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# Underwater high frequency noise: Biological responses in sea urchin *Arbacia lixula* (Linnaeus, 1758)

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ABSTRACT

Marine life is extremely sensitive to the effects of environmental noise due to its reliance on underwater sounds for basic life functions, such as searching for food and mating. However, the effects on invertebrate species are not yet fully understood. The aim of this study was to determine the biochemical responses of *Arbacia lixula* exposed to high-frequency noise. Protein concentration, enzyme activity (esterase, phosphatase and peroxidase) and cytotoxicity in coelomic fluid were compared in individuals exposed for three hours to consecutive linear sweeps of 100 to 200 kHz lasting 1 s, and control specimens. Sound pressure levels ranged between 145 and 160 dB re 1μPa. Coelomic fluid was extracted and the gene and protein expression of HSP70 with RT-PCR was evaluated on coelomocytes. A significant change was found in enzyme activity and in the expression of the HSP70 gene and protein compared to the control. These results suggested that high-frequency stimuli elicit a noise-induced physiological stress response in *A. lixula*, confirming the vulnerability of this species to acoustic exposure. Furthermore, these findings provide the first evidence that cell-free coelomic fluid can be used as a signal to evaluate noise exposure in marine invertebrates.

## 1. Introduction

Every marine ecosystem is characterized by its own, very individual soundscape (Radford et al., 2010; Ceraulo et al., 2018), made up of sounds generated by abiotic components (water currents, geophysical processes, waves, rain, ice), biotic components (sounds emitted voluntarily or involuntarily by marine species) and by human activities (Kennedy et al., 2010; Piercy et al., 2014; Buscaino et al., 2016). In recent decades, anthropogenic activities have increased underwater acoustic energy globally (Ross, 2005; Slabbekoorn et al., 2010; Hildebrand, 2009), thereby changing the acoustic characteristics (signature) of marine ecosystems at coastal, pelagic, and deepwater levels. Noise pollution, generated by commercial traffic, offshore and inshore construction, seismic explorations and sonar, has been recognized as a pollutant for aquatic ecosystems and a threat to marine fauna (World Health Organization, 2011; European Framework Directive 2008/56/EC - Marine Strategy (MSFD)).

All aquatic species, both vertebrates and invertebrates, are able to hear sounds (Slabbekoorn et al., 2010; Simpson et al., 2011; Filiciotto et al., 2014; Celi et al., 2015; Nedelec et al., 2016; Hawkins and Popper, 2017) and use this ability to carry out vital biological activities, such as foraging, predation, mating and habitat selection (Fay and Popper, 1998; Popper, 2003; Eggleston et al., 2016).

In this context, anthropogenic noise can disturb animal behaviour and physiology with potential consequences at population level (Sun et al., 2001; Slabbekoorn et al., 2010; Buscaino et al., 2010; Wale et al., 2013; Voellmy et al., 2014; Radford et al., 2014; Popper et al., 2014; Kunc et al., 2016; Magnhagen et al., 2017; Hawkins and Popper, 2017; Weilgart, 2018).

Reactions to boat or to seismic noise (Wardle et al., 2001; Engås and Løkkeborg, 2002; Slotte et al., 2004; Parry and Gason, 2006) were the most explored, given the fact that low frequencies can influence species at greater distances from the source than higher frequencies. However, echo sounders, fishing net control sonar, CHIRP sonar, side-scan and multi-beam sonar, deterrent devices and small, fast boats produce high-frequency sounds up to 800 kHz (Bonanno et al., 2006; Hildebrand,

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2009; Buscaino et al., 2009; Hawkins et al., 2015).

Despite the fact that absorption of high-frequency sounds is greater than that of low frequencies (resulting in an attenuation over shorter distances), their use is so extensive that they can contribute to increasing environmental noise (Hawkins et al., 2015), determining potential impact, especially on sessile species that cannot move quickly to avoid a noisy area. In particular, many sonars are designed to explore the sea bottom and, therefore, to reach the bottom at high intensity in order to receive backscattering. The upper hearing limit for fish and marine mammals is variable and can be as high as 160 kHz, suggesting possible effects of high frequency systems on animals at behavioural and physiological level (Dunning et al., 1992; Mann et al., 1997; Deng et al., 2014). However, effects of anthropogenic noise differ from species to species due to individual characteristics, behavioural responses, disturbance duration and emission characteristics. Furthermore, some animals can recover over time, while others suffer irreversible damage (Kight and Swaddle, 2011). A number of scientific studies describe the occurrence of behavioural and physiological changes in the presence of noise (Fewtrell and McCauley, 2012; Aguilar de Soto et al., 2013), including effects on immune response (Filiciotto et al., 2014; Celi et al., 2016; Vazzana et al., 2016) and on growth and development rates (Wysocki et al., 2007; Davidson et al., 2009) in marine organisms. For example, mussels exposed to low-frequency band treatment showed significantly higher values for cellular and biochemical stress parameters, such as glucose, total proteins, total number of haemocytes (THC), expression of heat shock proteins 70 (HSP70) and acetylcholinesterase (AChE) activity, measured in plasma and tissues (Vazzana et al., 2016). It is known that cellular stress response (in addition to molecular chaperone synthesis and the activation of the DNA repair system) also includes the enhancement of enzyme activities, which play an important role in environmental stress resistance (Sørensen et al., 2003). Alkaline phosphatase (AKP) is a metalloenzyme which catalyses the nonspecific hydrolysis of phosphate monoesters (Zhang et al., 2004). When exposed to different environmental stresses, lysosomal enzymes, in addition to AKP, are involved in the degradation of foreign proteins, lipids and carbohydrates (Ottaviani, 1984; Pipe et al., 1993; Xue and Renault, 2000), and can be used to assess immune status in invertebrates (Mou et al., 1999; Liu et al., 2000, 2004; Sarlin and Rosamma, 2011; Parisi et al., 2017). Esterase is one of the most common biomarkers of environmental exposure used in aquatic organisms (Galloway et al., 2002; Forget et al., 2003; Rickwood and Galloway, 2004; Barata et al., 2004; Hannam et al., 2008; Ren et al., 2015; Parisi et al., 2017). Another enzyme, peroxidase, is also involved in disease resistance and stress response, and changes in peroxidase levels can due to contaminants and other environmental stressors (Mydlarz and Harvell, 2007).

