Effects of acoustic stimulation on biochemical parameters in the digestive gland of Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819)

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JASA ARTICLE

Effects of acoustic stimulation on biochemical parameters in the digestive gland of Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819)[a)]

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ABSTRACT:

Underwater sounds generated by anthropogenic activity can cause behavior changes, temporary loss of hearing, damage to parts of the body, or death in a number of marine organisms and can also affect healing and survival. In this study, the authors examined the effects of high-frequency acoustic stimulations on a number of biochemical parameters in the Mediterranean mussel, *Mytilus galloprovincialis*. During the experiment, animals were placed in a test tank and exposed to acoustic signals [a linear sweep ranging from 100 to 200 kHz and lasting 1 s, with a sound pressure level range of between 145 and 160 dBrms (re 1μParms)] for 3 h. Total haemocyte count was assessed and glucose levels, cytotoxic activity and enzyme activity (alkaline phosphatase, esterase and peroxidase) in the digestive gland were measured. For the first time, this study suggests that high-frequency noise pollution has a negative impact on biochemical parameters in the digestive gland. © *2020 Acoustical Society of America*.

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I. INTRODUCTION

Due to growing levels of anthropogenic activities over the last few years, underwater acoustic energy increased on a global scale (Ross, 2005; Slabbekoorn *et al.*, 2010; Hildebrand, 2009; Hawkins and Popper, 2017) leading to changes in the acoustic characteristics (signature) of marine ecosystems (Smott *et al.*, 2018; Buscaino *et al.*, 2016; Lecchini *et al.*, 2018) and interfering with animal physiology, behavior, and communication (Payne and Webb, 1971; Wale *et al.*, 2013; Wong and Candolin, 2015; Papale *et al.*, 2015; Hawkins and Popper, 2017).

Low frequency (LF) noise, (continuous or impulsive) generated by boats and seismic surveys has recently been categorized as a potential threat to marine fauna by the Marine Strategy Framework Directive (Directive 2008/56/EC). Its ability to propagate over long distances makes LF sound harmful to many marine species (Romano *et al.*, 2004; Wysocki *et al.*, 2006; Graham and Cooke, 2008; Rolland *et al.*, 2012). Furthermore, its persistence over time, characterized by fluctuations in amplitude and frequency, could compromise food acquisition, migration, reproduction, intraspecific communication (Slabbekoorn *et al.*, 2010; Stanley *et al.*, 2017; Buscaino *et al.*, 2019) and blood parameters in fish (Graham and Cooke, 2008; Vazzana *et al.*, 2017), mammals (Rolland *et al.*, 2012) and invertebrates (Celi *et al.*, 2013; Filiciotto *et al.*, 2016).

However, an increasing number of human activities in the sea are carried out with acoustic instrumentation which produces sound at frequencies as high as 200 kHz. The sound produced consists of extremely high-power tones or broadband frequency-modulated signals, typically used for navigation, detection, localization, communication, mapping and surveillance, and ocean floor studies (Hildebrand, 2009; Bonanno *et al.*, 2006; Buscaino *et al.*, 2009; Hawkins *et al.*, 2015). Given its widespread use for marine and civilian purposes, high-frequency (HF) sound is considered ubiquitous and can impact greatly on sessile species that are unable to move away at speed from a noisy area. Physical and physiological effects were observed in marine mammals (Wright *et al.*, 2007; Fernández *et al.*, 2005; Ketten *et al.*, 1993; Rolland *et al.*, 2012) and fish (Halvorsen *et al.*, 2012; Cox *et al.*, 2018) as a result of both low and high acoustic frequencies.

Despite the amount of scientific literature published on invertebrates, most refer to mobile invertebrates and few data are available on sessile specimens, which may be affected by human-generated noise to an even greater degree. Many man-made sources are deployed in shallow waters (i.e., air and water-guns), producing waves and vibrations within the substrate which may harm those organisms living near or in the substrate (Popper and Hawkins, 2018). There is evidence of negative effects from noise exposure in

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invertebrates from larval to adult stages, with increasing mortality levels, developmental delays (Nedelec *et al.*, 2014; McCauley *et al.*, 2017), and changes in hemato-immunological parameters (Celi *et al.*, 2013; Celi *et al.*, 2015; Filiciotto *et al.*, 2014; Filiciotto *et al.*, 2016; Vazzana *et al.*, 2020). In particular, with regard to the mussel, Roberts *et al.* (2015) showed that tone-signal vibrations at frequencies in the range of 5–410 Hz affected the overall fitness of *Mytilus edulis*, with negative effects on natural valve periodicity. In the Mediterranean mussel, *Mytilus galloprovincialis*, Vazzana *et al.* (2016) recently evaluated physiological sensitivity to underwater noise, showing significant change in plasma glucose, total protein and total haemocyte (THC) levels, and tissue change in the expression of heat shock protein 70 (HSP70) and in acetylcholinesterase (AChE) activity.

