to develop a final RF model (Liaw and Wiener 2002) and to estimate the classification of the individuals. This classification was then compared to the different treatment groups using a confusion matrix - control treatment: 0 days, C t (0d); control treatment: 10 days, C t (10d); vessel noise treatment: 0 days, Vn t (0d); and vessel noise treatment: 10 days, Vn t (10d). The scaling coordinates of matrix 1- proximity from the final RF model - were used to obtain Multi Dimensional Scaling (MDS) plots in two dimensions. The RF analysis provided the proximities between each pair of specimens, which indicated how close they were to each other.

All the statistical analyses were carried out using the statistical software R (R 3.0.1).

Results

All the fish used in the present study were healthy, as indicated by the observations carried out on their behaviour and external examinations.

The PERMANOVA showed that there were no significant differences in all the biometric and plasma parameters ($p>0.05$) among the replicated trials of each treatment.

Despite the fact that there were no significant differences (PERMANOVA, $p>0.05$), the weights and fork lengths of the control fish were obtained after 10 days. Meanwhile, no increases were observed in the specimens exposed to vessel noise after 10 days of sound treatment. In particular, the weight values increased from 132.66 ± 26.91 to 137.6 ± 29.93 and the fork lengths from 20.13 ± 1.35 to 20.19 ± 1.27 (Mean ± SD) in the sea bream belonging to the control treatment groups at 0 days and 10 days, respectively. In the fish exposed to the vessel noise, values of 146.83 ± 31.44 and 145.19 ± 29.74 for weight, and 20.85 ± 1.49 and 20.82 ± 1.55 for fork length, were recorded after 0 and 10 days, respectively.

The PERMANOVA highlighted significantly increased values ($p<0.05$) for ACTH, cortisol, glucose, lactate,

haematocrit, Hsp70, cholesterol, triglycerides, and osmolarility in the fish exposed to vessel noise for 10 days. However, the PC did not show any differences ($p>0.05$) in the sea bream in the experimental treatment groups. No differences ($p>0.05$) were observed in the plasma parameters of the fish groups among the vessel noise treatment groups after 0 days and the control treatment groups after 0 days and 10 days.

All the plasma value results and the differences among the experimental treatments are set out in Table 1.

The variables chosen to discriminate between the individuals belonging to the experimental treatments were ACTH, cortisol, glucose, lactate, haematocrit and Hsp70 (RF analysis), and were evaluated as the most important variables using the mean decrease in the accuracy criterion (Fig. 3). The confusion matrix (Table 2) shows that the RF model and data agree in 42.67% of classified cases. Individuals in the vessel noise treatment group at 10 days were all correctly classified, while those in the control treatment groups at 0 and 10 days, and the vessel noise group at 0 days, are very confused (see Table 2).

The plot of scaling coordinates of the proximity matrix from the RF analysis revealed that there were two clusters, the first represented by the specimens belonging to the vessel noise treatment at 10 days and the second by the other three experimental treatments (control treatment: 0 days; control treatment: 10 days; and vessel noise treatment: 0 days) (Fig. 4).

Discussion

The aim of this study was to investigate the effects of anthropogenic noise generated by vessel traffic on gilthead sea bream (*Sparus aurata*).

Anthropogenic activities, and in particular shipping traffic, have changed many marine ecosystem soundscapes globally, with world seas getting noisier (Ross, 2005). Although other studies have evidenced that anthropogenic noise can negatively affect the behaviour and physiology of a wide range of organisms (Slabbekoorn et al. 2010; Kight and Swaddle 2011; Wale et al. 2013; Bruintjes and Radford 2014; Filiciotto et al. 2014; Morley et al. 2014), the biochemical changes observed in the present research show a clear stress response in adult gilthead sea bream after brief exposure to noise generated by human activities and, in particular, maritime vessel traffic.

In order to evaluate the impact of noise exposure, biometric values and several plasma parameters were measured in the species.

