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Noise elicits hematological stress parameters in Mediterranean damselfish (*Chromis chromis*, perciformes): A mesocosm study

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ABSTRACT

In the last few decades, technological developments and the widespread rise of anthropic activities have increased the exposure of organisms to noise pollution, thus evoking great interest in its biological effects, particularly on the immune system. The aim of the present work was to investigate some of the biochemical parameters in the blood of *Chromis chromis* (Linnaeus, 1758) following in vivo exposure to noise levels of 200 and 300 Hz. Our results revealed that, compared to the control specimens, the fish exposed to noise had significantly increased levels of stress biomarkers such as glucose, lactate and total proteins in plasma, as well as a rise in the expression of heat shock protein 70 (HSP70).

1. Introduction

The noise of boats is one of the most recognized sources of human disturbance in the marine environment [1], and is able to divert the natural behaviour of fish species [2,3] and affect their use of their habitat [3–5]. In Mediterranean coastal habitats, the most common and abundant infralittoral fish is the damselfish (*Chromis chromis* L.), which is the only species belonging to the Pomacentridae family. *Chromis chromis* has is maximum of sensibility to sound stimuli in the range between 200 and 500–600 Hz [6], which falls well within the range of the noise generated by boats [3,7]. Specifically, Picciulin et al. [8], demonstrated that a frequency of about 400 Hz could have a detrimental effect on damselfish behaviour, masking particular sounds emitted by males during courtship (e.g. pops). Furthermore, a previous companion paper [5] showed that low frequency noise (viz. 100 and 1000 Hz), such as that generated by boats, elicited in damselfish the modification of: group behaviour, the time taken to reach the sea floor and natural feeding rates [5]. Accordingly, the sound window of auditory capability between 200 and 300 Hz seems to be crucial for the individual fitness of this species.

Buscaino et al. [9] demonstrated that noise exposure produced a significant sub-organismal response, as shown by the increase in swimming activity and significant changes in lactate, glucose and hematological levels in coastal fish (sea bream and sea bass).

Moreover, it has been demonstrated [10] in the European spiny lobster (*P. elephas* Fabricius, 1787) that, after exposure to acoustic pollution, there are increases in glucose and total plasma protein concentrations, as well as an increase in HSP70 expression.

These studies that the acoustic stimulus can be perceived as noise and can activate stress responses. The stress response involves a wide range of physiological mechanisms, including metabolism and immune-response that will be useful to overcome the imbalance condition triggered by stressful or primary stress response. With these reactions the animal tries to avoid dangerous situations and the risk to life and body integrity, and subsequently

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to cope with the allostatic load produced by the stressor and reintegrate the balance throughout physiological systems in order to regain homeostasis.

Several resources are committed to meet the challenge and metabolic reorganization affecting the efficiency of other functions, including the immune system; this is a common trait of the stress response that may transiently affect the immune system and resistance to pathogens.

When a response to stress develops it can be assumed that the result will depend on the intensity of stress and its duration. However it is not possible make a prediction of the effects of a particular stress on a particular immune or inflammatory function even if you know the cellular and molecular effects [11]. Vazzana et al. [12] showed in sea bass, *Dicentrarchus labrax*, that crowding stress (the high density and confinement) modulated the cellular immune response. Indeed, the cytotoxic activity and respiratory burst activity of peritoneal cavity cells were lower compared to control specimens. Furthermore, were found a high level of cortisol, glucose and osmolarity in the plasma. The effect appeared correlated to cortisol because administration of exogenous cortisol decreased the above mentioned cellular immune-activity [13].

In stress condition, as molecular responses, an increase in heat shock proteins is demonstrated from invertebrates to fish [14–21].

It is now known that HSP70 also functions under normal physiological conditions as a molecular chaperone. It does this by interacting with the hydrophobic regions of nascent polypeptides to prevent aberrant folding or aggregation into insoluble complexes [22–24].

However, while it is well-recognized that the production of chaperones is highly a-specific, as they are produced both by thermal stress and a variety of noxious stimuli (e.g. viral infections, xenobiotics, heavy metals, free radicals) [25], there is no experimental evidence in fish for the proposal that such proteins can be produced under stressful noise conditions. HSP70 is one of the HSPs which increases rapidly after stress and is very sensitive to every kind of stressor [26]. It is thus widely utilized in the literature as a very good indicator of general stress [27,28], which justifies its use in the present study.

HSP70 mRNA expression can be induced by psycho-physical stress [29], but little research has been conducted on the expression of HSP70 under different sound stress exposure durations.

In this study, we investigated if pure sounds inside an auditory window (200, 300 Hz) elicited plasmatic and cellular stressful responses.

The experiments were conducted in mesocosms with semi-captive specimens of *Chromis chromis* collected from a Sicilian MPA (Isola delle Femmine and Capo Gallo, Palermo, Northern Sicily). The stress effects were evaluated by measuring the concentrations of glucose, lactate and total proteins in the plasma and the cellular expression of HSPs. These are considered to be common indicators of stressful responses, and have been adopted in several studies discussed in the relevant literature [15,18].