Moreover, cytotoxicity can be an efficient natural defence system in vertebrates and invertebrates; lysins can be secreted in the body fluids or act at the membrane level of the effector cell. Cytotoxicity was reported as a rapid and effective response to the K562 tumour cell line and rabbit erythrocytes in the fish *Dicentrarchus labrax* (Cammarata et al., 2000) and in the sea urchin *P. lividus* (Arizza et al., 2007), and against rabbit and sheep blood cells in sea cucumber *H. tubulosa* (Vazzana et al., 2018).

Although boat noise has been reported to influence behaviour and haemato-immunological parameters, such as THC, total protein concentration (PC), phenoloxidase (PO) activity, DNA integrity and HSPs protein expression (Celi et al., 2013, 2015; Filiciotto et al., 2014, 2016) in crustaceans, nothing is known about the influence of acoustic stimulus on different enzymes and on cytotoxic activity in invertebrates.

Therefore, knowledge on acoustic perception in invertebrates is little or entirely lacking (even more so in echinoderms) and more information is necessary to understand the potential negative effects on these marine organisms.

The aim of this study was to evaluate the effects of high frequency noise, from 100 to 200 kHz, on *Arbacia lixula* sea urchin in order to better understand the effects of this frequency-band noise on invertebrates. More specifically, the biochemical effect at cell-free coelomic fluid level was evaluated for the first time, including total protein concentrations, levels of alkaline phosphatase, esterase and peroxidase, degree of hemolysis (cytotoxicity), and the gene and protein expression of heat shock proteins 70 (HSP70).

## 2. Materials and methods

### 2.1. Animals

The experiment was carried out in the Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF) at the University of Palermo (Italy) according to current regulations regarding animal experimentation in Italy. Sea urchins, of the species *Arbacia lixula,* (weight ranging between 16 ± 1 g) were collected from Terrasini, Gulf of Palermo, from environments characterized by rocky coves and steep cliffs hanging over the sea. The animals were acclimatized for one week in square tanks (1 m × 1 m, depth: 1 m, water height: 80 cm, volume: 800 L at 18 °C) with: re-circulated, filtered seawater and a flow-through of dissolved oxygen at 8 mg $l^{-1}$ at 38‰ salinity. The organisms were fed commercially available invertebrate food (Azoo, Taikong Corp. Taiwan). For the experimental plan, the animals were chosen randomly from those available.

### 2.2. Experimental plan

Two rectangular tanks (85 cm × 43 cm, depth: 49 cm, water height: 27 cm, volume: 250 L) were used for the experiment: a test tank and a control tank (Fig. 1). A projector and a calibrated hydrophone were placed both in the control and the experimental tanks. 16 individuals of *A. lixula* were randomly divided in the two tanks, 8 specimens for each. Only the individuals in the experimental group were exposed to acoustic stimulus for 3 h, while no stimulus was applied to the control group. The control and experimental treatments were conducted at the same time.

The two tanks were located in two different cabinets without any contact points and no noise could be transmitted through vibration of the walls. Moreover, the different acoustic conditions (with and without acoustic stimulus) of the two tanks were monitored before trials, using the calibrated hydrophones located inside each tank (see “Acoustic stimulus, recording and analysis” paragraph for details).

The experiment was repeated three times, under the same environmental conditions using a total of 48 animals (24 control and 24 experimental). The animals were not fed in the 24 h prior to the experiment. All sea urchins, treated and not, were then taken to extract cell-free coelomic fluid for subsequent biological assays.

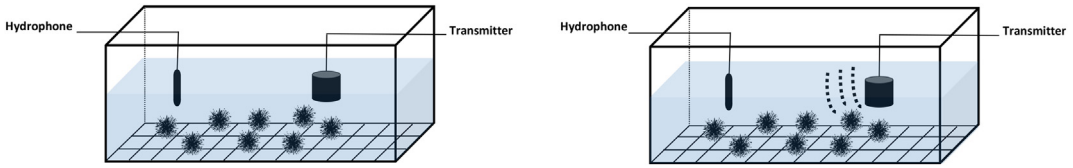


**Fig. 1.** Schematic drawing of the control and experimental tanks. Each tank was equipped with one hydrophone and one transmitter.