Even though no statistically significant differences were found in the weight and fork length values between the experimental treatment groups (PERMANOVA, $p>0.05$), the control fish had higher values after 10 days, but there were no increases in the specimens exposed for 10 days of noise exposition. Although these results are evidence of a biometric response, it is possible to conclude that body parameters need medium-long term stimulation to undergo significant modifications, as recently reported by Filiciotto et al. (2013) in juvenile gilthead sea bream exposed to noise stimuli from aquaculture systems for 120 days.

Generally, stress can disturb the normal physiological equilibrium or homeostasis of a fish by forcing a reallocation of energy within its system. The degree to which stress affects any particular fish is largely determined by the severity of the stress, its duration and the health of the specimen.

It is known that fish respond to stress on three integrated levels (primary, secondary and tertiary) involving the Hypothalamic-Pituitary-Interrenal (HPI) axis. The primary response is neuroendocrinological, and is manifest in the activation of two major systems: the Sympathetico-Chromaffin (SC) and HPI axis, which are responsible for the, respective, release of catecholamines and corticosteriods into the bloodstream. The secondary response is activated by

these hormones and is manifest as changes in a range of biochemical, haematological and immunological factors (Barton and Iwama 1991).

The tertiary response extends beyond the cellular level to the entire animal, inhibiting the immune response, reproduction, growth and the ability to tolerate additional stressors (Barton et al. 1986; Maule et al. 1987; Pickering 1987; Mesa 1994). The results of this study confirm the above stress responses. In particular, individuals subjected to acoustic stress after 10 days showed an increase in ACTH and cortisol that is directly responsible for altering the metabolism, including glucose, lactate, triglyceride and cholesterol levels.

Although the PC level in the plasma of fish is widely used to monitor stress situations, this modulation seems to depend on the kind and duration of the stress (Coeurdacier et al. 2011), and we did not observe a significant change in PC in the fish subjected to the acoustic stimulus. However, following a study on shellfish subjected to acoustic stress (Celi et al. 2013; Filiciotto et al. 2014; Celi et al. 2015), we highlighted a significant increase in the plasma levels of Hsp70, also suggesting an alteration to the cellular level. The alteration of homeostasis is also evidenced by increased osmolarity, indicating a disturbance of the osmotic balance (Cammarata et al. 2012). In other teleosts, stress-induced increases in haemoglobin concentrations and haematocrit levels can occur: as a result of increased muscle activity and the concomitant movement of water from the plasma to the muscles (Jones and Randall 1978; Buscaino et al. 2010); or for the induction of splenic contractions and the subsequent mobilization of stored erythrocytes (Yamamoto et al. 1980; Wells and Weber 1990).

In this study, we also recorded an increase in lactate and haematocrit values in fish exposed to the acoustic stimulus, indicating, as according to Buscaino et al. (2010), a correlation between these parameters and increases in muscle activity.

These results may be particularly relevant when considering the potential effect of acoustic pollution on some biological and ecological activities of marine fish in general and this species in particular. In fact, long-term stress exposure could consequently compromise other elements, such as egg survival and reproductive and growth rates (Banner and Hyatt 1973; Lagardère 1982). In addition, boat noise could have a negative effect not only on the adult stages, but also on fish larvae, with implications for settlement and population dynamics, as demonstrated by Holles et al. (2013) in coral reef habitats.

Although almost all the plasma parameters measured showed significant changes in the fish exposed to the vessel noise, an RF analysis highlighted how ACTH, cortisol, glucose, lactate, haematocrit and Hsp70 were the most discriminant variables.

These results reveal that the plasma parameters mentioned above could be regarded as the most reliable for *S. aurata* in particular, and for fish in general, as already reported by other authors (Pickering 1981; Wells and Pankhurst 1999; Martínez-Porchas et al. 2009; Gronquist and Berges 2015). The reliability of the parameters allows them to be identified as useful indices for the application of a standardized measuring system of stress in this species. Moreover, the characterization of these different stress responses in a target marine species may be an early indicator of the degradation of environmental health.

In conclusion, this study clearly highlights both that fish are likely to be susceptible to the impact of anthropogenic noise and that they are valuable targets for detailed investigations into the effects of this global pollutant.

