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Are semi-terrestrial crabs threatened by human noise? Assessment of behavioural and biochemical responses of *Neohelice granulata* (Brachyura, Varunidae) in tank



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ABSTRACT

This study examined the effects of human lab-generated noise (sweep tone) on the behaviour and biochemistry of a semi-terrestrial crab (*Neohelice granulata*). The experiment was carried out in tanks equipped with video- and audio-recording systems on a total of seventy-eight specimens. In total, 42 experimental trials with sweep-tone exposure and control conditions were performed using crabs in single and group layouts. After a habituation period of 30 min, the locomotor and acoustic (sound signals emitted by the crabs) behaviours were monitored for 30 min. During this time, the animals in sweep-tone conditions were exposed to ascending sweeps in a bandwidth range of 2.5–25 kHz. Exposure to sweep-tone noise produced significant changes in the number of signals emitted, locomotor behaviours and plasma parameters, such as haemolymph total haemocyte count and glucose, lactate and total protein concentrations, revealing that human noise could represent a disturbance for this crustacean species.

1. Introduction

Anthropogenic noise is currently widely recognized as a global pollutant and in particular, the European Marine Strategy Framework Directive (Directive 56/2008 CE), in addition to other International legislations such as the United Nations Convention on the Law of the Sea (UNCLOS, 1982), includes the human-generated noise in the qualitative descriptors for determining a Good Environmental Status (GES) of marine waters so that “the Introduction of energy, including underwater noise, is at levels that do not adversely affect the marine environment”.

Over recent decades, a variety of anthropogenic sounds have been introduced into marine environments, changing the acoustic characteristics of marine ecosystems globally (Hildebrand, 2009). Main anthropogenic activities that contribute to acoustic pollution include: military sonar, coastal development, marine traffic, fishing activity (e.g., trawling, gillnetting, and seine fishing), scientific exploration, seismic surveys, sonar equipment, underwater explosions and offshore

construction (Debusschere et al., 2016). These noises occupy a wide frequency band, ranging from 20 to 5000 Hz (e.g., vessel traffic or seismic surveys) to hundreds of kHz (e.g., military sonar or scientific exploration). Acoustic pollution generated by these sounds may affect large areas and could pose a serious hazard not only to individual animals but also entire populations (Celi et al., 2016).

Many marine animals use acoustic signals to perform a wide range of biological activities. In particular, they use sound to communicate, protect themselves, locate food, navigate underwater, and adapt to their environment (Amorim et al., 2015; Sebastianutto et al., 2008; Stimpert et al., 2015; Vasconcelos and Ladich, 2008). Despite their relative abundance and central role in many aquatic ecosystems, the ability of marine invertebrates to detect and potentially use sound is poorly understood (Budelmann, 1992).

Underwater sound is characterized by pressure variations (sound pressure) and the associated oscillation of water molecules, referred to as particle motion. The particle motion component of an underwater sound may propagate not only via the water column, but also by the

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seabed (Hazelwood, 2012; Hazelwood and Macey, 2016) and, given their benthic lifestyle, crustaceans appear sensitive to the particle motion component of substrate vibration (Roberts et al., 2016; Roberts and Elliott, 2017). Awareness of sound is believed to be associated with mechanical disturbances of the surrounding water/sediment being detected by organs such as pairs of statocysts in the cephalothorax, chordotonal organs associated with joints in antennae, legs and an array of internal and external hair-like mechano-receptors (Budelmann, 1992; Breithaup, 2001; Popper et al., 2001). Filiciotto et al. (2016) studied the effects of noise pollution on *Palaemon serratus* specimens kept in tanks and highlighted that the signal structures and trends of the particle motion velocity (dB re nb/s) are related to those of the sound pressure level (SPL) (see also Nedelec et al., 2016), concluding that the propagation behaviour of these two acoustic wave components are similar in tank conditions.

Crustaceans are known to produce sounds as signals in many contexts, such as conspecific territorial interactions, warning signals, prey stunning and predator deterrence (Buscaino et al., 2015, 2011; Coquereau et al., 2016; de Vincenzi et al., 2015). Moreover, sound emission is important for sexual display in many crustacean species (Salmon and Horch, 1972; Von Hagen, 1984). It has been reported that anthropogenic noise pollution can adversely affect acoustic communication by decreasing the signal-to-noise ratio of a signal and limiting the broadcast area or active space (Brumm and Slabbekoorn, 2005).

A growing body of literature suggests that crustaceans respond to noise pollution by varying their physiological parameters (Celi et al., 2013; Filiciotto et al., 2016). These variations may be followed by the mobilization of energetic reserves and alteration of resource allocation (Wale et al., 2013). Wale et al. (2013) have demonstrated that oxygen consumption in *Carcinus maenas* crabs in response to ship-noise playback is related to a higher metabolic rate that can indicate increased cardiovascular activity arising from stress, and chronic stress can reduce fitness through detrimental effects on reproductive success and growth (Kight and Swaddle, 2011).

When combined with physiological assessments, behavioural observations can provide a more complete understanding of the impact of an external stimulus on an organism, population or species (Celi et al., 2013; Filiciotto et al., 2016). Filiciotto et al. (2014) reported that *Palinurus elephas* specimens, when exposed to boat noise in a closed and controlled environment (tank), significantly increased their locomotor activities and haemolymphatic bioindicators of stressful conditions such as plasma glucose, total proteins, Hsp70 expression and total haemocyte count. These authors concluded that the quantification of these different stress responses could contribute to define early indicators of the wellness of the specie and consequently as sign of a potential degradation of the environmental health.

The semi-terrestrial burrowing crab *Neohelice granulata* (Varunidae) is a keystone species of the South Western Atlantic saltmarshes (Bortolus and Iribarne, 1999; Escapa et al., 2004). This species is distributed in high densities from northern Patagonia, Argentina (42°25'S, 64°36'W) to Río de Janeiro, Brazil (22°57'S; 42°50'W) (Spivak, 2010). Throughout its extensive geographical distribution, this species faces variable semi-diurnal tide amplitudes (from a few cm up to 9 m), water salinities (from nearly 0 up to 60 g/l) and substratum types (from a soft bottom to cobble beaches). As reported by Filiciotto et al. (2018), this crab is able to produce two distinct acoustic emissions and one of these, the multi-pulses rasp, could convey messages involved in the sexual interactions.