2. Materials and methods

2.1. Animals

The experiment was carried out from July to September 2008 at the Istituto per l'Ambiente Marino Costiero of Consiglio Nazionale delle Ricerche (IAMC-CNR) – Laboratories of Capo Granitola (Trapani, Italy). A total of 135 adult *C. chromis* specimens were collected from the A-zone of the MPA of Capo Gallo and Isola delle Femmine (38° 13′ 00" N; 13° 19′ 00" E and 38°11′40″20 N; 13°14′58″92 E, respectively) by a circular net (50 × 6 m) directly maneuvered from a small fishing boat [5]. Once collected and selected for size on board, adult individuals (10–12 cm) were kept alive in tanks of 50 L and transported to the CNR-IAMC Institute of Capo Granitola (Trapani, Sicily). They were then maintained in circular submerged pools (diameter: 3 m, depth: 1 m, volume: 5000 L) for at least two weeks for acclimation purposes. Pure oxygen was administered when necessary and a mixture of natural (zooplankton cultivated in the CNR-IAMC premises) and artificial food (aquaculture pellets) was used to feed the specimens twice a day (4–6% of total weight) following simple aquaculture rules for maintaining wild fish in captivity [30]. The CNR-IAMC Institute is located in an old tuna trap facility where there is a small, private harbour (diameter: about 200 m, max. depth: about 4 m) that is closed and quiet, and where the passage of boats is forbidden. Such conditions were ideal for placing the experimental cage where the pure sounds were to be administered [9]. The fish were exposed to the natural photoperiod and fed daily with commercial dry pellets. Feeding was stopped at least 48 h before the start of the experiment.

2.2. Acoustic stimuli

Most fish are able to detect sound, and their range of hearing is from 100 to 500 Hz [31]. Since acoustic energy produced by vessel traffic is more intense at low frequencies [32], a decision was made to use an acoustic stimulus with a frequency band of 200–300 Hz pure tones. The tones were generated by a waveform generator (Model 33220A, Agilent Technologies, Santa Clara, CA, USA), and were amplified (Model PA-4000 Inkel, Chonan City, Korea) and emitted using an underwater moving coil loudspeaker with a 100 Hz–10 kHz rated frequency response (Model UW30, Lubell, Columbus, Ohio, USA; transmit response: 125 ± 5 dB re 1 micro Pa/V re 1 m in the frequency range 100–1000 Hz; 120 ± 10 dB re 1 microPa/V re 1 m in the frequency range 1–20 kHz). The acoustic signals emitted were acquired to measure the intensity and map the sound pressure spectrum level (see Fig. 1). This was done using an omni-directional calibrated hydrophone (TC4034, Reson, Slangerup, Denmark) positioned inside the cage, 5.5 m from the underwater speaker.

Each group was exposed to one treatment only [33]. The sound administration lasted for 2 min. It was then interrupted and blood samples were collected after 5 min.

2.3. Sample preparation and experimental protocols

The experiments were performed in triplicate in full compliance with national rules (D. Lgs 116/92 and subsequent amendments) and the international European Commission guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC). In total, we used 135 specimens distributed in 9 groups of 15 specimens each. Three groups were assigned to the tests with 200 Hz, three groups for the test with 300 Hz, and three groups for the control test (no sound emission). In order to avoid any multiple subministration of noise stimulus each group of 15 specimens was used in sequential way in the mesocosm (there was only one experimental cage in the harbor).

For each test, a group of 15 specimens was randomically choose from the maintenance tank and transferred in the cage in the harbor (mesocosm). After 30 min of acclimation in the experimental cage, the groups of fish were passed randomly through the treatments exposing the fish to 200 or 300 Hz or in the case of control group no stimulus was applied. Then the fish were then captured with a net to take blood samples. After this, the fish were transferred into a small tank and then released after recovery.

After the sound administration, the fish were gently collected from the mesocosm and blood samples were obtained. This was achieved by way of a cardiac puncture in the presence of heparin,