THC is a bioindicator that refers to the number of haemocytes in circulation. It is used to evaluate the health status of aquatic organisms as the immune system is based on circulating haemocytes, which play an important role in immune surveillance. Numerous studies have used THC to evaluate the effects on invertebrates of stressful conditions (Le Moullac *et al.*, 1998; Sánchez *et al.*, 2001; Filiciotto *et al.*, 2014), including bivalves (Malagoli *et al.*, 2007; Vazzana *et al.*, 2016).

Blood glucose is another important biomarker widely used to evaluate stress response in invertebrates (Hannan *et al.*, 2016). Stress factors lead to alterations in homeostasis in animals and mechanisms required to restore balance use energy stored in tissues (Lu *et al.*, 2015). The immune system is also involved in homeostatic perturbance in animals and an important role is played by cytotoxic response, which occurs when lysines are released into body fluids or act directly on effector cell membranes.

These lytic molecules carry out a cytotoxic action on a variety of foreign agents in order to protect the animal and restore homeostasis (Arizza *et al.*, 2007; Vazzana *et al.*, 2018; Cammarata *et al.*, 2000). Regarding humoral factors, enzymes such as alkaline phosphatase (AKP) and esterase (both of the hydrolase classes), and antioxidant enzymes also play an important role in stress resistance (Sørensen *et al.*, 2003).

Alkaline phosphatase removes the phosphate groups and is involved in the degradation of foreign proteins, lipids, and carbohydrates (Ottaviani, 1984; Xue and Renault, 2000). Esterase enzymes perform the hydrolysis of the ester bond and are present in different forms for different substrates (Hannam *et al.*, 2008). In the presence of contaminants or other environmental stressors, antioxidant enzymes are also used to determine the health of marine invertebrates (Mydlarz and Harvell, 2007) and are able to reduce the state of oxidative stress due to increases in reactive oxygen species (ROS) (Livingstone, 2003).

In the mussel, a key sentinel organism in the biomonitoring of environmental pollution, immune responses were studied in the digestive gland; a tissue which is affected by a number of environmental stress conditions (Parisi *et al.*, 2017; Campillo *et al.*, 2019; Shaw *et al.*, 2019).

The aim of this study was to gain a better understanding of the effects of high-frequency (100–200 kHz) noise pollution on Mediterranean mussel *Mytilus galloprovincialis*. In order to test if acoustic stimulation triggered and alteration in the health status of the animals, THC was determined. Furthermore, due to the role played by the digestive gland in intracellular and extracellular digestion, in the storage of nutritive substances (Bayne *et al.*, 1976) and in reproductive activities (Robledo and Cajaraville, 1997), the mussel was used as a biological matrix, for the first time, to evaluate the impact of underwater noise by analysing glucose levels, cytotoxic activity, and enzyme activity (alkaline phosphatase, esterase, and peroxidase).

II. MATERIALS AND METHODS

A. Experimental plan

A total of 36 adult specimens of *Mytilus galloprovincialis* were obtained for the experiment from a local supplier in Palermo.

The mussels were placed in a holding tank (1 m × 1 m, depth: 1 m, water height: 80 cm, volume: 800 L at 13 °C ± 3 °C) equipped with an oxygenated and filtered-seawater recirculation system. All animals were acclimatized for one week and fed on commercially available invertebrate food (Azoo, Taikong Corp. Taiwan). Mean (±standard deviation) weight of the mussels was 18.30 g ± 1.36 and length 4.55 cm ± 0.21.