In this study, the locomotor and acoustic behaviours and changes in haemolymphatic indexes of stress such as protein concentrations (PCs) and lactate and glucose levels in a semi-terrestrial burrowing crab (*N. granulata*) were investigated in different social layout trials after short-term exposure to sweep noise stimuli. Moreover, as further objective, we examined whether the sound exposure affected differently these parameters in (1) sexually mature females (receptive and unreceptive) and males held individually; and (2) in different mating context

involving grouped specimens (one or two males with receptive or unreceptive female).

Crabs in single and group trials were exposed to a 2.5–25 kHz stimulus, a bandwidth range that encompasses most of the frequencies produced by human activities.

2. Materials and methods

2.1. Animal collection

Crabs were collected in the Mar Chiquita coastal lagoon (MCL) located in the Buenos Aires Province, Argentina (37°45'S, 57°19'W). MCL is a body of brackish water (46 km²) with a low tidal amplitude (ca. 1 m) that is permanently connected to the sea (Reta et al., 2001). The upper intertidal zone is a mixed *Spartina densiflora* and *Sarcocornia perennis* marsh, and the mid- and lower-intertidal zones are mud flats (Isacch et al., 2006). Crabs occupy the upper- and mid-intertidal zones, generating extensive beds of burrows (Luppi et al., 2013; Spivak, 2010).

A total of 60 male and 60 female adult *N. granulata* crabs (> 19 mm carapace width [CW]) (López Greco and Rodríguez, 1999) were randomly selected and transported to the laboratory at Nagera Marine Station of the National University of Mar del Plata. Males and females were isolated and placed in natural seawater aquaria (30 × 35 × 25 cm, 26 l capacity, filled with 3 l sea water) with a ratio of 4 crabs per aquarium under a controlled photoperiod of 12:12 h, 23 g/l of salinity, 20.4 ± 2.5 °C (mean ± SD) of water temperature and continuous aeration. Crabs were fed daily *ad libitum* with rabbit pellet feed (15% protein content), and the water was changed twice a week. The crabs were maintained for ten days, and no mortality was observed. Experimental trials were conducted for one week from the end of November to early December 2015 always during the daytime. Seventy-eight crabs, 42 males and 36 females (18 receptive and 18 unreceptive), were used in the experiment. Since both receptive and unreceptive females were involved in the study, the receptive period of the females was monitored daily in the laboratory by pushing the operculum to check for mobility under a binocular stereoscope. The pleon was lifted, and the opercula were gently probed using fine forceps. When the opercula are mobile, they can be pushed inwards, indicating that the females are receptive (Brockerhoff and McLay, 2005; Sal Moyano et al., 2012). Unreceptive females are always characterized by non-mobile opercula.

The crabs were measured with callipers (accurate to 0.1 mm) using the maximum carapace width (CW) as the reference variable (42 males CW = 30.56 ± 2.6 mm and 36 females CW = 27.06 ± 3.1 mm (mean ± SD)).

2.2. Experimental conditions

The experimental study was performed underwater in a PVC circular tank (1.5 m diameter with a water deep of 50 cm). Both the holding aquaria and experimental tank were equipped with an independent flow-through seawater system from a common source. Salinity and temperature were monitored over the entire study period using a multiparametric probe (EC300, VWR International). The crabs were not fed for two days before the start of the trials.

The study was performed under two experimental conditions: [A] control and [B] sweep-tone exposure. Each experimental condition consisted of the subsequent social layout trials: Single trials: 1 male (M) (1), 1 unreceptive female (F) (2), and 1 receptive female (RF) (3); group trials, 1 male and 1 receptive female (M + RF) (1), 1 male and 1 unreceptive female (M + F) (2), 2 males and 1 receptive female (M + M + RF) (3), and 2 males and 1 unreceptive female (M + M + F) (4).

For both experimental conditions, three replicates were conducted for each trial. The experimental design of the study is reported in Table 1. A total of forty-two 30-minute trials were performed

Table 1

Schematic view of the experimental conditions design. Animals in single: M: male; RF: receptive female; F: unreceptive female; Animals in group: M + F: male + unreceptive female; M + RF: male + receptive female; M + M + F: male + male + unreceptive female; M + M + RF: male + male + receptive female.

Experimental condition	Experimental trial	Social layout	No specimens per trial	No replicates	TOT specimens involved	TOT trials
Control	Animals in single	M	1	3	9	9
		RF	1	3		
		F	1	3		
	Animals in group	M + F	2	3	30	12
		M + RF	2	3		
		M + M + F	3	3		
M + M + RF		3	3			
Sweep tone exposition	Animals in single	M	1	3	9	9
		RF	1	3		
		F	1	3		
	Animals in group	M + F	2	3	30	12
		M + RF	2	3		
		M + M + F	3	3		
		M + M + RF	3	3		
	TOT					78

sequentially inside the experimental tank adopting a random sequence in terms of experimental conditions and associated social layout trials during which the crabs' locomotor and acoustic behaviours were monitored and recorded. Each crab was used in only one trial to meet the assumption of experimental independence. After the end of each trial the water content from the experimental tank was entirely removed and subsequently the same water volume was replaced before considering the tank ready for the next trial. The trials started after 30 min of habituation.

2.3. Sweep-tone generation and acoustic recording system

Acoustic sweep tones (sounds characterized by a linear ascending spectral shape, see Fig. 1C) in a bandwidth range of 2.5–25 kHz were produced using a signal generator (model 33220, Agilent Technologies, Santa Clara, CA, United States) connected to an underwater loudspeaker (LL916C, Lubell, Columbus, Ohio, USA) with a frequency response between 0.2 and 23 kHz. A calibrated hydrophone (model 8104, Brüel & Kjær, Nærum, Denmark) with a sensitivity of 205.6 ± 4.0 dB re 1 V/ μ Pa at the 0.1 Hz and 80 kHz frequency bands was used to obtain acoustic recordings of the sweeps and acoustic signals emitted by the crabs. The hydrophone was used with a preamplifier (VP1000, Reson) with a 1-MHz bandwidth single-ended voltage that had a high-pass filter set at 10 Hz and a 32-dB gain. The equipment was connected to a digital acquisition card (USGH416HB, Avisoft Bioacoustics, set with no gain) managed by the Avisoft Recorder USGH software (Avisoft Bioacoustics). Signals from the crabs, sweeps and tank background were acquired at 300 k samples s^{-1} with 16 bits and analysed by the Avisoft-SASLab Pro software (Avisoft Bioacoustics). This high sampling rate was chosen considering the spectral shape of *N. granulata* acoustic emissions. In fact, as showed by Filiciotto et al. (2018), the impulsive acoustic emissions of this crab have a large bandwidth ranging from 2547 ± 131 Hz (mean \pm SD) to $85,251 \pm 37,477$ Hz (mean \pm SD).