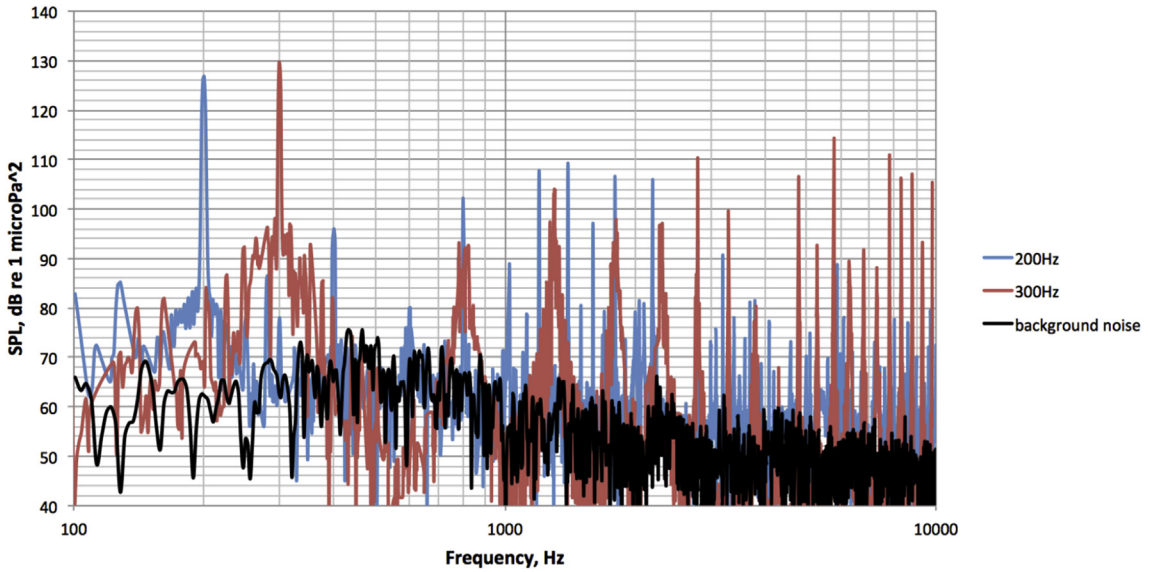

Fig. 1. Sound Pressure Spectrum Level (SPL. dB re 1 microPa2) of the acoustic stimuli recorded during the experimental trials. (FFT size 2096; sampling frequency 44 kHz).

according to Vazzana et al. [34], after the administration of anaesthesia with 0.05% w/v MS222 (3-aminobenzoic acid ethylester – Sigma Aldrich) in seawater. The blood samples were centrifuged at 400 g × 10 min to obtain both plasma and cellular components. Plasma was utilized to determine the glucose, lactate and total protein levels. Meanwhile, the cells were crushed in 1 ml of a lysis buffer RIPA: 0.5% sodium deoxycholate minimum 97%; 1% NP40; 0.1% SDS with PBS-T (Na_2HPO_4 1M, NaH_2PO_4 1 M, NaCl 1.5 M, 0.1% Tween 20) at a pH of 7.5 and supplemented with a cocktail of protease inhibitors: 2 μg/μl of antipain, leupeptin and bestatin; 1 μg/μl of aprotinin and pepstatin; 1 mM of benzamidine; and 0.1 mM of AEBSF. The samples were then centrifuged at 15,000 g for 30 min at 4 °C, and cellular lysate supernatants (CLS) were collected and dialyzed against a 50 mM Trizma base (Tris[hydroxymethyl] aminomethane) at a pH of 7.5.

2.4. Biochemical blood parameters

The glucose plasma levels were determined using the Accutrend GC kit (Roche) according to the manufacturer's instructions. The lactate plasma levels were determined using a commercially available kit (Roche), also according to the manufacturer's instructions [35]. Briefly, a drop of plasma was placed on Accutrend Glucose and BM-Lactate strips. The results were recorded on the instrument after 12 and 60 s, respectively. The CLS and plasma protein concentration (PPC) of *C. chromis* was estimated on 1 μl of plasma or CLS using a Quibit 2.0 Fluorometer (Invitrogen) that, as suggested by the instructions, must first be calibrated using fluorescent standards with known concentrations included in the kit.

2.5. SDS–PAGE and western blot

The HSP70 protein expression was performed using Western-blot analyses [36]. The protein pattern of the CLS (20 μg) was obtained using 7.5% SDS-PAGE gels at 200 V for 30 min at 27 °C. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) at 30 V for 24 h in a transfer buffer (48 mM tris, 39 mM glycine, 20% v/v methanol, pH 8.3) and a wet transfer apparatus (Bio-Rad, Mini-Protean II Cell). After blocking the PVDF blots, HSP70 was detected by incubation for 1 h with anti-mouse HSP70 mAbs (Sigma-Aldrich).

The analysis of molecular weights and the quantifying of the HSP70 expression were determined by a densitometric method carried out with the AlphaEaseFC software. The densitometry data were expressed as the mean values of three different experiments and reported as a percentage of the integrated optical density value.

2.6. Statistical analysis

The results are expressed as a mean ± S.E. The data collected were analyzed using a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests by fixing the significance at a $P < 0.05$ level. Assumptions of ANOVA, heteroscedasticity and normal distribution were not violated as confirmed by proper testing.