Following initial acclimatization, 12 individuals were extracted randomly and transferred to two tanks (85 cm × 43 cm, depth: 49 cm, water height: 27 cm, volume: 250 L; 6 mussels per tank) one tank was used as the control and the other for acoustic testing (individuals subjected to acoustic stimulation). The animals were given a second 72-h acclimatization period and were not fed in the 24 h prior to testing. The control and acoustic tanks were located in two separate cabinets with no contact (no noise could be transmitted through vibration in the walls); a workstation was set up near the tanks to house the sound-generator and recording systems. Figure 1 illustrates the layout of the tanks. In the acoustic tank, animals were exposed to acoustic stimulation for 3 h. Haemolymph and the digestive gland were extracted from each individual for biochemical assays at the end of acoustic stimulation. The experimental plan was

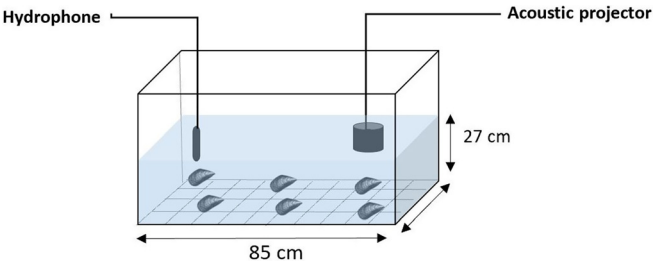

FIG. 1. (Color online) Schematic view of tank. Six individuals of *M. galloprovincialis*, a sound projector and a hydrophone were positioned in each tank (control and acoustic). In the control tank, the projector did not emit any acoustic stimulation.

repeated three times, using different individuals for each replica and under identical experimental conditions. All staff handling the animals and all experiments were conducted according to current regulations regarding animal experimentation in Italy.

B. Acoustic stimulation

Acoustic stimulation consisted of a linear sweep with lower/upper frequencies of 100–200 kHz and a duration of 1 s. High-frequency band signals are mainly used by sonars in shallow water for the location of small objects with detailed resolution (Hildebrand, 2009). In our experiment, a laboratory-built projector driven by a signal generator (Agilent 33210A, USA) (Fig. 1) was used. An identical silent projector was also positioned in the control tank to ensure no differences in conditions between the two tanks. A 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), positioned at half tank-depth, was connected to an analogical/digital card (Avisoft USGH416b, Germany; sampling frequency 500 kHz) to enable characterization of the background noise in both tanks and stimulation in the acoustic tank. Specific software (Avisoft recorder USGH software, Germany) was used to manage the acquisition system. Acoustic stimulation was emitted for 3 h and was monitored during testing for 30 s every 30 min.

The power spectral density of the tank background noise and of the recorded acoustic stimulation in the test tank is shown in the Fig. 2. The sound pressure level in the test tank during acoustic stimulation ranged between 145 and 160 dB (re 1μPa$_{rms}$, time average size 30 ms), by contrast, the level was found to be 140 ± 1 dB (re 1μPa$_{rms}$, time average size 30 ms) in the control tank.

C. Haemolymph collection

The mussels (control and experiment) were removed from the aquaria following acoustic emission. Haemolymph (800 μl) was collected from the adductor muscle of each mussel using a 1 ml sterile syringe in the presence of 200 μl of anticoagulant solution (0.45 M NaCl, 30 mM sodium citrate, 26 mM citric acid and 10 mM EDTA). THC per ml was determined using a Neubauer haemocytometer chamber.

D. Digestive gland extraction

The digestive gland was removed from each specimen using a scalpel and stored at −80 °C until time of assays. The glands were homogenized separately in 1 ml of PBS solution (NaCl 137 mM, KH_2PO_4 1.76 mM, Na_2HPO_4, 8.1 mM, KCl 2.7 mM, $CaCl_2$ 1.19 mM, and $MgCl_2$ 1.05 mM). Tissue homogenate was centrifuged at 9000xg at 4 °C for 25 min and supernatant used to evaluate protein concentration, glucose levels, cytotoxic activity, and enzyme activity (alkaline phosphatase, esterase, peroxidase).

E. Glucose evaluation

Glucose levels in the digestive gland were determined using Accutrend Plus instrument (Roche). Photometric measurement of reflectance was performed using Accutrend Glucose test strips. One drop (approximately 15 μl) was used for each sample.