The maximum sound pressure level of all the sweeps was 136 dB re 1 μ Pa. The experimental tank background noise had a lower SPL than the sweeps with a maximum SPL of 102 dB re 1 μ Pa. Here, SPL refers to the peak of pressure calculated in the time window of the pulse duration. The power spectrum and spectrograms of 5 s of the experimental tank background noise, the generated acoustic sweeps and a

multi-rasp acoustic signal are shown in Fig. 1.

2.4. Video monitoring system and analysis

A video system was used to monitor the crabs' locomotor behaviour and synchronized with the acoustic recording system. Videos for behavioural monitoring were recorded with a camera (AVTech, KPD136ZALTP model, connected to a generic digital recorder H.264 V2.08) placed on top of the experimental tank. The videos were analysed with EthoVision XT 9.0 (Noldus Information Technology, Wageningen, Netherlands) to measure the following locomotor behavioural parameters: distance moved, mobility state, movement and velocity. The analysis was performed on each frame (five per second) to distinguish the animals from the background based on their grey scale/brightness values, and the coordinates of the geometric centre and surface area were extracted for each object per frame. We pooled the events in 1-min intervals to obtain thirty values from every 30-minute trial in order to generate a higher number of data points for analysis. All behavioural events automatically evaluated in all the trials are described in Table 2.

2.5. Biochemical analysis

At the end of the experimental phase in the tank, the animals from [A] and [B] conditions were captured with a net, sacrificed and placed on crushed ice until death and sampling for haemolymph extraction. The protocols for animal husbandry and experimentation were reviewed and approved in accordance with Directive 86/609/EEC of the Council of European Communities regarding the treatment of animals used for scientific purposes.

2.5.1. Bleeding procedure

Haemolymph was collected from each animal. The specimens were anaesthetized by placing them at -20 °C for 10 min before the haemolymph was withdrawn from the dorsal vessel with a 1-ml sterile syringe fitted with a 27 G needle. Approximately 50 μ l of haemolymph from each animal was collected in a sterile Eppendorf tube in the presence (v/v) of an anticoagulant solution (0.45 M NaCl, 30 mM sodium citrate, 26 mM citric acid, and 10 mM EDTA). The haemolymph was centrifuged at 1000g for 10 min at 4 °C, and the plasma was obtained.

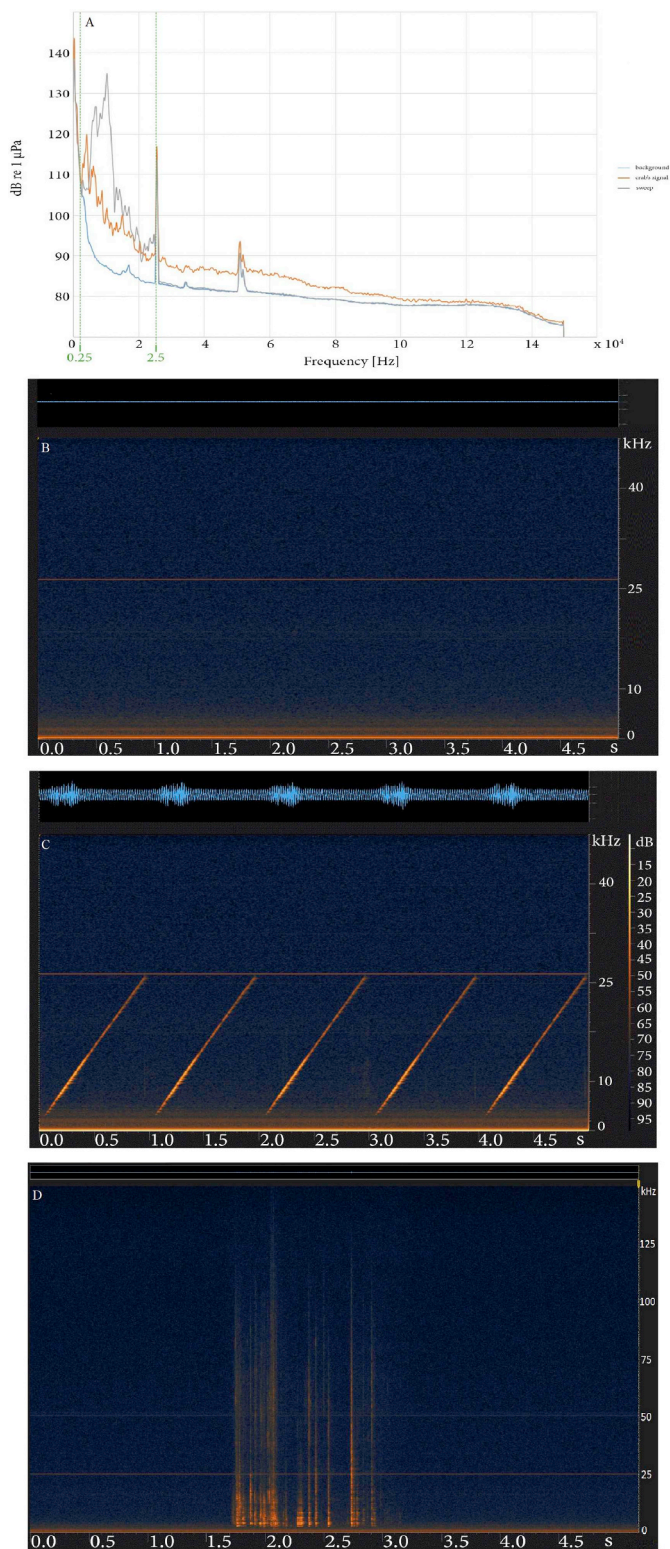


Fig. 1. (A) Power spectrum of 10 sweep tones (2.5–25 kHz) compared with that of *N. granulata* multi-rasp signals and the background noise of the experimental tank. Green values report the frequency bands of the sweep tones. (B) The oscillogram and spectrogram of background noise from the experimental tank. (C) The oscillogram and spectrogram of the sweep tone generated during the experimental phases. (D) The oscillogram and spectrogram of one multi-rasp acoustic emission by a *N. granulata* male. The oscillogram and spectrogram images were created using RX (iZotope, Massachusetts) software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Description of *N. granulata* behaviours measured during the different trials. Each description of the behaviours is supported by the corresponding figure showing the analysis process with dedicated software. In particular, for the acoustic emissions, the spectrogram created by Avisoft-SASLab Pro software is shown, while for the locomotor events recorded in each specimen (distance moved, mobility, movement and velocity), the continuous data acquisition analysed by EthoVision XT 9.0 software are shown.