Our experiment was performed in tanks, making it possible to carefully control several variables, and allowing us to only assess the effect on the animals of the selected noise stimulus. However, the acoustics of small spaces are very complex, and playbacks cannot perfectly replicate natural sound sources (Parvulescu 1964; Parvulescu 1967; Okumura et al. 2002). Accordingly, field-based studies using real noise sources would enable the potential impact of

anthropogenic noise to be assessed fully, especially from the perspective of policy-making and management. Moreover, although our results suggest that short-term exposure to noise pollution is able to induce an acute stress response in fish, in the near future further studies should be performed over medium and long-term time expositions in order to measure the effects of chronic noise exposure and its potential negative impact on biological and ecological factors such as reproduction, growth, population viability and resilience in the face of anthropogenic change.

Finally, the growing awareness of the need to consider anthropogenic sounds as a source of stress in aquatic organisms has led to the establishment of policies aimed at stemming this particular pollutant phenomenon. Indeed, the Marine Strategy Framework Directive, which aims to achieve a Good Environmental Status of marine waters (GES), states that the "introduction of energy (including underwater noise) does not adversely affect the ecosystem". The improvement in the quality of policies related to the management plans for maritime spaces is closely related to studies in mesocosms. Therefore, these experimental activities must represent part of the scientific basis that is able to vertically integrate with monitoring and actuating policies, with a view to achieving a GES of coastal zones, seas and their resources (Breen et al. 2012).

Abbreviations

ACTH Adrenocorticotropic Hormone
ELISA Enzyme-Linked Immunosorbent Assay
GES Good environmental status
HPI Hypothalamic‑Pituitary‑Interrenal axis
Hsp Heat shock protein
MDS Multi Dimensional Scaling
MSFD Marine Strategy Framework Directive
(OOB) classification accuracy for out-of-bag
PC Total Protein Concentrations
PERMANOVA Univariate Permutational Multivariate Analysis of Variance
SC Sympathetico-Chromaffin
SPL Sound Pressure Level
RF Random Forest analysis

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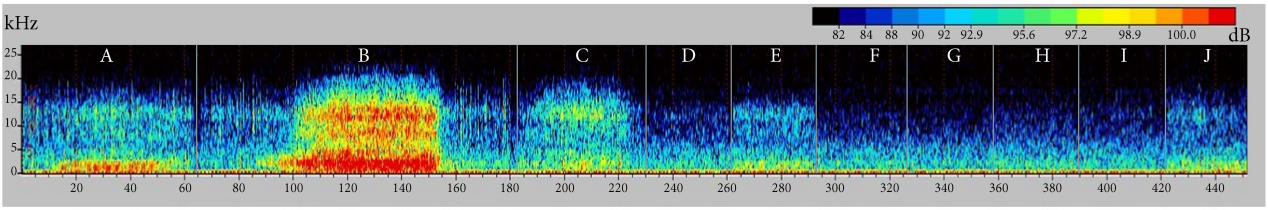

Fig. 1 Spectrogram of all the played back vessel noise stimuli: frequency (kHz) vs. time (s). The intensity is reflected by the colour scale (dB re 1 µPa rms, 1024-sample FlatDown window, sampling frequency 92 kHz). Spectrograms representing the noise from the following vessels: A) hydrofoil; B) ferry boat; C) fishing boat; D) recreational boat 1; E) recreational boat 2; F) recreational boat 3; G) recreational boat 4; H) recreational boat 5; I) recreational boat 6; and L) recreational boat 7.