F. Cytotoxic assay

Cytotoxic activity of the mussel plasma (v/v) was evaluated using sheep erythrocytes at 1% in TBS Ca^{2+} as target cells. After incubation at 37 °C for 1 h, the samples were centrifuged at 400 g and 4 °C for 10 min. Optical density (O.D.) was measured at 540 nm using a microplate reader (GloMax®-MultiDetection System; Promega Corporation, Madison, Wisconsin, USA). The following formula was used to calculate hemolysis:

$$\text{Degree of hemolysis} = \frac{\text{O.D. measured release} - \text{O.D.T. spontaneous release}}{\text{O.D. complete release} - \text{O.D.T. spontaneous release}} \times 100.$$

G. Enzyme assay in *M. galloprovincialis* digestive gland

Following total protein estimates in the digestive gland for each sample, enzyme activities were determined using the Bradford method (1976).

Alkaline phosphatase activity was measured by incubating an equal volume of sample with 4 mM p-nitrophenyl phosphate (Sigma, Italy) in 100 mM ammonium bicarbonate buffer containing 1 mM $MgCl_2$, pH 7.8 at 30 °C, as described by Ross *et al.* (2000). Increases in O.D. were measured continuously at regular 5-min intervals over 1 h at 405 nm by a microplate reader (GloMax®-Multi Detection System; Promega Corporation, Madison, Wisconsin, USA). Enzyme activity was expressed in U/μg of protein.

Esterase activity was assessed by incubating an equal volume of sample with 0.4 mM of p-nitrophenyl myristate substrate in 100 mM of ammonium bicarbonate buffer containing 0.5% Triton X-100, pH 7.8 at 30 °C. Activity was determined in the same way as alkaline phosphatase but using p-nitrophenyl myristate as a substrate (Sigma, Italy), as described by Ross *et al.* (2000). O.D. was read at 405 nm for 1 h. Enzyme activity was expressed in U/μg of protein.

Peroxidase activity was determined by modifying the Quade and Roth (1997) method, incubating 50 μl of sample in 100 μl of TMB (3.3′, 5.5′ tetramethylbenzidine) (TMB, Sigma, Italy) for 30 min in 96-microwell plates. The reaction was stopped with 2 M of sulphuric acid (H_2SO_4).

The amount of enzyme was measured using O.D. values read at 450 nm, expressed as a unit U/μg of protein in the sample.

H. Statistical analysis

Results were expressed as a mean value ±SD. Data were analysed using a non-parametric test (Mann-Whitney

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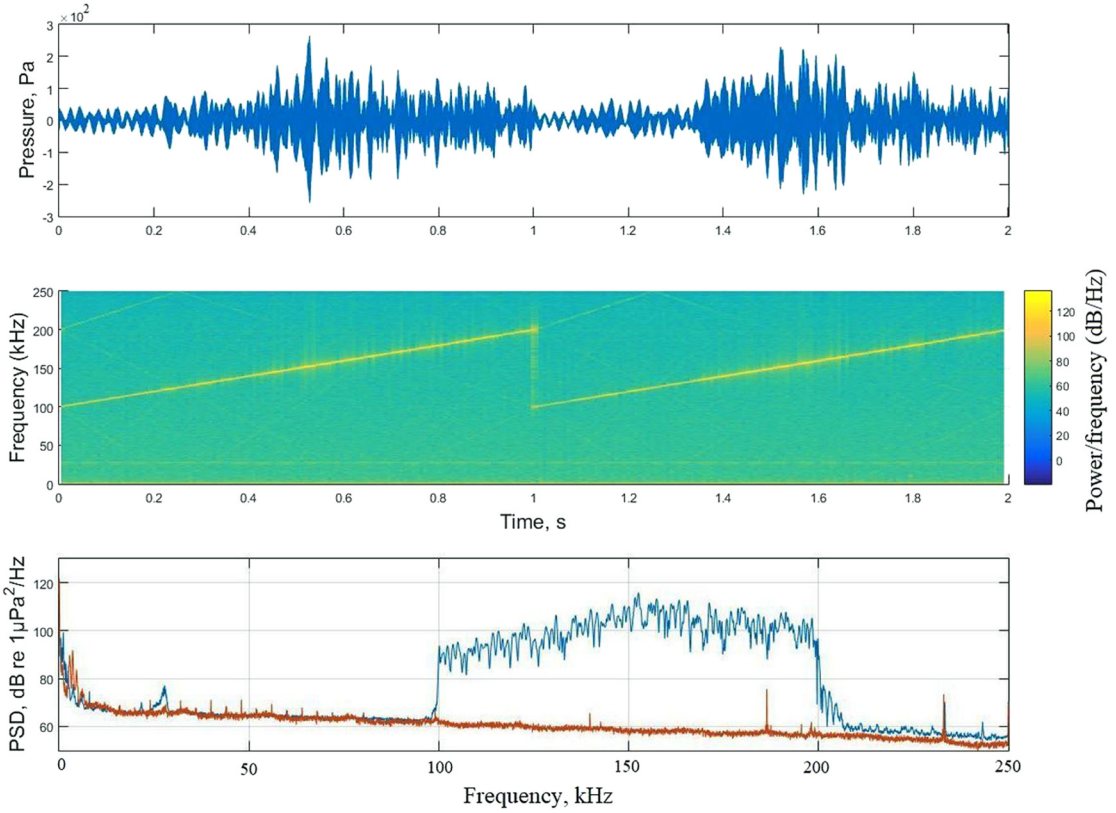