Behaviours (unit)	Behavioural description	Graphical representation of the behaviour
Acoustic signal (number of emissions)	Number of wide-band frequency signals emitted by specimens of <i>Neohelice granulata</i>	
Distance moved (cm)	Distance travelled by the subject during the experimental trials (cumulative values)	
Mobility (s)	Duration of animal movements (cumulative values), even if the centre point (colour point in the geometric centre of body animal) remains the same	
Movement (cm/s)	Movements of animal that consider its change of position inside the tank. The state becomes “in movement” if the subject running mean velocity exceeds the start velocity of 1.25 cm/s, while becomes “not in movement” if the subject’s average running velocity drops below 1.00 cm/s	
Velocity (cm/s)	Mean distance moved by the subject per unit time	

2.5.2. Total and differential haemocyte counts

The total haemocyte count (THC; number of haemocytes per mm³) was determined using a Neubauer haemocytometer chamber. Haemocytes were classified according to Lanz et al. (1993) using the presence or absence of cytoplasmic granules as simple criteria.

2.5.3. Glucose, lactate and protein concentration (PC) assessments

The glucose and lactate plasma levels were determined using the Accutrend Plus instrument (Roche) according to the manufacturer's instructions. A measurement by photometric reflection was performed using the Accutrend test strips developed specifically for each of these parameters with a single drop of blood for each analysis.

Total plasma protein concentration (PC) was determined using a Quibit® 2.0 Fluorometer (Invitrogen). Obtained data were calibrated against standards.

2.6. Statistical analysis

Data were tested for goodness-of-fit to a normal distribution using a chi-square test. Different distributions in behavioural data were observed; thus, nonparametric tests were applied to compare the different values among the trials.

To determine if the number of acoustic signals counted was different between [A] and [B] conditions, a general linear model (GLM) (McCulloch and Neuhaus, 2001) was performed using the Poisson distribution and log-link function.

A Mann-Whitney *U* test was employed to assess differences in behavioural and biochemical parameters between [A] and [B] conditions. The results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Acoustical and behavioural responses in single trials

The acoustic analysis did not show a significant difference in the number of crab signal emissions between [A] and [B] conditions ($\chi^2_{(3)} = 1.335$; $p > 0.05$) (Fig. 2A).

The behavioural analysis showed that the crabs under [B] conditions had clear behavioural responses to acoustic stimuli exposure, showing significant differences with respect to the crabs under [A] conditions. Specifically, unreceptive females exposed to sounds had significantly lower distance moved (Mann-Whitney *U* test; $p < 0.05$), mobility (Mann-Whitney *U* test; $p < 0.001$), movement (Mann-Whitney *U* test; $p < 0.001$) and velocity (Mann-Whitney *U* test; $p < 0.001$); otherwise, the receptive females and males exposed to sounds had significantly higher values of distance moved (Mann-Whitney *U* test; $p < 0.001$) and velocity (Mann-Whitney *U* test; $p < 0.001$) but lower values of mobility (Mann-Whitney *U* test; $p < 0.001$) and movement (Mann-Whitney *U* test; $p < 0.001$) (Fig. 3).

3.2. Acoustical and behavioural responses in group trials

The acoustic analysis showed a significant difference in the number of crab signal emissions between [A] and [B] conditions. Specifically, under [B] conditions the crabs emitted a significantly lower number of acoustic events compared to [A] conditions ($\chi^2_{(3)} = 185.185$; $p < 0.001$) (Fig. 2B).

The behavioural analysis showed significant behavioural responses between the animals under [B] and [A] conditions. Specifically, in all the trials, crabs exposed to sounds had significantly higher values of distance moved (Mann-Whitney *U* test; $p < 0.001$), movement (Mann-Whitney *U* test; $p < 0.001$) and velocity (Mann-Whitney *U* test; $p < 0.001$) but lower values of mobility (Mann-Whitney *U* test; $p < 0.001$) (Fig. 4).

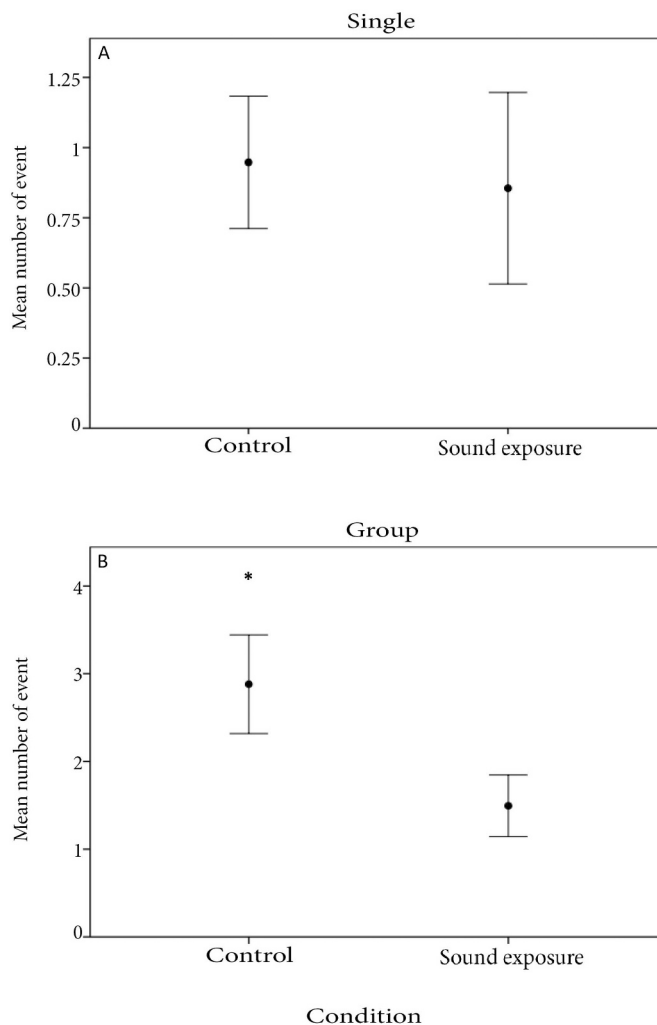


Fig. 2. Mean (\pm SD) number of acoustic emissions of *N. granulata* in the control [A] and sweep-tone exposure [B] conditions. (A) Single trials; (B) group trials. The asterisk (*) indicates significant differences among the conditions ($p < 0.05$).