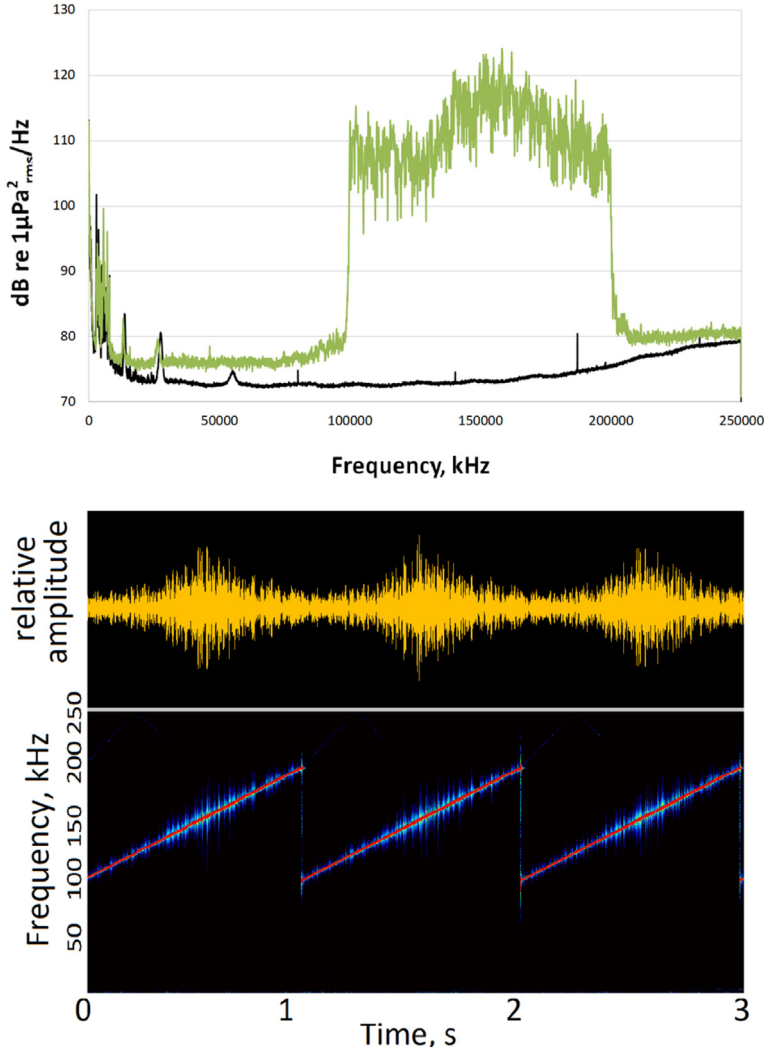


**Fig. 2.** On the top: Power Spectrum of 3 consecutive sweep and of the median background noise in the tanks (FFT size 16,384, Resolution 30.5 Hz); below: oscillogram and spectrogram (FFT 1024, window Hamming), sampling frequency 500 kHz.

### 2.3. Acoustic stimulus, recording and analysis

A linear sweep ranging from 100 to 200 kHz for the duration of 1 s and emitted in continuous mode was used as acoustic stimulus. To generate the acoustic stimulus in the experimental tank, a signal generator (Agilent 33210A, USA) was used coupled with a projector (Transmitter, Fig. 1). The characterization of background noise in both tanks and of acoustic stimulus in the experimental tank, were performed using the hydrophone (ResonTC 4034–3, Denmark; receiving sensitivity of −218 dB re 1 V/µPa +2 dB and -4 dB in the range 1 Hz - 250 kHz) located in each tank (Fig. 1) connected to an analogical/digital card (Avisoft USGH416b, Germany; sampling frequency 500 kHz, Gain 20 dB). This acquisition system was handled using specific software (Avisoft recorder USGH software, Germany). During the trials, the acoustic stimulus was monitored for 30 s every 30 min.

The background noise sound pressure level in the tanks was 140 ± 1 dB (re 1µPa$^2_{rms}$). Sound pressure during stimulus in the test tank ranged between 145 and 160 dB. The Power Spectrum and the spectrogram of the recorded acoustic stimulus are shown in Fig. 2.

### 2.4. Coelomic fluid withdrawal

The sampling of coelomic fluid was carried out with isosmotic anticoagulant medium (ISO-EDTA: 20 mM Tris, 0.5 M NaCl, 30 mM EDTA, pH 7.5) in a centrifuge tube. Cell counts were performed with Neubauer chamber and each sample was centrifuged at 1650 rpm for 10 min at 4 °C. The supernatant (cell-free coelomic fluid) was removed and stored at −20 °C until the time of assaying. The pellets were separated into two aliquots (one for RT-PCR and one for western blot analysis) and stored at −80 °C for subsequent analysis.

### 2.5. Protein concentration measure

Protein concentration was measured using the Bradford method (Bradford, 1976), both in the cell-free coelomic fluid and the pellet samples at 595 nm.

### 2.6. Alkaline phosphatase activity

Alkaline phosphatase activity was measured according to Ross et al. (2000), incubating 50 µl of *A. lixula* cell-free coelomic fluid with 50 µl of buffer (4 mM *p*-nitrophenyl liquid phosphate in 100 mM ammonium bicarbonate containing 1 mM $MgCl_2$, pH 7.8, 30 °C). Optical density (OD) was read at 405 nm for 1 h.

Enzyme activity is expressed in U/µg and calculated as: {(Abs/min) × (1000/Eb) × (Vf/Vi)}, with Eb = 18.4. One unit of activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol produced in 1 min.

### 2.7. Esterase activity

Esterase activity was evaluated by incubating 50 µl of *A. lixula* cell-free coelomic fluid with 50 µl of buffer (0.4 mM *p*-nitrophenyl-myristate substrate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100, pH 7.8, 30 °C) (Ross et al., 2000). OD was read at 405 nm for 1 h. Enzyme activity is expressed in U/µg and calculated as: {(Abs/min) × (1000/Eb) × (Vf/Vi)}, Eb =16.4. One unit of activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol produced in 1 min.

### 2.8. Peroxidase activity

Peroxidase activity was measured modifying the Quade and Roth (1997) method. Cell-free coelomic fluid (50 µl) was incubated for 30 min with 100 µl of TMB (3.3′, 5.5′ tetramethylbenzidine) (Sigma, Italy). The reaction was stopped with 50 µl of 2 M sulfuric acid ($H_2SO_4$). OD was read at 450 nm. Peroxidase activity was expressed as unit U/µg. One unit of activity was defined as the amount of enzyme required to release 1 µmol of substrate produced in 1 min.