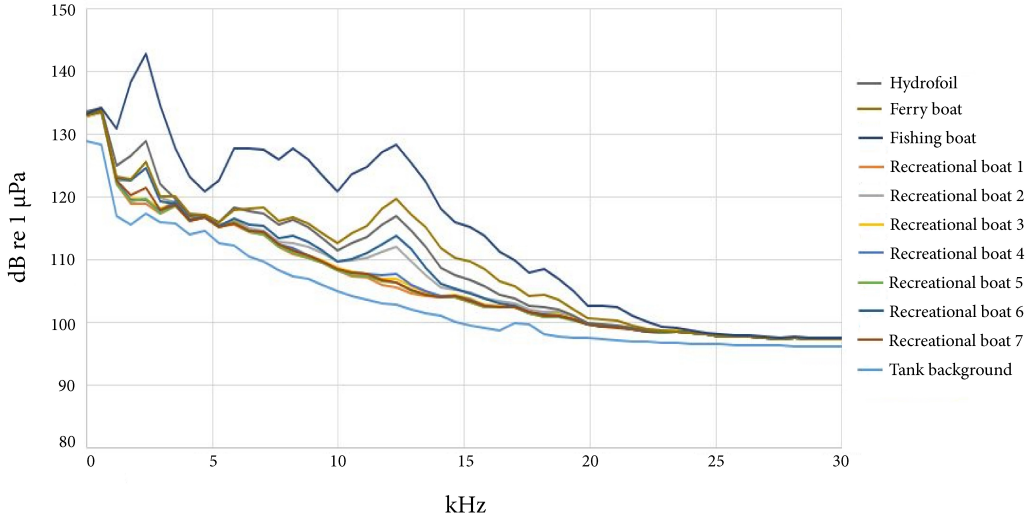

Fig. 2 Acoustic characteristics of the playback files. Power spectrum of the vessel stimuli and experimental tank background noise (each stimulus is represented by different coloured lines). Sound Pressure Level (SPL) expressed in dB re 1 µPa rms (wave files were under-sampled to 96 kHz, FFT size 4096, bandwidth 30.47 Hz, resolution 23.44, Hamming window) versus the frequency expressed in kHz.

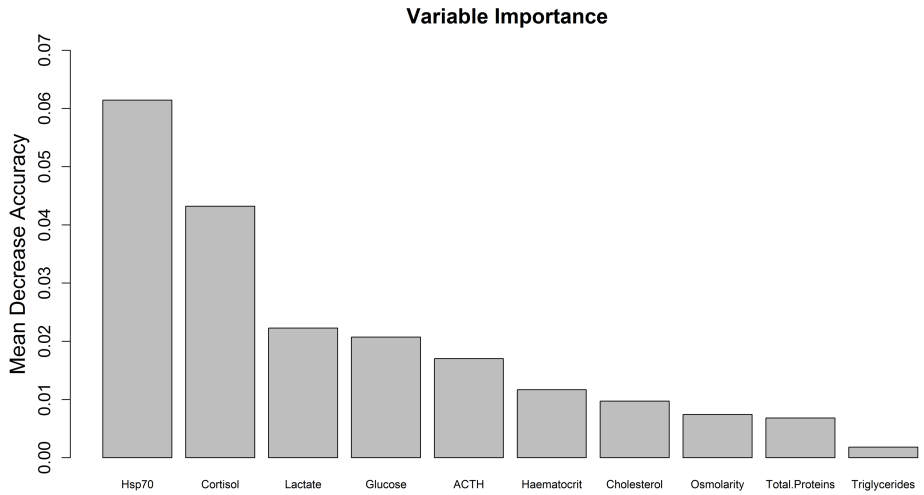

Fig. 3 Bar plot of the mean decrease in accuracy from the RF analysis of the plasma parameter levels.