3.3. Determination of the plasma parameters

The Mann-Whitney *U* test highlighted significant differences in the values of the plasma parameters in the crabs exposed to [B] conditions. In particular, both in the single and group trials, the animals under [B] conditions showed significantly lower levels of THC (Mann-Whitney *U* test; $p < 0.001$), while significantly higher levels of glucose (Mann-Whitney *U* test; $p < 0.001$), lactate (Mann-Whitney *U* test; $p < 0.001$) and PC (Mann-Whitney *U* test; $p < 0.001$) with respect to those of the animals under [A] conditions (Fig. 5).

4. Discussion

This study aimed to detect differences in locomotor/acoustic behaviours and the haemolymphatic parameters of *N. granulata* specimens kept in a tank. The sound dynamics and associated particle displacement in tank were subjected to the reflectiveness constraints, and the abiotic and biotic pressures have less impact compared to a natural environment. Taking into account that the study was conducted in a closed and controlled environmental context, the perturbations due to the sweep tone [B] have to be considered in a strict relationship and comparative prospective with the control conditions [A] and not in terms of responses observable in nature.

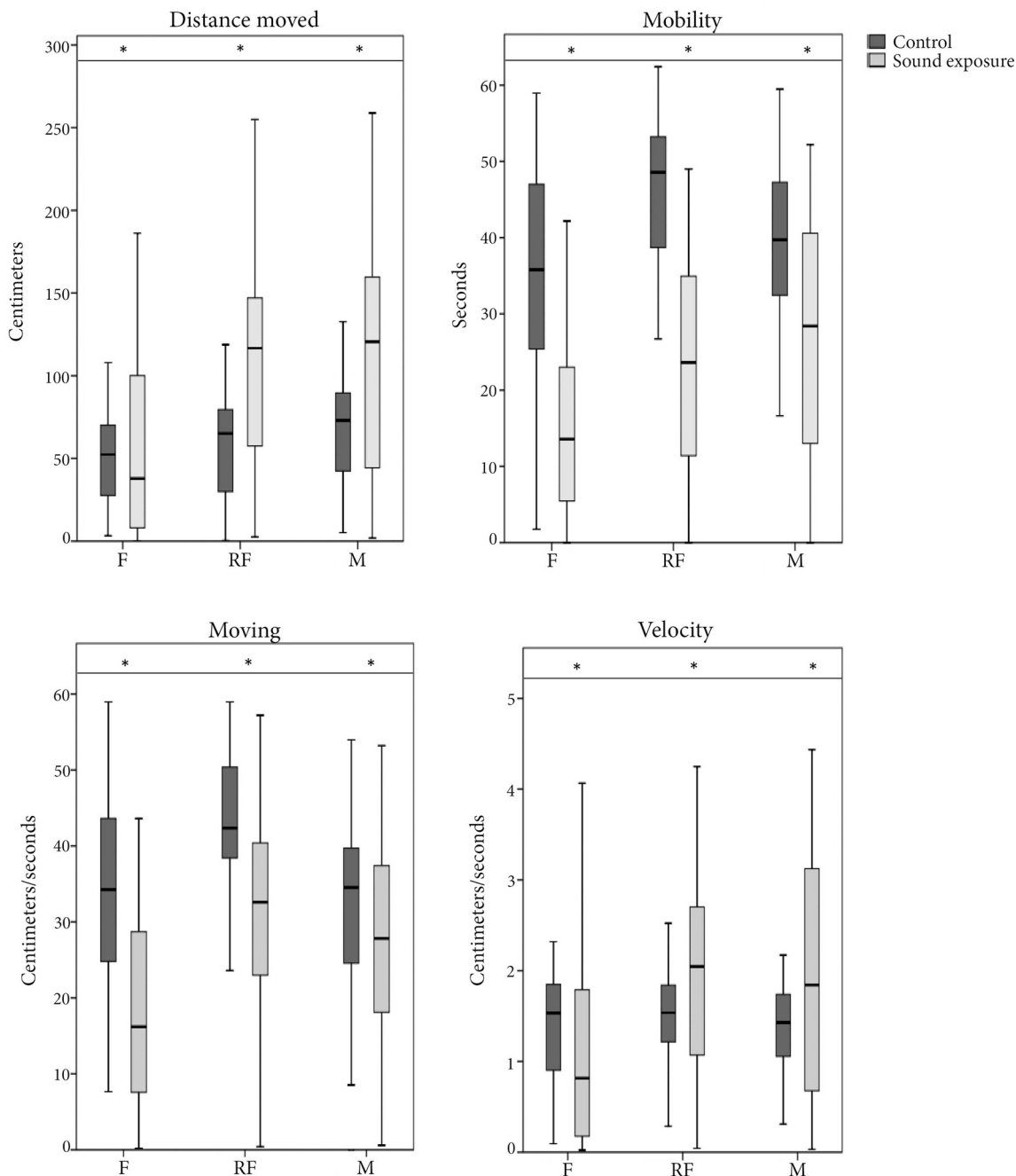


Fig. 3. Behavioural changes in crabs. Values (median ± 25th to 75th percentiles; error bars: 95% CI) of the behavioural parameters in the single trials during silence and sound exposure. M: male; F: unreceptive female; RF: receptive female. The asterisk (*) indicates significant differences among the conditions ($p < 0.05$).