3. Results

The glucose, lactate and PPC levels recorded for the different sound treatments are listed, respectively, in Figs. 2–4. The results revealed that when the specimens of *C. chromis* were exposed to acoustic stimuli at 300 Hz, the glucose, lactate and PPC values were significantly higher ($p < 0.001$) compared to those of the controls

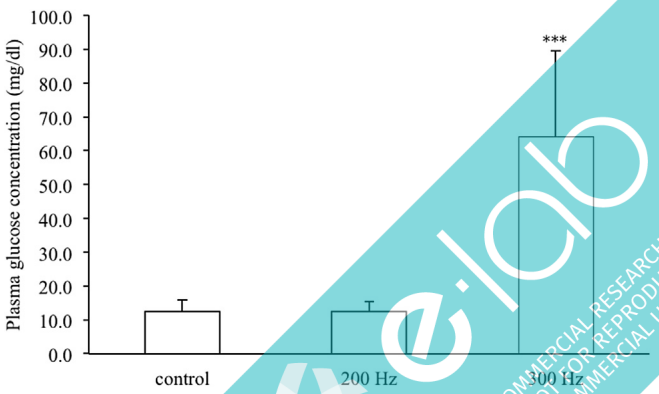

Fig. 2. Means values (n = 15) ± standard deviation of plasma glucose levels (mg/dl) for *C. chromis* exposed to different noise treatments. Asterisks represent significant differences between control and test groups (*** = $P < 0.001$).

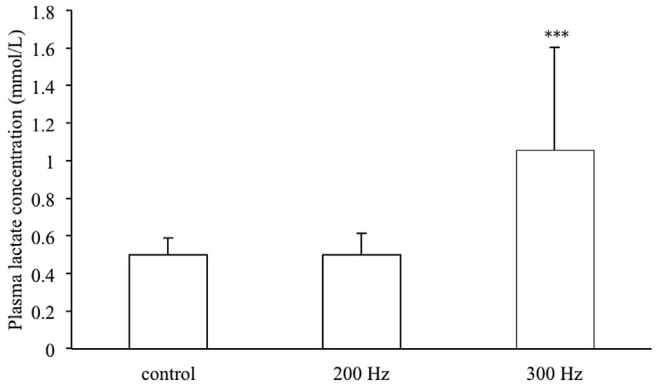

Fig. 3. Means values (n = 15) ± standard deviation of plasma lactate levels (mmol/L) for *C. chromis* exposed to different noise treatments. Asterisks represent significant differences between control and test groups (*** = $P < 0.001$).

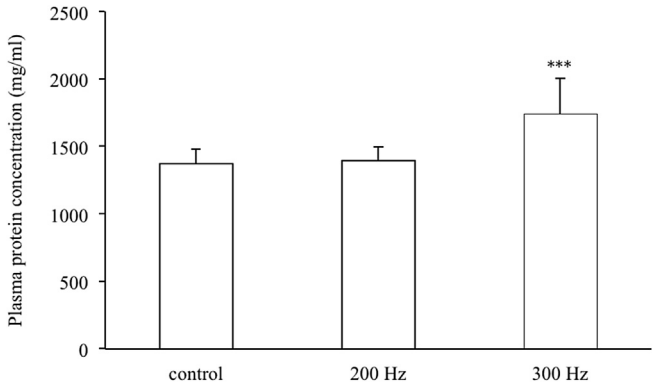

Fig. 4. Means values (n = 15) ± standard deviation of protein levels (mg/ml) for *C. chromis* exposed to different noise treatments. Asterisks represent significant differences between control and test groups (*** = $P < 0.001$).

(Figs. 2–4). Indeed, the plasmatic concentrations of glucose, lactate and PCC measured in unexposed *C. chromis* (the fish controls) changed from mean values of, respectively, 12.3 ± 3.5, 0.5 ± 0.1 and 1370 ± 110 to significantly higher mean values of 64.1 ± 25.5 (5.3-fold higher respect control), 11.1 ± 05 (2.1-fold higher respect control) and 1741 ± 260 (1.2-fold higher respect control), respectively (Figs. 2–4). The fish treated with a noise at 200 Hz did not have a significant ($p > 0.05$) increase in glucose, lactate and PPC plasma concentrations (Figs. 2–4).

As shown in Fig. 5A, the anti-mouse HSP70 mAb only cross-reacted with a 70 kDa band in the pattern resolved by the CLS. As is clear in Fig. 5A, the Hsp70 is constitutively expressed in the blood cells of *C. chromis*. The densitometric analysis of Hsp70 protein levels (Fig. 5B) showed that when the *C. chromis* specimens were subjected to the acoustic stimuli (300 Hz), the blood levels of HSP70 were significantly ($P < 0.001$) higher compared to the controls. Indeed, at an intensity of 300 Hz, the IDV value of the controls went from 3.22 ± 3.7 to a value of 33.52 ± 7.3. As reported previously for the plasmatic indicators, the HSP70 concentrations did not show significantly ($p > 0.05$) higher values compared to the controls (5.57 ± 0.7) in the specimens exposed to the noise at an intensity of 200 Hz (Fig. 5B).

4. Discussion

In fish, diverse stressors activate a wide spectrum of physiological functions that guide behavioural and biological responses.