### 2.9. Cytotoxicity assay

To analyse the cytotoxic activity of cell-free coelomic fluid, rabbit and sheep erythrocytes, provided by the Istituto Zooprofilattico Sperimentale della Sicilia A. Mirri (IZS, Palermo), were used as target cells. Erythrocytes were washed three times in PBS and suspended at 1% ($8 \times 10^6$ fresh erythrocytes) in ISO–$Ca^{2+}$ (0.5 M NaCl, 20 mM Tris-HCl, 10 mM $CaCl_2$; pH 7.4). The 100 µl samples were incubated with 100 µl of erythrocyte solution for 1 h at 37 °C. At the end of the incubation period, the samples were centrifuged for 10 min at 1500 rpm at 4 °C and the amount of released haemoglobin in the supernatant was measured at 540 nm using a microplate reader (GloMax; Promega Corporation, USA). To obtain the degree of hemolysis, the following formula was used: [(measured release-spontaneous release)/(complete release- spontaneous release)]x100. Complete haemoglobin release values were obtained by preparing an erythrocyte in distilled water at room temperature; a control erythrocyte suspension was prepared under identical experimental conditions using the same medium and incubation in order to measure haemoglobin release values.

### 2.10. RNA extraction from Arbacia lixula coelomocyte

The HSP70 gene expression was evaluated using one aliquot of cellular pellet. RNA extraction was performed using an RNA MiniPrep kit (Direct-zol™). To lyse the samples, $5 \times 10^6$ cells were resuspended in 600 µl of trizol. The samples were then centrifuged at 10,000 ×*g* for 30 s to remove particulate debris and the supernatant transferred to a new tube. An equal volume of ethanol (95%) was added to the supernatant and mixed thoroughly. This was transferred into a Zymo-Spin IIC Column within a Collection Tube before centrifugation; the flow-through was discarded. Subsequently, 400 µl of RNA wash buffer was added to samples and centrifuged. In a new RNase-free tube, 5 µl of DNaseI and 75 µl of DNA digestion buffer were mixed. This solution was added directly to the column. Samples were incubated at room temperature (20 °C) for 15 min. 400 µl of Direct-zol™ RNA Pre Wash was then added to the column and centrifuged. Flow-through was discarded before repeating this step and discarding flow-through once again. At this point, 700 µl RNA Wash Buffer was added to the column and centrifuged with an extended time of 2 min (to ensure total removal of wash buffer). The column was transferred into a new RNase-free tube. RNA was eluted by adding 50 µl DNase/RNase-Free Water to the column and centrifuging. At the end, 1 µl of RNA was extracted from *A. lixula* ceolomatic liquid cell samples and used in real-time polymerase chain reaction.

### 2.11. Real time PCR of Arbacia lixula RNA for HSP70

HSP70 gene expression was detected by Real-Time PCR (RT-PCR). This was performed using the Rotor-gene Q system (Qiagen) with QuantiTechSYBR Green RT-PCR kit. The QuantiTechSYBR Green RT-PCR kit provides accurate, real-time quantification of RNA targets in an easy to handle format. Use of 2xQuantiTechSYBR Green RTPCR master mix together with QuantiTechSYBR Green RT PCR mix allowed both reverse transcription and PCR to take place in a single tube; converting extracted RNA into cDNA. The products supplied for this Real-Time included 1 µl of each RNA sample, 0.5 µl of forward primer and 0.5 µl of reverse primer, 2 × 12.5 µl of QuantiTechSYBR Green RT-PCR Master Mix, 0.5 µl of QuantiTectRT mix and 10.75 µl of distilled water added to complete the amounts. Specific primers set for HSP70 (accession no. X61379), according to Marrone et al. (2012), were used. RT-PCR was run as follows: reverse transcription 1× cycle at 50 °C for 30 min, PCR initial activation step at 95 °C for 15 min and 40 cycles, denaturation 15 s 95 °C, annealing at 60 °C and 30 s at 72 °C for extension.

### 2.12. SDS-PAGE and western blot

The remaining aliquot of the cellular pellet was homogenized for 8 min in a Potter-Elvehjem tissue grinder using 1 ml of trizma base (20 mM, pH 7.5) with 300 mM NaCl and 10% SDS; this was followed by sonication for 60 s. Samples were centrifuged at 9000 rpm for 10 min at 4 °C and protein concentrations in the cell lysate supernatants (CLS) were evaluated.

Using western blot analyses (Towbin et al., 1979) we performed HSP70 protein expression. The equivalent of 20 µg/ml of *Arbacia lixula* cellular lysate supernatants were separated on 7.5% SDS-PAGE gels at 60 V for the first 10 min and at 120 V for 1 h. The separated proteins were subsequently transferred to polyvinylidenedifluoride (PVDF) membranes (Bio-Rad) at 15 V for 1 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); correct transfer was confirmed by Ponceau red-staining. After blocking PVDF blots, HSP70 was detected by overnight incubation at 4 °C with antimouse HSP70 (primary antibody dilution was 1:7500, Sigma-Aldrich). After incubation, PVDF blots were washed twice with TBS-T 1× and incubated with secondary antibody alkaline phosphatase-conjugated goat antimouse IgG (1: 3000 dilution, Sigma-Aldrich). Development was finally done with BCiP-NBT.

Densitometric analysis of the immunoblotted bands was performed using ImageJ software. Densitometry data were expressed as the mean values of different experiments and reported as a percentage of the integrated density value. The integrated density value (I.D.V.) of the relevant bands was normalised to the I.D.V. of the beta-actin bands (data not shown).

### 2.13. Statistical analysis

All the data were statistically analysed, using Statistica software 8.0, to assess possible differences between the control and experimental groups. To determine statistical differences, the data were analysed using a non-parametric test (Mann-Whitney *U* test) as our data did not fall into a normal distribution (verified by a Shapiro-Wilk tests $p < .05$). Statistical differences between control and experiment are shown on the error bars for each graph as $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$.