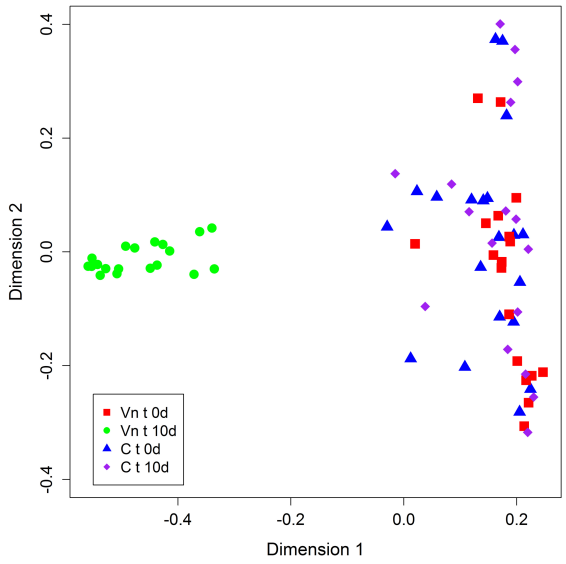

Fig. 4 The Multi-Dimensional Scaling (MDS) plot in two dimensions using the RF proximity matrix. Group treatments are: vessel noise treatment: 0 days (Vn t 0d); vessel noise treatment: 10 days (Vn t 10d); control treatment: 0 days (C t 0d); and control treatment: 10 days (C t 10d).

Table 1. Average rms octave Band Pressure Level (BPL) in field and tank for 9 octave: 62.5 (44-88), 125 (88-177), 250 (177-355), 500 (355-710), 1000 (710-1420), 2000 (1420-2840), 4000 (2840-5680), 8000 (5680-11360), 16000 (11360-22720).

Central frequency (Hz)	Field (dB)	Tank (dB)
62.5	113.9	131.2
125	119.8	127.1
250	134.2	123.5
500	141.2	125.8
1000	136.7	123.1
2000	134.6	134.9
4000	134.6	125.0
8000	133.4	136.0
16000	128.6	131.7

Table 2. Plasma parameters measured in treatment groups. Mean values (±SEM) of the plasma parameters in gilthead sea bream from the control treatment groups (C t) after 0 (0d) (N=10) and 10 days (10d) (N=10); and the vessel noise treatment groups (Vn t) after 0 (0d) (N=10) and 10 days (10d) (N=10).
Means with the same superscripts are not significantly different (PERMANOVA; $p>0.05$).

Experimental Treatment	ACTH (pg/ml)	Cortisol (ng/ml)	Glucose (mg/dl)	Lactate (mmol/L)	Haematocrit (%)	Hsp70 expression	Total Proteins (µg/µl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	Osmolarity (mOsm/kg)
C t (0d)	114.40^{a} (±20.36)	75.97^{a} (±26.31)	42.45^{a} (±10.12)	0.58^{a} (±0.14)	27.80^{a} (±4.22)	155.30^{a} (±28.59)	1.43^{a} (± 0.20)	215.40^{a} (±40.20)	182.70^{a} (±60.92)	323.60^{a} (±21.11)
C t (10d)	123.00^{a} (±15.11)	75.58^{a} (±25.46)	41.17^{a} (±9.73)	0.60^{a} (±0.08)	26.31^{a} (± 3.11)	147.47^{a} (±28.51)	1.34^{a} (±0.12)	200.28^{a} (±32.12)	205.37^{a} (±92.17)	318.89^{a} (±14.39)
Vn t (0d)	119.25^{a} (±22.31)	69.67^{a} (±16.86)	33.30^{a} (±14.33)	0.57^{a} (±0.10)	27.75^{a} (±2.71)	134.15^{a} (±30.18)	1.43^{a} (±0.19)	198.95^{a} (±35.11)	200.65^{a} (±78.39)	311.90^{a} (±21.64)
Vn t (10d)	213.05^{b} (±74.47)	163.35^{b} (±37.01)	68.60^{b} (±19.58)	2.00^{b} (±1.13)	32.70^{b} (±3.06)	265.60^{b} (±35.65)	1.29^{a} (±0.19)	280.25^{b} (±72.64)	293.55^{b} (±53.62)	343.60^{b} (±39.75)

575 **Table 3: Classification matrix of the treatment groups**. Vessel noise treatment: 0 days (Vn t 0d); vessel noise
576 treatment: 10 days (Vn t 10d); control treatment: 0 days (C t 0d); and control treatment: 10 days (C t 10d).
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	C t 0d	C t 10d	Vn t 0d	Vn t 10d	Classification error
C t 0d	7	8	5	0	0.6500000
C t 10d	8	4	5	0	0.7647059
Vn t 0d	4	4	10	0	0.4444444
Vn t 10d	0	0	0	19	0.0000000

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