FIG. 2. (Color online) Upper: Oscillogram of two sweeps. Middle: spectrogram of two sweeps (sampling frequency: 500 000 sample per second, FFT size 8192 sample, time segments overlap 50%). Lower: mean power spectral density (dB re 1 $\mu Pa^2/Hz$) of two sweeps (blue) and the background noise (red).

U test) as our data did not showed a normal distribution (verified by a Shapiro-Wilk tests $p < 0.05$).

III. RESULTS

Mussel *M. galloprovincialis* exposed to 3 h of noise at high frequency (100–200 kHz) showed changes in a number of biochemical parameters.

THC in *M. galloprovincialis* (Fig. 3) showed higher values in sound-exposed animals than in the control, although differences were not significant.

However, glucose levels in the digestive gland of animals exposed to acoustic stimulation for 3 h were significantly lower ($p = 0.05$; $Z = 1.96$) than those of the control groups, as shown in Fig. 4. More specifically, glucose levels in the control were 367 ± 96.3 mg/dl whilst found to be 301 ± 69 mg/dl in sound-exposed animals.

The mean percentage of cytotoxic activity in the digestive gland was significantly lower ($p = 0.02$; $Z = 2.32$) in mussels subjected to acoustic exposure for 3 h compared to controls, as shown in Fig. 5.

Enzyme activity of alkaline phosphatase, esterase and peroxidase (Fig. 6) in the digestive gland of mussels showed significantly lower values in sound-exposed animals than in the control [alkaline phosphatase in exposed animals 68.9 ± 15.44 U/μg and in control 99.9 ± 61.6 U/μg ($p = 0.05$; $Z = 1.97$), esterase in exposed animals 27.5 ± 9.61 U/μg and in control 37.2 ± 15.51 U/μg ($p = 0.05$; $Z = 1.99$), and peroxidase in exposed animals 8.9 ± 4.56 U/μg and in control 13.63 ± 5.79 U/μg ($p = 0.01$; $Z = 2.54$)].

IV. DISCUSSION

It is known that anthropogenic activities in the sea and inland waters introduce noise at various frequencies and it has been demonstrated that low-frequency noise causes negative responses in many marine organisms

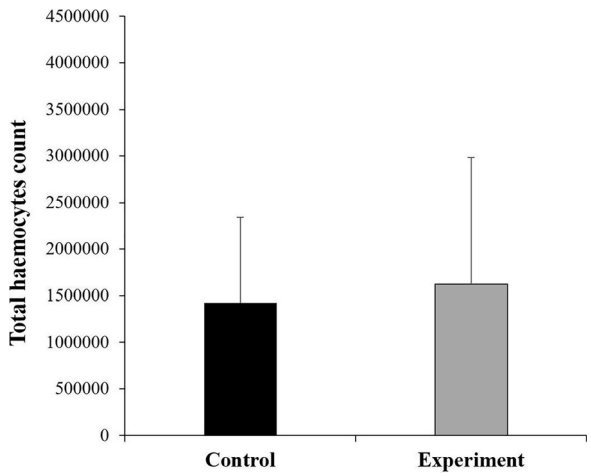

FIG. 3. THC of *M. galloprovincialis* haemolymph, presented as a mean ±SD. The control is represented by the black bar while the acoustic-exposed animals by the grey bar. No statistical differences between control and acoustic-exposed animals are apparent.