## 3. Results

No statistical differences were found between the control and experimental values ($p = .657533$; $Z = 0.44$) for total protein levels in cell-free coelomic fluid. Fig. 3 shows approximately the same values in the control and experimental samples, 54.51 ± 4.6 (mean ± SD) µg/

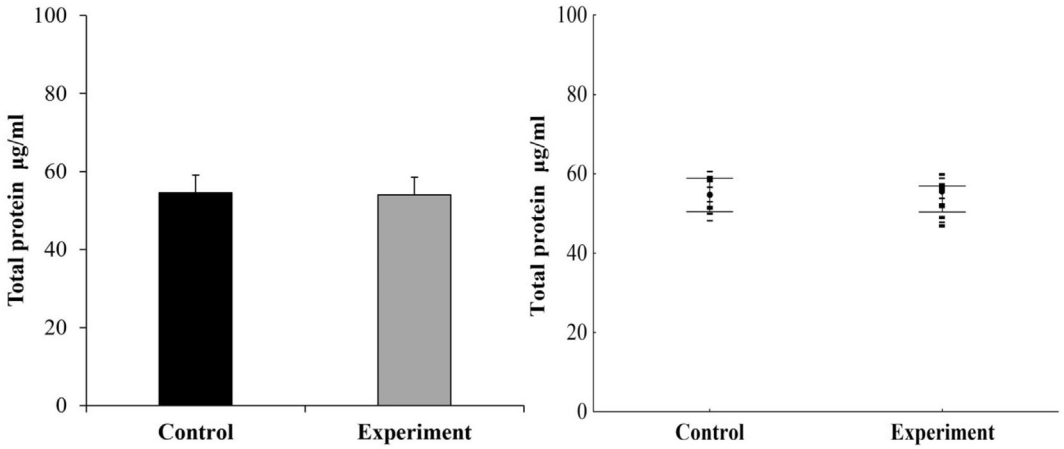


**Fig. 3.** Bar-graph (left) and dot-plot (right) of protein concentration (µg/ml) of cell-free coelomic fluid expressed as means ± SD, (−) raw data, (•) median values and (⊥) percentiles values.

ml and 53.95 ± 4.6 µg/ml respectively.

Our experimental plan also included an evaluation of the different enzyme activity involved in immune responses. The alkaline phosphatase activity of experimental cell-free coelomic fluid was significantly influenced ($p$ = .000012; Z = -4.38) by high frequency noise, showing higher values (7.6 ± 4.84 U/µg) compared to the control (2.38 ± 1.90 U/µg) (Fig. 4).

As shown in Fig. 5, the esterase activity in cell-free coelomic fluid was significantly higher ($p$ = .000000; Z = -5.57) in the experimental samples (71.96 ± 50.61 U/µg) compared to the control (21.68 ± 12.31 U/µg).

Cell-free coelomic fluid of the experimental groups had a significantly ($p$ = .000553; Z = -3.45) higher amount (6 ± 3.76 U/µg) of peroxidase activity compared to the control (2.70 ± 1.85 U/µg, Fig. 6).

Regarding cytotoxicity (Fig. 7) in the control samples, the degree of hemolysis was found to be 4.99 ± 1.91% against sheep erythrocytes and 3.93 ± 1.72% against rabbit erythrocytes. Significant increases were observed in the degree of hemolysis of experimental samples against rabbit erythrocytes with values of 11.91 ± 2.68% ($p$ = .000037; Z = -4.12) and against sheep erythrocytes with values of 9.92 ± 4.46% ($p$ = .001652; Z = -3.14).

Evaluation of the gene expression of HSP70 (Fig. 8) in *A. lixula* with primers of *P. lividus* highlighted that the experimental group had significantly higher (p = .000037; Z = -4.12) relative quantification (7.18) compared to the control. Relative HSP70 expression was calculated by dividing the normalised value of HSP70 by the normalised value obtained from the control sample. Densitometric analysis (Fig. 9) showed significant lower ($p$ = .008616; Z = 2.62) values of HSP70 protein in the experimental group.

## 4. Discussion

In coastal environments, background noise at high frequencies is influenced by human-generated noise, such as sonars, CHIRP, multibeam devices, etc. The use of this instrumentation is widespread, mostly in coastal areas and is linked to fishing activities, recreational boats and large vessels using sonars for safe navigation in shallow waters. Impact, in terms of low and mid-frequency noise, was investigated and noise presence could be heard continuously in the area, reaching at least 30% of daytime presence (Buscaino et al., 2016; Ceraulo et al., 2018). Source levels of sonars can reach high values, even > 220 dB (re 1µPa @1 m), and acoustic stimulus, tested with an SPL of 160 dB re 1µPa, represents a realistic sound that an animal attached to the bottom could receive. However, despite the fact that the vast majority of animals, including aquatic species, rely on sound (Bregman, 1990) for communicative and survival purposes, no studies have reported the impacts of high frequency sound and potential effects on benthic species which are unable to move rapidly to get away from noise. In this study, the *A. lixula* sea urchin was used for the first time to investigate the impact of high frequency noise at cellular, enzyme, cytotoxic and gene/protein expression levels.