The results showed that the sweep tones generated with a bandwidth between 2.5 and 25 kHz clearly affect the behavioural and biochemical parameters of *N. granulata* specimens kept in a controlled environment. These artificial signals were generated considering bandwidths encompassing the main frequencies produced by anthropic activities such as shipping-related continuous noise or high-intensity impulsive noise (e.g., pile driving, seismic testing, and active sonar application) and considering the physics of underwater acoustic propagation. In fact, despite the bandwidth of these anthropogenic noises can include also a frequency component lower than 2.5 kHz, the generation of signals below 2 kHz in a tank 1.5 m in diameter would be unreliable, as shown by Filiciotto et al. (2016) who studied the effects of vessel noise on *Palaemon serratus* specimens kept in a tank. The

behaviour of sound (e.g., propagation, decay and reflectiveness) in controlled and open environments is obviously different, as well as the way it affects animal perception. Despite this, Filiciotto et al. (2016) recognized similar signal structures and trends when comparing the SPL and particle velocity level (dB re nb/s) (see Nedelec et al., 2016 for a detailed description of the phenomenon). Considering that the tank utilized in the present study was similar to that used by Filiciotto et al. (2016), we can say that the particle velocity levels were related to those expressed by the sound pressure levels, although, there are still uncertain which component of sound propagation (e.g. particle motion, substrate vibration, pressure) crustacean are able to detect (Breithaupt and Tautz, 1990; Cohen and Dijkgraaf, 1961; Popper et al., 2001).

In the present study, the behavioural and biochemical responses

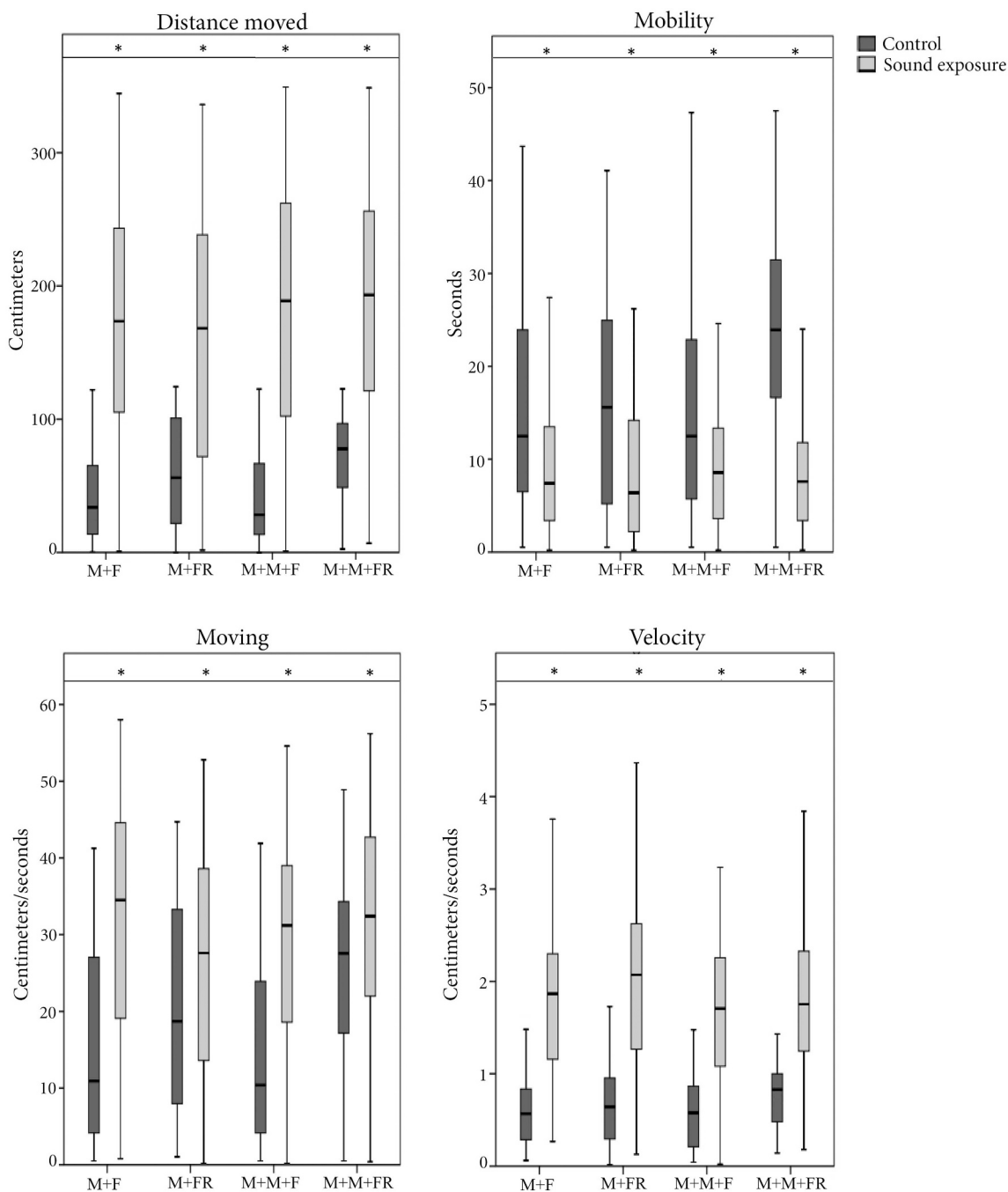


Fig. 4. Behavioural changes in crabs. Values (median ± 25th to 75th percentiles; error bars: 95% CI) of the behavioural parameters in the group trials during silence and sound exposure. M + F: group with 1 male and 1 unreceptive female; M + RF: group with 1 male and 1 receptive female; M + M + F: group with 2 males and 1 unreceptive female; M + M + RF: group with 2 males and 1 receptive female. The asterisk (*) indicates significant differences among the conditions ($p < 0.05$).

obtained in the tank indicate that *N. granulata* is able to perceive part of the particle motion of acoustic stimuli (in term of substrate- and water-born propagation) projected within the wider bandwidth of their underwater rasps characterized by signals with energy mostly concentrated in the 2.5–85 kHz range (Filiciotto et al., 2018). This result partially agrees with Horch (1971), who showed the hearing curves of two *Ocyropsis* species and reported sensitivities from approximately 800 Hz to 3000 Hz with a maximum sensitivity in the 1–2 kHz range (Horch, 1971).

Several authors showed that anthropogenic noise can influence marine organisms in several ways, including spectral overlapping (Weilgart, 2007). In this context, increasing levels of noise pollution may result in negative impacts on species depending on acoustic

communication (Caorsi et al., 2017). In this study a scanty number of acoustic signals were observed, this condition may be related to a peculiar characteristic of the specie or to tendency to increase sound emission rate at night as reported for other crustacean species (Buscaino et al., 2015, 2012). Regarding this last aspect, in animals that are active nocturnally, such as *Ovalipes* spp. (Takahashi and Kawaguchi, 2001), visual signals are of limited value, and thus vocal and chemical cues could be used in mate attraction.