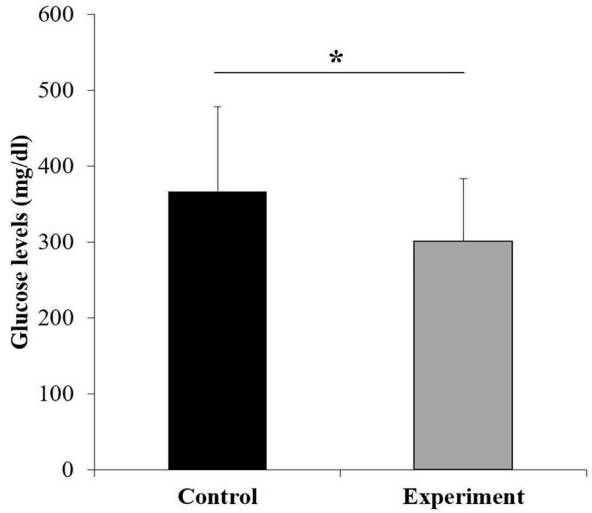

FIG. 4. Means ± SD of glucose levels of mussels exposed to acoustic stimulation for 3 h. Statistical differences between control and acoustic-exposed animals are shown as *p < 0.05.

(Slabbekoorn *et al.*, 2010; Hawkins and Popper, 2017). Bivalve organisms are also sensitive to human-generated noise exposure. In fact, Roberts *et al.* (2015) and Peng *et al.* (2016) highlighted negative behavioral responses as a result of acoustic noise (500 Hz sine wave and 1000 Hz sine wave) and substrate vibrations. In particular, noise stimulations generated an increase in digging behavior (Peng *et al.*, 2016) and valve-closure acceleration (Roberts *et al.*, 2015).

In this study, for the first time, we exposed the mussel *M. galloprovincialis* to high frequency noise in the 100–200 kHz band for 3 h.

Sound pressure levels and time of exposition being tested (140 dB re 1μPa for 3 h) represent realistic noise exposure that an animal attached to the sea bottom could be subjected to. In shallow waters, human activities use sonar equipment that can reach high values, as powerful as >220 dB (re 1μPa @1 m), and frequencies up to 700 kHz. We are fully aware that the acoustic field in small tanks in a laboratory setting, such as the tanks in our experimental setup, is considerably different from the acoustic field that occurs in the sea (Rogers *et al.*, 2016). In addition to the water soundscape, the acoustic field of the substrate, especially in regard to benthic organisms such as the mussel, must also be considered (Popper and Hawkins, 2018). However, this study based its evidence on a comparison between control and test trials exposed to a different acoustic-scape (which in water differs mainly according to the energy in the band 100–200 kHz, see Fig. 2), and represents a model in order to further explore the effects of noise in natural environments.

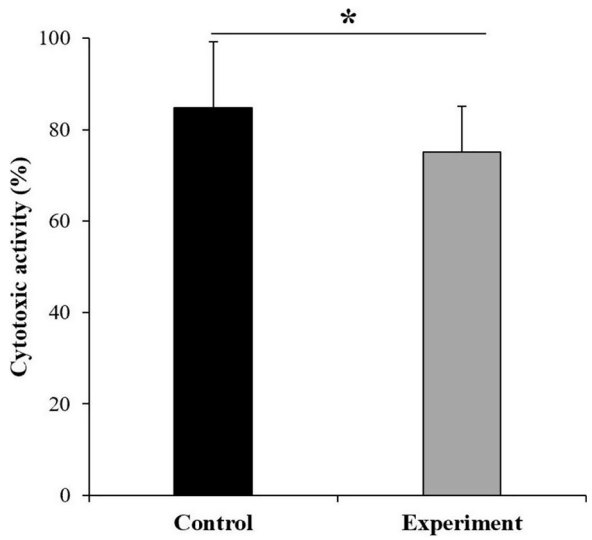

FIG. 5. Cytotoxic activity of *M. galloprovincialis* digestive gland expressed as a mean percentage ± SD. Statistical differences between control and acoustic-exposed animals are shown as *p < 0.05.