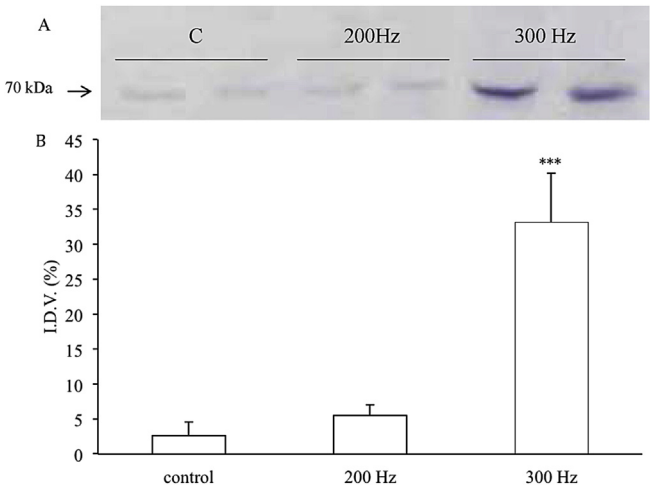

Fig. 5. Effect of the acoustic stimuli on expression levels of the Hsp70 in *C. chromis*. (A) Representative western blot of Hsp70 levels (two sample for each experimental trial). (B) Integrated optical density histogram (IDV) of the Hsp70 protein bands. Data are means ± s.d. (n = 15 control and n = 15 specimens for each test). Asterisks represent significant differences between control and test groups (*** = $P < 0.001$).

These coordinated responses, which are often referred to as "stress responses", are comprised of alterations in behaviour and autonomic functions and the production of hormones and chaperones.

The primary stress responses include an increase in plasma corticosteroids, and these have a wide range of metabolic effects such as the modulation of how carbohydrates are metabolized through gluconeogenesis and increases in protein turnover [37–39].

In particular, the increase in plasma cortisol typically causes rises in glucose and lactate plasma levels [18,37,40]. Meanwhile, Filiciotto et al. [10,38] and Celi et al. [17] have demonstrated that ambient noise can influence the primary, secondary and tertiary stress responses in crustaceans.

In this study, we demonstrate that the specimens of *C. chromis* are able to hear the acoustic stimuli delivered through a waveform generator. At low values of acoustic stimulus (≤200 Hz) do not appreciate significant changes in stress parameters evaluated. This indicates that, although, *C. chromis* are sensitive to sound from 200 Hz, as shown by Wysocki and colleagues [6], however these frequencies are not perceived as stressful stimuli. Only at frequencies of 300 Hz were able to activate the stress response. Indeed, we find that all measured biochemical parameters (Glucose, Lactate, PPC and HSP70) reached the highest value in fish stimulated at a frequency of 300 Hz compared with the 200 Hz treated and control animals.

From invertebrates to fish and higher vertebrates the stress response is accomplished at three integrated levels (primary, secondary and tertiary) involving the hypothalamic–pituitary–interrenal (HPI) axis. The primary response is neuroendocrinological responsible for the respective release of catecholamines and corticosteroids into the bloodstream. The secondary response is activated by these hormones and is manifest as changes in a range of biochemical, hematological and immunological factors [41]. 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 [42–45]. In this study we have not evaluated the blood cortisol levels, however, our results confirm the activation of secondary stress responses. In particular, individuals subjected to acoustic stress showed an altering metabolism with an increase of glucose,

lactate, and PC in the plasma.

Proteins are the main serum components [46]. The use of total proteins as a stress parameter in fish remains contradictory and unconvincing, although it seems to be a good parameter of stress in crustaceans [47]. The recent literature shows that there are increases in total protein concentrations in crustaceans exposed to acoustic stimuli [10,17]. In the present study, the total protein plasma trend of the exposed fish is in accordance with other stress markers. The significant increase of PT could be indicative of an impaired physiology.

Heat shock proteins can be induced by noise and ototoxic drugs [48]. Moreover, when induced in response to moderate non-traumatic sound levels, they can condition the ear to withstand the effects of loud noise and protect it from hearing loss, although there is noticeable individual variability [16,17,49,50]. HSPs function as molecular chaperones, and the HSP70 proteins are well known to have functions related to stress tolerance. In the present study, we show for the first time that acoustic stimuli induce HSP70 over-expression in fish. Across the current literature, there is no evidence of the effects of noise stress on the expression of HSPs in fish. Wu et al. [51] and Konings et al. [52], however, have shown that HSP70 increases after exposure to stress noises in humans and birds. This latter study reported that only HSP70 (but not HSP 30, HSP 60, or HSP 90) significantly increased in avians after exposure to loud noises [53]. Celi et al. [18] and Filiciotto et al. [10] have demonstrated changes in agonistic behaviour and HSP70 expression levels in red swamp crayfish (*Procambarus clarkii* Girard, 1852) after exposure to 30 min of an acoustic stimulus.

The main outcome of the current study indicates that traditional biochemical parameters such as glucose, lactate protein concentrations in plasma and HSP70 [54,55] could be regarded as molecular biomarkers, including of acoustic stress. This opens up a vast area of research for studying the effects of anthropogenic noises on both behaviour and biology in wild fish populations.