However, the results of crab acoustic emissions showed a significant decrease in the biological signals of grouped animals exposed to sweep noise. Several authors have proposed that the acoustic emissions of different crustacean species can transmit information to conspecifics regarding: warning of predators (de Vincenzi et al., 2015), sexual

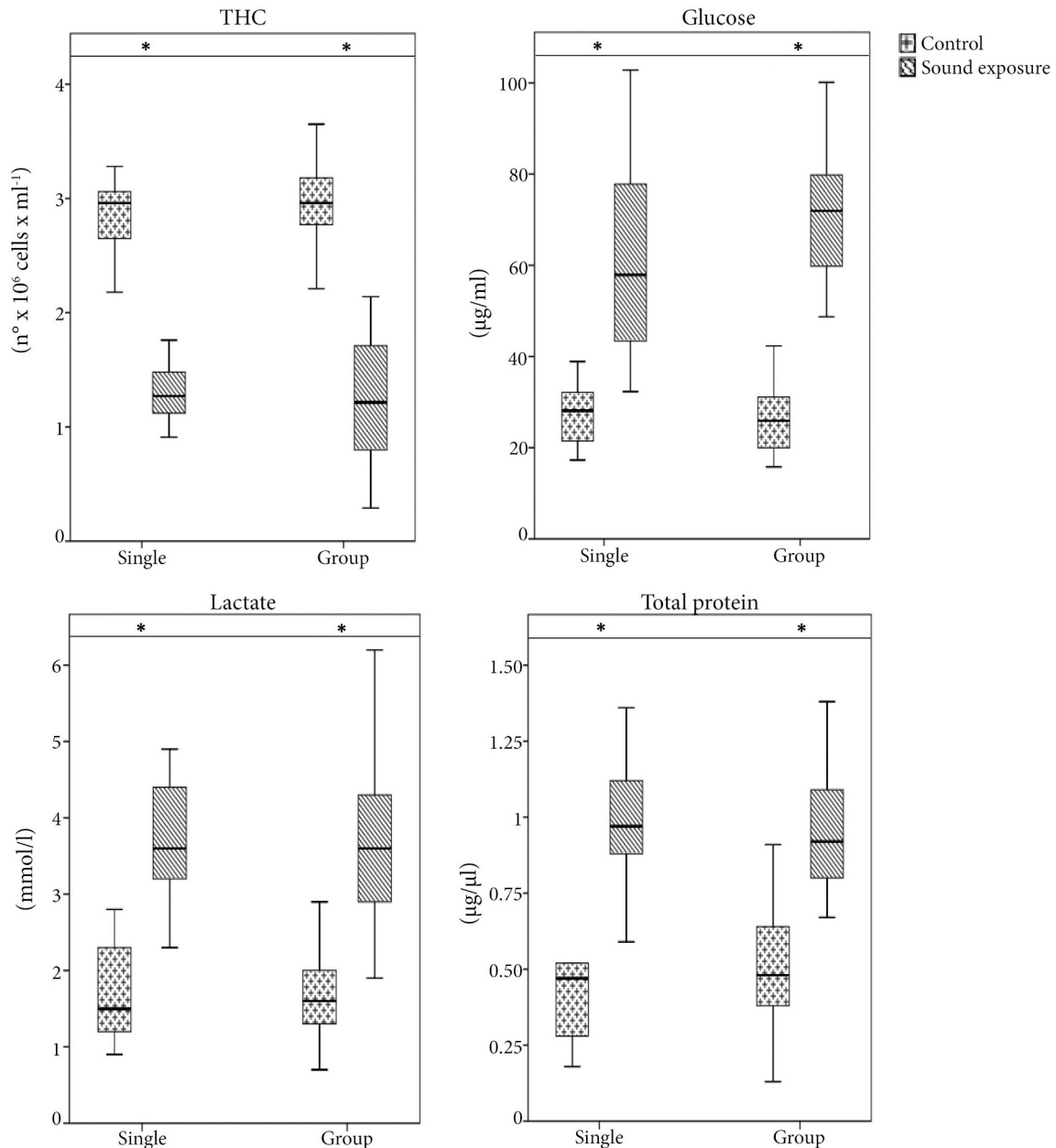


Fig. 5. Total haemocyte count (THC), glucose, lactate and total protein content (PC) of *N. granulata* under [A] and [B] conditions. Values (median \pm 25th to 75th percentiles; error bars: 95% CI) were measured in the haemolymph from the single and group tests. The asterisk (*) indicates significant differences between the conditions ($p < 0.05$).

interactions (Buscaino et al., 2015; Von Hagen, 1984) and anti-predator strategies (Bouwma and Herrnkind, 2009). Filiciotto et al. (2018) studied the role of acoustic emissions from *N. granulata* specimens in sexual behaviour. The authors characterized two distinct signals in terms of their spectral shape (the single rasp and the multi-pulse rasp) showing that the use of multi-pulse rasp (occupying a bandwidth in the range 2.5–85 kHz) can be tied to coupling formation. In view of these considerations, one can hypothesize that sweep tones occupied a significant bandwidth within the frequency range utilized by the crabs for intraspecific communication. This spectral overlapping can compromise the reliability of the acoustic messages conveyed (Samarra et al., 2009), limiting the use of acoustic communication and potentially adversely impacting reproductive success (McGregor et al., 2013). Another interesting result, coming from the visual comparison of the mean number of acoustic events between single and group trials in control condition (Fig. 2), concerns the higher number of emitted signals by

crabs in group. This difference could be related to the behaviour of some crustacean species such as thus belonging to Ocypodidae and Polybiidae families that, when stimulated by sounds of other crabs or by the nearby movements of neighbouring specimens, can more than double the rates of sound production, as reported by other Authors (Buscaino et al., 2015; Popper et al., 2001).