A. Noise effects on haemolymph

An important role regarding immunity in invertebrates is played by the cellular component. Circulating cells are fundamental actors in a defence response against non-self in the animal (Ottaviani, 2005; Rowley and Powell, 2007). THC is used to evaluate the effects of stressful conditions and the health status of aquatic organisms (Sánchez *et al.*, 2001; Filiciotto *et al.*, 2014; Celi *et al.*, 2015). In general, immune efficiency depends on homeostatic balance in individuals and many authors have demonstrated that the number of circulating haemocytes in the mussel increases in response to environmental changes. Malagoli *et al.* (2007) reported an increase in THC levels as a consequence of stress stimuli (air exposure, mechanical stress, high temperature, and extreme salinity conditions) and the same results were observed in mussels subjected to acute heat and cold conditions (Yao and Somero, 2013), and heavy-metal exposure (Lopes-Lima *et al.*, 2012). Vazzana *et al.* (2016) showed that THC values increased significantly in mussels exposed to acoustic stimulation. In agreement with these authors, THC of *M. galloprovincialis* increased in our study, although not significantly, suggesting that exposure to acoustic noise increased the number of total circulating cells.

B. Noise effects on the digestive gland

The digestive gland is considered a reliable target-tissue when investigating the effects of pollutants at cellular, biochemical, and molecular levels (Faggio *et al.*, 2018). In this study, glucose levels in the digestive gland of mussels exposed to acoustic stimulation was evaluated. It was previously shown that haemolymphatic glucose levels increased in *M. galloprovincialis* after exposure to acoustic stimulation (Vazzana *et al.*, 2016); probably due to a decrease in glycogen content in the digestive gland as a result of high-glycolytic activity (Pekkarinen and Suoranta, 1995; Ngo *et al.*, 2011). In fact, Sonawane and Sonawane (2018) reported that during acute and chronic stress exposure, a significant reduction in glycogen content in the digestive gland was occurred. Our results showed that animals exposed to

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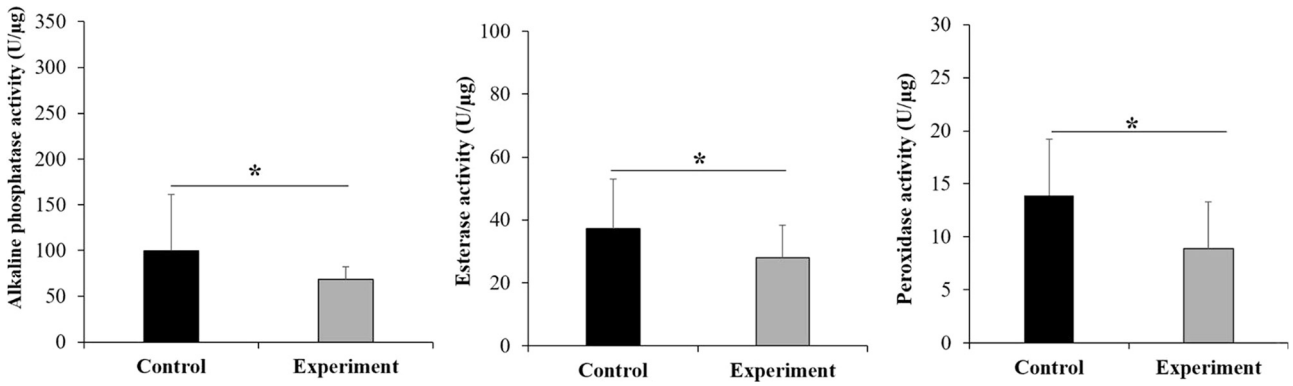

FIG. 6. Alkaline phosphatase, esterase and peroxidase activity of *M. galloprovincialis* digestive glands expressed in U/ml and presented as means ± SD. Statistical differences between control and acoustic-exposed animals are shown as *$p < 0.05$.

HF had lower glucose levels in the digestive gland compared to control animals. It is likely that the molecule was released into the blood circulation immediately following higher energy demand, determined by a greater breakdown of glycogen in acoustic exposed animals.