5. Conclusions

We show that the anthropogenic sound in the marine environment can be considered a physical pollutant, that such as chemical and biological pollutants, generate a stress condition that brings the animals to react vs the stimulus modulating the cellular and biochemical immune-responses. The impairment of the immune system, could increase susceptibility to disease and thus, decrease animal welfare. Is highly probable that the chronicity of the conditions of noise pollution, present in impacted areas, can cause serious damage not only to individuals but also to the exposed fish populations. Therefore, it would be desirable to pay more attention to all the human activities that may produce noise pollution and harm to marine animal communities.

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References

[1] J.A. Hildebrand, Anthropogenic and natural sources of ambient noise in the ocean, Mar. Ecol. Prog. Ser. 395 (2009) 5–20.
[2] N.O. Handegard, K. Michalsen, D. Tjøstheim, Avoidance behavior in cod (*Gadus morhua*) to a bottom - trawling vessel, Aquat. Living Resour. 16 (2003) 265–270.
[3] G. Sarà, J. Dean, D. D'Amato, G. Buscaino, A. Oliveri, S. Genovese, S. Ferro, G. Buffa, M. Martire, S. Mazzola, Effect of boat noise on the behaviour of bluefin tuna *Thunnus thynnus* in the Mediterranean Sea, Mar. Ecol. Prog. Ser. 331 (2007) 243–253.
[4] R.A. Kastelein, W.C. Verboom, N. Jennings, D. de Haan, Behavioral avoidance threshold level of a harbor porpoise (*Phocoena phocoena*) for a continuous 50kHz pure tone, J. Acoust. Soc. Am. 123 (2008) 1858–1861.
[5] C. Bracciali, D. Campobello, C. Giacoma, G. Sarà, Effects of nautical traffic and noise on foraging patterns of Mediterranean damselfish (*Chromis chromis*), PLoS One 7 (2012) e40582.
[6] L.E. Wysocki, A. Codarin, F. Ladich, M. Picciulin, Sound pressure and particle acceleration audiograms in three marine fish species from the Adriatic Sea, J. Acoust. Soc. Am. 126 (2009) 2100–2107.
[7] A.N. Popper, J. Fewtrell, M.E. Smith, R.D. McCauley, Anthropogenic sound: effects on the behavior and physiology of fishes, Mar. Technol. Soc. J. 37 (2003a) 35–40.
[8] M. Picciulin, L. Sebastianutto, A. Codarin, A. Farina, E.A. Ferrero, In situ behavioural responses to boat noise exposure of *Gobius cruentatus* (Gmelin, 1789; fam. Gobiidae) and *Chromis chromis* (Linnaeus, 1758; fam. Pomacentridae) living in a Marine Protected Area, J. Exp. Mar. Biol. Ecol. 386 (2010) 125–132.
[9] G. Buscaino, F. Filiciotto, G. Buffa, A. Bellante, V. Di Stefano, A. Assenza, et al., Impact of an acoustic stimulus on the motility and blood parameters of European sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.), Mar. Environ. Res. 69 (2010) 136–142.
[10] F. Filiciotto, M. Vazzana, M. Celi, V. Maccarrone, M. Ceraulo, G. Buffa, et al., Behavioural and biochemical stress responses of *Palinurus elephas* after exposure to boat noise pollution in tank, Mar. Pollut. Bull. 84 (2014) 104–114.
[11] S.B. Pruett, Stress and the immune system, Pathophysiology 9 (2003) 133–153.