From a behavioural point of view, several studies showed that acoustic noise can influence animal behaviour (Celi et al., 2016; Filiciotto et al., 2013; Sabet et al., 2016; Simpson et al., 2016, 2015; Voellmy et al., 2014). Although no studies have been carried out to assess the influence of noise on *N. granulata*, the changes in behavioural parameters observed here may indicate a behavioural disturbance as a result of increasing ambient noise in the tank. In fact, the locomotor behaviours in single and group trials showed significant changes between the two experimental conditions. Animals react to cues from predators, and avoiding predators can obviously prevent harmful

encounters. This reaction is actuated by detecting the surrounding environment, using visual, chemical and acoustical cues (Basil and Sandeman, 2000; Díaz and Thiel, 2004; Montgomery et al., 2006), and the response to new stimuli causes a need to investigate or a readiness to escape, described as an orientating response (Barrows, 2011). Here, the crabs, when exposed to sweep noise, showed higher values for all locomotor behaviours both in single (except for distance moved and velocity in the unreceptive female; see the part discussed below) and group trials except for lower values of movement in the single trials. Crustaceans are mobile species, and their movements are highly related to ecological and biological aspects. From a functional perspective, moving further distances at faster velocities enables the crab to escape from unfavourable conditions. For these reasons, the results of this study can be linked to the hypothesis of the behaviour responses of crabs in a realistic scenario where they potentially adopt an orientating response with the aim to investigate an optimal refuge to cope with adverse situations. From this perspective, an important role could be probably played by the low values of attenuation and high reflectiveness of the signals in the tank. In this sense, the displayed locomotor behaviours may be related to the fact that the specimens prolonged the time spent investigating a less impacted refuge to cope with the severe noise conditions as they would have done in their natural environment.

Regarding the significant decrease in the mobility behaviour in both single and group specimens, it is necessary to highlight that the mobility state parameter consists of changes in the animal body mass, including rotating around its axis and not necessarily changing position. Stressful events can lead to changes in the intensity, duration or frequency of particular activities as part of an allostatic response (Broom and Johnson, 1993; McEwen and Wingfield, 2003; Wingfield, 2005), and this response includes a reduction or cessation of normal activity (Metcalf et al., 1987). From this viewpoint, the low mobility values may indicate a disturbance response due to the increasing ambient noise, validating the results reported by other authors (Filiciotto et al., 2016; Wale et al., 2013).

Another result concerns the manner in which the noise condition affected the locomotor behaviour responses of unreceptive female with respect to the receptive ones and the males. Some species of crabs, such as Ocypodid, exhibit a mixture of males and receptive females mate searching (Crane, 2015), and different factors, such as ecological, physiological and sexual conditions may determine the effort involved in this behaviour (de Rivera et al., 2003). Otherwise, the active search for partner occurs through visual, chemical, tactile and acoustical cues, which may act synergistically (Sal Moyano et al., 2014; Sal Moyano and Gavio, 2012). In this study, the receptive females and the males, when exposed to the noise condition, showed a significant increase in distance moved and velocity behavioural parameters, differently to what occurred in the unreceptive females. Given the consideration stated above, it is possible hypothesize that failing the suitable characteristics for the transmission of reliable acoustic information, considering the unfavourable condition due to the noise, these specimens have shown an increased predisposition to the locomotor behaviour to maximize the effort for mate searching.

In addition to behavioural responses due to a potential threatening stimulus, there are many studies in the literature that examine the effects of noise on biochemical aspects (Filiciotto et al., 2014; Kight and Swaddle, 2011; Santulli et al., 1999; Vazzana et al., 2016). The combination of behavioural and physiological assessments could have significant relevance in crustaceans, where the behavioural and biochemical patterns in response to stress conditions are not yet well known (Celi et al., 2013). Solan et al. (2016) studied the effects of anthropogenic noise on several invertebrate specimens and showed significant changes in behaviour and glycolytic activity due to variations in tissue glucose and lactate concentrations. These authors concluded that anthropogenic noise has the potential to alter the way in which species interact with their environment, compromising the regulation of important ecosystem properties. Our results highlighted the

effects of sweep-tone noise on glucose and lactate mobilization in *N. granulata* specimens, showing a significant increase in the plasma in both single and group trials. Marine invertebrates are important members of the marine habitat, food web, and ecosystem. Noise can have indirect impacts on crabs, such as increased vulnerability to diseases and predation due to, for example, immune system depletion (Moriyasu et al., 2004). In the current study, the assessed THC values seemed to be modulated as a result of noise exposure. In crustaceans, the haemolymph reflects the physiological status as well as environmental fluctuation through the regulation of its components. The effects of exposure on the cellular component of the haemolymph, which is responsible for mediating immune function, was quantified through the THC. The acoustic stimulus in the single and group trials induced a significant reduction in the THC, suggesting the possibility of immune depletion as well as an increased risk of infection (Celi et al., 2013) when subjects are exposed within reflective tank constraints.

Finally, the changes in the behavioural and biochemical parameters observed in crabs can be interconnected as a result of a higher metabolism rate in both single and group trials with sweep tones. Lactate is the major end product of anaerobic metabolism in crustaceans and is also responsible for enhancing haemocyanin O₂-binding affinity during hypoxia (McMahon, 2001; Morris and Callaghan, 1998; Qiu et al., 2011), and its accumulation is related to the duration and severity of hypoxia (Albert and Ellington, 1985). Hyperglycaemia is commonly recognized as a stress response to hypoxia in decapod crustaceans (crab: Zou et al., 1996; shrimp: Racotta et al., 2002; lobster: Patiño and Ramí, 2003; and crayfish: da Silva-Castiglioni et al., 2010). During hypoxia, glycogenolysis enhances haemolymph glucose concentrations in order to provide increased substrate levels for anaerobic glycolysis (Marqueze et al., 2011; Oliveira et al., 2001). Behaviourally, to maximize oxygen availability, crabs often move to the air-water interface to take advantage of higher oxygen concentrations and may leave the water completely. Consequently, haemolymph lactate, glucose, and protein concentrations in *N. granulata* have been shown to fluctuate, possibly in response to lower oxygenation due to acoustic stress and increased locomotor activity, as detailed in other studies (Bonvillain et al., 2012; da Silva-Castiglioni et al., 2010; Fujimori and Abe, 2002; Morris and Callaghan, 1998).

5. Conclusion

In conclusion and in view of all the behavioural and biochemical changes due to sweep-tone perturbation, we propose that noise could develop as a real source of stress for the semi-terrestrial crab *N. granulata*, adversely affecting biological parameters and, consecutively, ecological interactions in the crab life cycle. However, to increase the available information about the effects of noise on behavioural and physiological responses, further studies should also be performed in an open natural environment where the acoustical field is not influenced by walls, as it is in small tanks. This study is particularly valuable considering that anthropogenic noise is recognized as a major 21st century pollutant, appearing in both the United Nations Convention on the Law of the Sea (UNCLOS) and European legislation such as the Marine Strategy Framework Directive 56/2008 CE. Nevertheless, the effects of noise on marine organisms, especially on invertebrates, and indirect repercussions at the ecosystem level are still largely unknown.

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