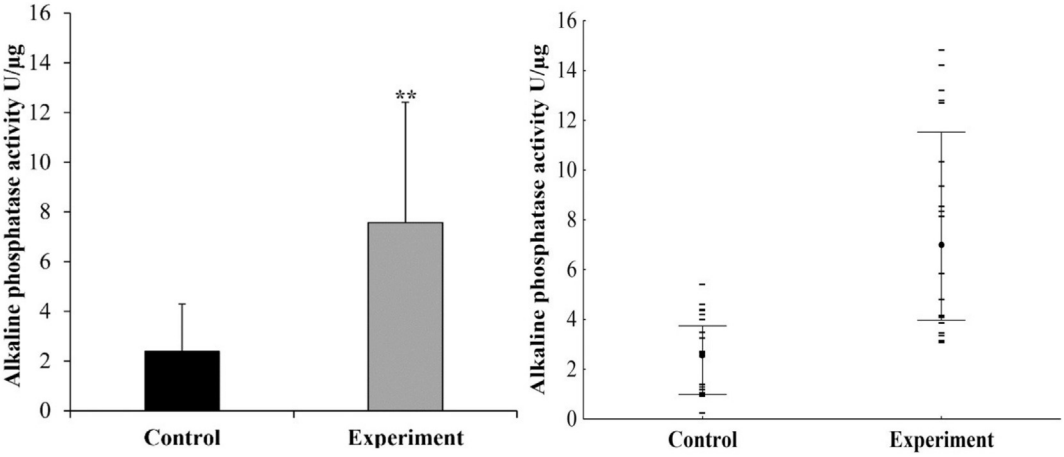


**Fig. 4.** Bar-graph (left) and dot-plot (right) of alkaline phosphatase activity (U/µg) of cell-free coelomic fluid expressed as means ± SD. The asterisks indicate the significant difference (**$p$ < .01), (−) raw data, (•) median values and (⊥) percentiles values.

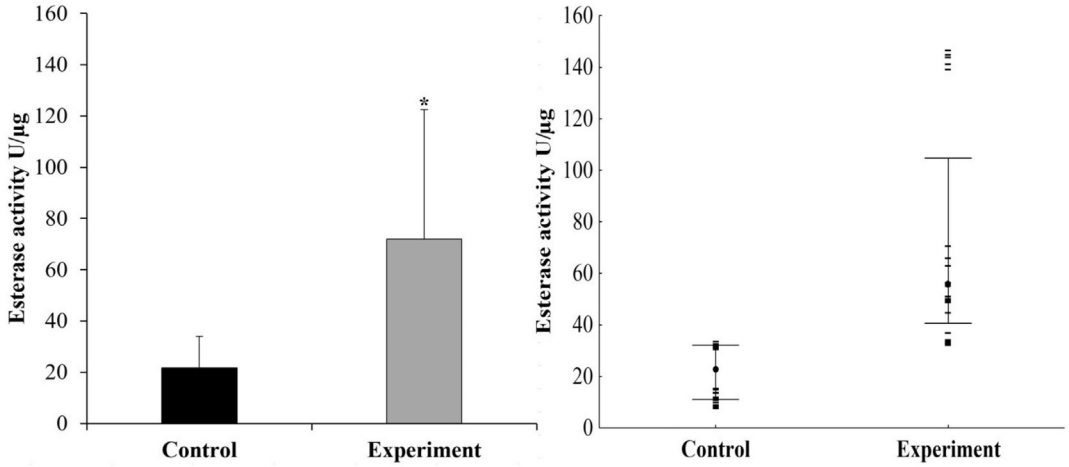


**Fig. 5.** Bar-graph (left) and dot-plot (right) of esterase activity (U/µg) of cell-free coelomic fluid expressed as means ± SD. The asterisk indicates the significant difference (*$p < .05$), (−) raw data, (•) median values and (⊥) percentiles values.

Total protein concentrations in cell-free coelomic fluid samples did not show significative differences between control and treated specimens. This result is in accord with data reported by Celi et al. (2013, 2016) on haemolymph of *Procambarus clarkii* and on plasma of *Sparus aurata* exposed to boat noise recordings.

In our study, the reason for this lack of response could be attributed to the acoustic frequencies of the stimulus used. It has been demonstrated, in fact, that the modulation of total protein concentration could depend on the type of frequency and the duration of acoustic stress Coeurdacier et al., 2011). Individuals of *Chromis chromis* exposed to acoustic stimuli at 300 Hz showed significantly higher plasma total protein levels compared to untreated fish; however, when exposed to 200 Hz, no significant differences were found (Vazzana et al., 2017). As an immune response indicator, a number of scientific studies have considered endogenous and antioxidant enzyme activities in haemolymph and in tissues of many bivalve species (Beckmann et al., 1992; Hine and Wesney, 1994; Carballal et al., 1997; Torreilles et al., 1997; Chen et al., 2007; Parisi et al., 2017). Esterase activity, for example, was chosen as a biomarker in mollusca exposed to organophosphorus and carbamate pesticides (Galloway et al., 2002; Valbonesi et al., 2003; Bolton-Warberg et al., 2007; Wheelock et al., 2008; Solé et al., 2010) and recently it has been used also in toxicological studies performed on echinoderm embryos and larvae (Torres-Duarte et al., 2019). The hydrolase alkaline phosphatase (AKP) was used to evaluate the state of health of the sea cucumber (Wang et al., 2008a, 2008b; Mazorra et al., 2002; Jing et al., 2006) and increased AKP activity was reported in sea urchin *Strongylocentrotus intermedius* gametes following exposure to environmental pollutants. This suggests a correlation between phosphatase concentration and environmental stress (Seitkalieva et al., 2016). In addition to hydrolase activities, cells have various mechanisms to reduce and repair damage; antioxidant enzymes are an example of this: the first line of defence against free radicals (Chainy et al., 2016).

Regarding echinoderms, Rabeh et al. (2019) showed an up-regulation of glutathione peroxidase (GPx) in *Holothuria forskali* intestine treated with mercury, and Telahiguea et al. (2019) confirmed this result in the body wall of the same species.

Our results showed significant increases in esterase, alkaline phosphatase and peroxidase activity in cell-free coelomic fluid of treatment animals, confirming that even noise modulates enzymes involved in immunity.