[12] M. Vazzana, M. Cammarata, E.L. Cooper, N. Parrinello, Confinement stress in sea bass (*Dicentrarchus labrax*) depresses peritoneal leukocyte cytotoxicity, Aquaculture 210 (2002) 231–243.
[13] A. Vizzini, M. Vazzana, M. Cammarata, N. Parrinello, Peritoneal cavity phagocytes from the teleost sea bass express a glucocorticoid receptor (cloned and sequenced) involved in genomic modulation of the in vitro chemiluminescence response to zymosan, Gen. Comp. Endocrinol. 150 (2007) 114–123.
[14] A. Nicosia, M. Celi, M. Vazzana, M.A. Damiano, N. Parrinello, F. D'Agostino, G. Avellone, S. Indelicato, S. Mazzola, A. Cuttitta, Profiling the physiological and molecular response to sulfonamidic drug in procambarus clarkii, Comp. Biochem. Physiol. Part C 166 (2014) 14–23.
[15] M. Celi, M. Vazzana, M.A. Sanfratello, N. Parrinello, Elevated cortisol modulates Hsp70 and Hsp90 gene expression and protein in sea bass head kidney and isolated leukocytes, Gen. Comp. Endocrinol. 175 (2012) 424–431.
[16] M. Celi, F. Filiciotto, D. Parrinello, G. Buscaino, M.A. Damiano, A. Cuttitta, S. D'Angelo, S. Mazzola, M. Vazzana, Physiological and agonistic behavioural response of *Procambarus clarkii* to an acoustic stimulus, J. Exp. Biol. 216 (2013) 709–718.
[17] M. Celi, F. Filiciotto, M. Vazzana, V. Arizza, V. Maccarrone, M. Ceraulo, et al., Shipping noise affecting immune responses of European spiny lobster (*Palinurus elephas*), Can. J. Zool. 93 (2015) 113–121.
[18] M. Celi, F. Filiciotto, G. Maricchiolo, L. Genovese, E.M. Quinci, V. Maccarrone, et al., Vessel noise pollution as a human threat to fish: assessment of the stress response in gilthead sea bream (Sparus aurata, Linnaeus 1758), Fish. Physiol. Biochem. 42 (2016) 631–641.
[19] M. Vazzana, M. Celi, C. Tramati, V. Ferrantelli, V. Arizza, N. Parrinello, In vitro effect of cadmium and copper on separated blood leukocytes of *Dicentrarchus labrax*, Ecotoxicol. Environ. Saf. 102 (2014) 113–120.
[20] M. Vazzana, T. Siragusa, V. Arizza, G. Buscaino, M. Celi, Cellular responses and HSP70 expression during wound healing in Holothuria tubulosa (Gmelin, 1788), Fish Shellfish Immunol. 42 (2015) 306–315.
[21] M. Vazzana, M. Celi, G. Maricchiolo, L. Genovese, V. Corrias, E. Maria Quinci, G. de Vincenzi, V. Maccarrone, G. Cammilleri, S. Mazzola, G. Buscaino, F. Filiciotto, Are mussels able to distinguish underwater sounds? Assessment of the reactions of *Mytilus galloprovincialis* after exposure to lab-generated acoustic signals, Comp. Biochem. Physiol. Part A 201 (2016) 61–70.
[22] C. Georgopoulos, W.J. Welch, Role of the major heat-shock proteins as molecular chaperones, Annu. Rev. Cell Dev. Biol. 9 (1993) 601–634.
[23] F.U. Hartl, Molecular chaperones in cellular protein folding, Nature 381 (1996) 571–580.
[24] B. Bukau, A.L. Horwich, The Hsp70 and Hsp60 chaperone machines, Cell 92 (1998) 351–366.
[25] R.I. Morimoto, A. Tissières, C. Georgopoulos, Progress and perspectives on the biology of heat shock proteins and molecular chaperones, in: R.I. Morimoto, A. Tissières, C. Georgopoulos (Eds.), The Biology of Heat Shock Proteins and Molecular Chaperones, Cold Spring Harbor Laboratory Press, Cold Spring