Regarding cytotoxicity, our results showed, for the first time, that cytotoxic activity is present in the digestive gland and that this was significantly influenced by acoustic stimulation. In particular, a decrease in activity in bivalves exposed to acoustic stimulation compared to controls was observed. Cytotoxic activity is one of the most important functions linked to immune responses (Franceschi *et al.*, 1991). This immune response has been conserved throughout evolution and described in both invertebrates and vertebrates (Ratcliffe *et al.*, 1985; Savary and Lotzová, 1986). Wittke and Renwrantz (1984) showed that circulating cells (immunocytes) in *Mytilus edulis* are able to produce cytotoxic substances which lyse human erythrocytes. A cytotoxic protein complex was also found in *M. galloprovincialis* hemolymph (Hubert *et al.*, 1997) and Malagoli *et al.* (2008) showed that a reduction in cytotoxic activity could compromise health in mussels. Overall, cytotoxicity is a dynamic parameter that can be used as an indicator of immune efficiency and, therefore, of health in mussels.

The digestive gland is an organ that also plays an important role in enzyme-production and secretion (Dimitriadis *et al.*, 2004); in addition to being involved in nutrient transport and digestion, it modulates the immunological processes of haemocytes, such as phagocytosis (Chen *et al.*, 2007). A number of scientific studies have showed that changes in hydrolase-class enzyme activities are correlated with the performance and survival of some invertebrate species (Galloway *et al.*, 2002; Rickwood and Galloway, 2004; Barata *et al.*, 2004; Hannam *et al.*, 2008; Ren *et al.*, 2015) and that a decrease of enzyme activity is a suitable biomarker to understand the effect of environmental conditions (Wang *et al.*, 2012) and pollutants (Douhri and Sayah, 2009). Despite this, these enzymatic responses in marine invertebrates subjected to acoustic stress are little known to date and have been analyzed only in mussels and echinoderms (Vazzana *et al.*, 2016; Vazzana *et al.*, 2020). In this study for the first time, levels of enzyme activity inside the digestive gland were analyzed in mussels exposed to HF acoustic emission. Parisi *et al.* (2017) showed that in mussels exposed to environmental stress conditions, alkaline phosphatase, and esterase activity in the digestive gland was lower than in the control group, highlighting the fact that environmentally stressful situations compromise this enzyme activity. The significant decrease in enzyme levels in the digestive gland observed in this study confirmed that HF emission can adversely affect these enzyme activities, as observed in other environmental conditions (Kopecka-Pilarczyk, 2010; Parisi *et al.*, 2017).

Many sources of environmental pollution can also induce oxidative stress in marine organisms which react to an increase in ROS production by regulating the activity of antioxidant enzymes (Livingstone, 2003).

Santovito *et al.* (2005) showed that in both the digestive gland and gills of *M. galloprovincialis* (with some tissue-specific differences), antioxidant enzymes, such as SOD, GPX, and CAT, are expressed. In this study, peroxidase activity in the digestive gland was assessed, highlighting a significant reduction in the activity of these enzymes in acoustic-exposed animals. These results were in agreement with Doyotte *et al.* (1997) who showed a decrease in antioxidant response in mussels when exposed to chemical pollutants. It has long been known that humoral and cellular immune-related parameters in bivalves are sensitive to stress factors, such as salinity, nutrient availability, water temperature, dissolved oxygen, and parasites (Auffret *et al.*, 2004; Giron-Perez, 2010).

V. CONCLUSION

Although scientific studies are available describing acoustic impacts on invertebrates, no data are reported in literature regarding acoustic effects on enzyme response and cytotoxicity activity in invertebrates.

In this study, for the first time, we showed a decrease in various immunological and biochemical parameters in the digestive gland of animals exposed to high frequency acoustic treatment (100–200 kHz), highlighting that noise pollution in the marine environment emitted from sound sources

at high frequencies (i.e., sonars) can be considered a pollutant capable of compromising the immune system. Furthermore, in addition to immunity, noise emissions affect a number of physiological responses in invertebrates (Carroll *et al.*, 2017).

Bivalves exposed to acoustic stimulation, for example, can respond with valve closure, altering water filtration capacity (Roberts *et al.*, 2015). This could require a great deal of energy and could affect respiratory activity, heart rate, excretion ability, and the fitness of the animal, with subsequent repercussions on the population and, eventually, the ecosystem (Widdows *et al.*, 1984).

In the future, further studies could be carried out to analyse biochemical responses in other invertebrate species exposed to the same acoustic frequencies of this study. This would enable understanding of whether other species are as sensitive as mussels or if responses are species-specific. More attention could be paid, therefore, to all anthropogenic activities that cause high-frequency noise pollution.

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