Many marine species exposed to stress conditions or potentially pathogenic microorganisms have defence responses based on immunocytes and humoral factors contained in coelomic fluid (Coffaro and Hinegardner, 1977; Smith, 1981; Chia and Xing, 1996; Smith et al., 1996; Pancer et al., 1999; Gross et al., 2000; Pancer, 2000; Kudriavtsev

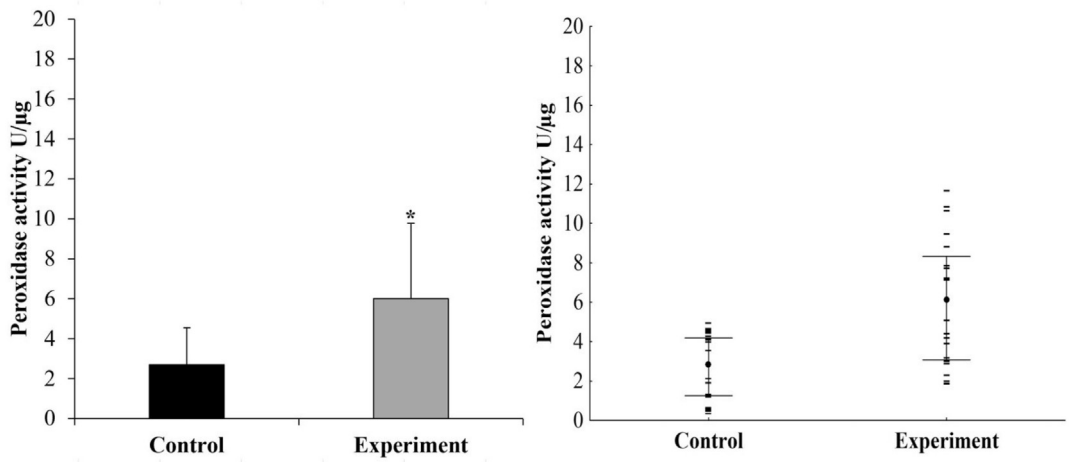


**Fig. 6.** Left: bar graph of peroxidase activity (U/µg) of cell-free coelomic fluid is expressed as means ± SD. The asterisk indicates the significant difference (*p < .05) calculated using Mann-Whitney *U* test to compare the means values. Right: dot plot of peroxidase activity (U/µg) represented like raw data (−), median values (•) and percentiles values (⊥).

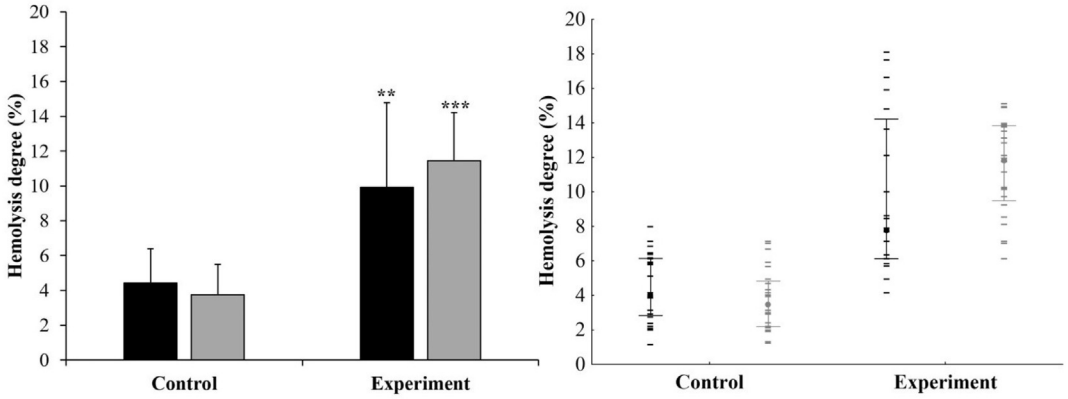


**Fig. 7.** Bar-graph (left) and dot-plot (right) of hemolysis degree (%) of cell-free coelomic fluid expressed as means ± SD. Cell-free coelomic fluid tested against sheep erythrocytes (■);. Cell-free coelomic fluid tested against rabbit erythrocytes (▒). The asterisks indicate the significant differences between control and experimental (**p < .01; ***$p$ < .001), (−) raw data, (•) median values and (⊏⊐) percentiles values.

and Polevshchikov, 2004). Focusing on invertebrates, in addition to the defence activity performed by enzymes in stress conditions, cytotoxic activity acts through the release of lysines in body fluids. In echinoids, in particular, it is known that amebocytes and spherulocytes are the main coelomocyte populations that perform cytotoxic activity (Bertheussen, 1979; Lin et al., 2001); coelomic fluid of *Paracentrotus lividus* sea urchin contains different types of celomocytes (including amebocytes and colourless spherulocytes) involved in immune defence. Furthermore, the cytoplasmic granules of this species show a calcium-dependent cytolytic activity (Canicattì, 1991; Pagliara and Canicattì, 1993; Arizza et al., 2007).