Harbor, NY, 1994, pp. 1–30.
[26] S. Fukudo, K. Abe, Y. Itoyama, S. Mochizuki, T. Sawai, M. Hongo, Psychophysiological stress induces heat shock cognate protein 70 messenger RNA in the hippocampus of rats, Neuroscience 91 (1999) 1205–1208.
[27] I. Varo, R. Serrano, E. Pitarch, F. Amat, F.J. Lopez, J.C. Navarro, Bioaccumulation of chlorpyrifos through an experimental food chain: study of protein HSP70 as biomarker of sublethal stress in fish, Arch. Environ. Contam. Toxicol. 42 (2002) 229–235.
[28] G.K. Iwama, L.O. Afonso, A. Todgham, P. Ackerman, K. Nakano, Are hsps suitable for indicating stressed states in fish? J. Exp. Biol. 207 (2004) 15–19.
[29] C.D. Delavega, A. Pujol, M.P. Viveros, Neonatally administered naltrexone affects several behavioral-responses in adult-rats of both genders, Pharmacol. Biochem. Behav. 50 (1995) 277–286.
[30] A. Mazzola, E. Favaloro, G. Sarà, Cultivation of the Mediterranean amberjack, *Seriola dumerili* (Risso, 1810), in submerged cages in the western Mediterranean sea, Aquaculture 181 (2000) 257–268.
[31] A.N. Popper, R.R. Fay, C. Platt, O. Sand, Sound Detection Mechanisms and Capabilities of Teleost Fishes Sensory Processing in Aquatic Environments, Springer, 2003b, pp. 3–38.
[32] D. Ross, Ship sources of ambient noise, IEEE J. Ocean. Eng. 30 (2005) 257–261.
[33] A.J. Underwood, Experiments in ecology and management: their logics, functions and interpretations, Aust. J. Ecol. 15 (1990) 365–389.
[34] M. Vazzana, D. Parrinello, M. Cammarata, Chemiluminescence response of beta-glucan stimulated leukocytes isolated from different tissues and peritoneal cavity of *Dicentrarchus labrax*, Fish. Shellfish Immunol. 14 (2003) 423–434.
[35] M. Cammarata, M. Vazzana, D. Accardi, N. Parrinello, Seabream (*Sparus aurata*) long-term dominant-subordinate interplay affects phagocytosis by peritoneal cavity cells, Brain Behav. Immun. 26 (2012) 580–587.
[36] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 4350–4354.
[37] T.P. Mommsen, M.M. Vijayan, T.W. Moon, Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation, Rev. Fish. Biol. Fish. 9 (1999) 211–268.
[38] F. Filiciotto, V.M. Giacalone, F. Fazio, G. Buffa, G. Piccione, V. Maccarrone, V. Di Stefano, S. Mazzola, G. Buscaino, Effect of acoustic environment on gilthead sea bream (*Sparus aurata*): sea and onshore aquaculture background noise, Aquaculture 414 (2013) 36–45.
[39] F. Fazio, V. Ferrantelli, G. Fortino, F. Arfuso, G. Giangrosso, C. Faggio, The influence of acute handling stress on some blood parameters in cultured sea bream (*Sparus aurata* Linnaeus, 1758), Ital. J. Food Saf. 4 (2015).
[40] K. Begg, N.W. Pankhurst, Endocrine and metabolic responses to stress in a laboratory population of the tropical damselfish *Acanthochromis polyacanthus*, J. Fish. Biol. 64 (2004) 133–145.
[41] Barton, G.K. Iwama, Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids, Ann. Rev. Fish. Dis. (1991) 13–26.
[42] B.A. Barton, C.B. Schreck, L.A. Sigismondi, Multiple acute disturbances evoke cumulative physiological stress responses in juvenile chinook salmon, Trans. Am. Fish. Soc. (1986) 115245–115251.
[43] A.G. Maule, C.B. Schreck, S.L. Kaattari, Changes in the immune system of coho salmon (*Oncorhynchus kisutch*) during the parr-to-smolt transformation and after implantation of cortisol, Can. J. Fish. Aquat. Sci. (1987) 44161–44166.
[44] A.D. Pickering, T.G. Pottinger, J. Carragher, J.P. Sumpter, The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout, Salmo trutta L, Gen. Comp. Endocrinol. (1987) 68249–68259.
[45] M.G. Mesa, Effects of multiple acute stressors on the predator avoidance ability and physiology of juvenile chinook salmon, Trans. Am. Fish. Soc. (1994) 123786–123793.
[46] S. Kumar, N. Sahu, A. Pal, D. Choudhury, S. Yengkokpam, S. Mukherjee, Effect of dietary carbohydrate on haematology, respiratory burst activity and histological changes in *L. Rohita* juveniles, Fish. Shellfish Immunol. 19 (2005) 331–344.
[47] S. Lorenzon, M. Martinis, E.A. Ferrero, Ecological relevance of hemolymph total protein concentration in seven unrelated crustacean species from different habitats measured predictively by a density-salinity refractometer, J. Mar. Biol. (2011) 7.
[48] H.H. Lim, O.H. Jenkins, M.W. Myers, J.M. Miller, R.A. Altschuler, Detection of HSP 72 synthesis after acoustic overstimulation in rat cochlea, Hear. Res. 69 (1993) 146–150.
[49] N. Yoshida, A. Kristiansen, M.C. Liberman, Heat stress and protection from permanent acoustic injury in mice, J. Neurosci. 19 (1999) 10116–10124.
[50] R.A. Altschuler, D. Fairfield, Y. Cho, E. Leonova, I.J. Benjamin, J.M. Miller, M.I. Lomax, Stress pathways in the rat cochlea and potential for protection from acquired deafness, Audiol. Neurootol. 7 (2002) 152–156.
[51] T. Wu, J. Ma, S. Chen, Y. Sun, C. Xiao, Y. Gao, R. Wang, J. Poudrier, M. Dargis, R.W. Currie, R.M. Tanguay, Association of plasma antibodies against the inducible Hsp70 with hypertension and harsh working conditions, Cell Stress Chaperon. 6 (2001) 394–401.
[52] A. Konings, L. Van Laer, S. Michel, M. Pawelczyk, P.I. Carlsson, M.L. Bondeson, E. Rajkowska, A. Dudarewicz, A. Vandevelde, E. Fransen, Variations in HSP70 genes associated with noise-induced hearing loss in two independent populations, Eur. J. Hum. Genet. 17 (2008) 329–335.
[53] K.A. Hoekstra, G.K. Iwama, C.R. Nichols, D.V. Godin, K.M. Cheng, Increased heat shock protein expression after stress in Japanese quail, Stress 2 (1998) 265–272.
[54] G.E. Hofmann, B.A. Buckley, S. Airaksinen, J.E. Keen, G.N. Somero, Heat-shock protein expression is absent in the Antarctic fish *Trematomus bernacchii* (family Nototheniidae), J. Exp. Biol. 203 (2000) 2331–2339.
[55] N. Basu, A. Todgham, P. Ackerman, M. Bibeau, K. Nakano, P. Schulte, G.K. Iwama, Heat shock protein genes and their functional significance in fish, Gene 295 (2002) 173–183.