In our study, significant increases in toxicity levels against rabbit and sheep erythrocytes were detected. The significant increase in cytotoxic activity of cell-free coelomic fluid against mammalian erythrocytes could be due to the release of lysins by competent cells circulating in the coelomic fluid as a response to acoustic stress. Another good biomarker to study environmental pollution in invertebrates is the HSP protein family, given their cytoprotective role (Hamer et al., 2004; Anestis et al., 2007; Vazzana et al., 2016). In particular, the heat shock proteins HSP70 protect cells from apoptosis by interacting directly with cellular signalling pathways and apoptotic factors (Lanneau et al., 2008). Both HSP70 genes and proteins are inducible and involved in immune response. Changes in pH, exposure to metals and UV-B radiation increase the expression levels of HSP70 in celomocytes of *P. lividus* (Matranga et al., 2000, 2002) and the use of these as a model to test whether environmental stress can affect HSP70 expression was evaluated (Matranga et al., 2000). Individuals exposed at 4 °C showed an HSP70 gene expression 5 times greater than the control group, while at 35 °C, a 2-fold expression was detected. The up-regulation of this gene is indicative of a link between expression and stress in the body, providing evidence for the use of HSP70 as a molecular marker in the study of stress factors in sea urchin. Pinsino et al. (2008) used *P. lividus* coelomocytes to assess environmental stress by evaluating the production of HSC70 proteins. Increased production of HSC70 is a defence mechanism of *P. lividus* coelomocytes used to cope with metal pollution. HSC70 is a protein part of the multi-gene family used as a molecular marker in long-term stress exposure, while HSP70 in short-term stress exposure (Ryan and Hightower, 1996). This validates the use of HSP70 in our study as a molecular marker in *A. lixula* exposed to short-term acoustic stimulus. Hamer et al. (2004) showed that the expression of HSP70 reached highest levels in the gills of *M. galloprovincialis* following exposure to environmental pollution, while Vazzana et al. (2016) showed how acoustic noise up-regulates HSP70 protein expression in the gills and mantle of *M. galloprovincialis*. The results of real-time PCR in this study showed a significant increase in HSP70 gene expression in coelomocytes of individuals treated with acoustic emission at 100–200 kHz. At high levels of gene expression, there is a decrease in the protein available in circulation in the experimental samples, probably due to the use of the protein produced in response to three hours of acoustic emission.

There are still very few studies in literature to describe the effects of noise on invertebrates and even fewer concerning echinoderms. In this study, we showed, for the first time, that acoustic emission between 100 and 200 kHz, lasting 3 h, significantly influences some immune

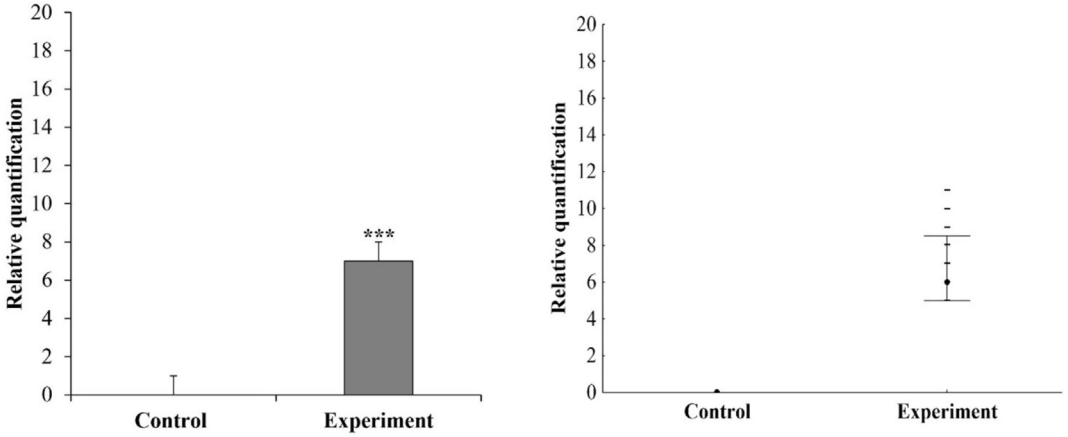


**Fig. 8.** Bar-graph (left) and dot-plot (right) of real-time PCR analysis of HSP70 expression. The statistical differences are indicated with the asterisks (***p < .001), (−) raw data, (•) median values and (⊏⊐) percentiles values.

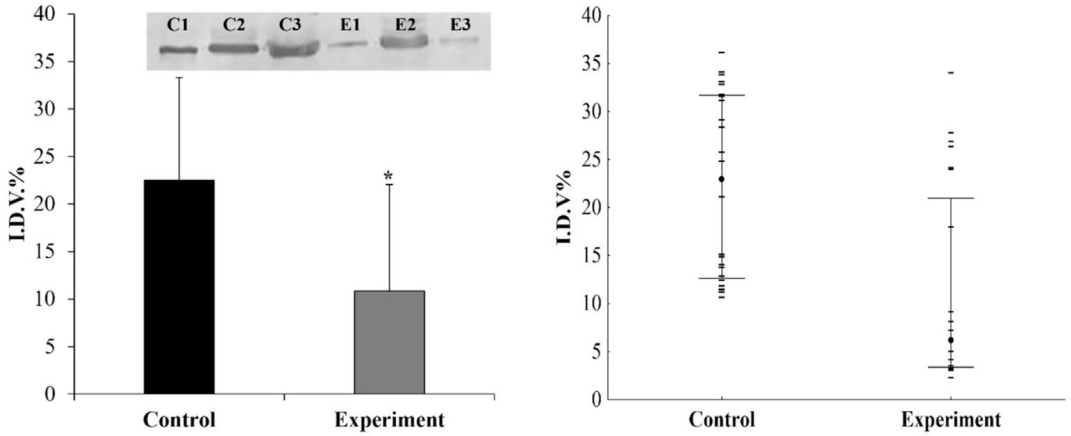


**Fig. 9.** Bar-graph (left) and dot-plot (right) of I.D.V. of the western blot HSP70 protein bands (inset). C1, C2, C3 were control samples; E1, E2, E3 were experimental samples. The statistical differences are indicated with the asterisks (*p < .05), (−) raw data, (•) median values and (⊥) percentiles values.

responses in *A. lixula* sea urchin, such as enzyme activity of cell-free coelomic fluid and HSP70 gene/protein expression levels, demostrating a perturbation of homeostasis. This highlights the fact that sea urchin could be an important species to study the effects of noise on marine organisms and that cell-free coelomic fluid could be considered a good matrix to evaluate animal welfare. This is reinforced by its wide distribution and by its unusual anatomical structure (an ovoid calcareous skeleton) which acts as a Helmholtz resonator (Radford et al., 2008). In conclusion, this study contributes to knowledge on the effects of high frequency noise on marine invertebrates. This is of considerable importance since negative impacts on marine animals could have considerable consequences, even at population level.

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## Declaration of Competing Interest

None

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