



TECHNICAL REPORT

Experimental procedure for the evaluation of behaviour and biochemical stress of *Palinurus elephas* exposed to boat noise pollution

Authors

Vincenzo Di Stefano, Vincenzo Maccarrone, Giuseppa Buscaino, Salvatore Mazzola, Francesco Filiciotto

IAMC-CNR Capo Granitola

March 2016

Many of the designations used by the manufacturers and sellers to promote their products are claimed as trademarks. Where those designations appear in the Report and CNR was aware of a trademark claim the designations have been printed in all caps. In addition, we have reported some of them in the Production.

The data presented hereafter is the property of CNR. Unauthorized use of the data would be considered unfair.

Many of the systems and programs used to generate data are 'free' because either they are either public domain or the licences are roughly equivalent to the GNU Public License. Some programs are either commercial or have more restrictive licenses and may require payment. Where known, programs and systems that are not 'free' are acknowledged.

Contents

1. INTRODUCTION AND BACKGROUND	4
1.1 Objectives.....	4
2. EXPERIMENTAL PROTOCOL	6
3. ACOUSTIC ACQUISITION AND PROJECTION SET-UP	7
4. VIDEO MONITORING AND ANALYSIS	9
5. BIOCHEMICAL STRESS ANALYSIS	12
6. REFERENCES	14

1. INTRODUCTION AND BACKGROUND

Over the past 50 years, anthropogenic activities, in particular shipping traffic, have produced increasing background sea noise pollution (Ross, 2005; Hildebrand, 2009). Although, many studies have evaluated the effects of anthropogenic acoustic disturbance on marine organisms (Santulli et al., 1999; Scholik and Hong Yan, 2001; Sarà et al., 2007). few studies focused the impact of noise on aquatic crustaceans (Christian et al., 2003; Andriguetto-Filho et al., 2005; Celi et al., 2013; 2015; Wale et al., 2013).

Behavioural observations, in combination with physiological assessment, may provide a more complete understanding of the homeostatic perturbations of an organism due to external or internal stress stimulus. For example, quantifiable behavioural changes in an organism that are associated with stress and toxicant exposure provide novel information that cannot be gained from traditional toxicological methods, including short-term and sub-lethal exposure effects and the potential for mortality (Bridges, 1997; Henry and Atchinson, 1986; Saglio and Trijasse, 1998).

A combination of behavioural and physiological approaches may have significant relevance in crustaceans, for which the behavioural patterns in response to stress conditions are not yet well known. Payne et al. (2007) found that lobsters exposed to very high as well as low sound levels experienced no effect on delayed mortality or damage to the mechanosensory system associated with animal equilibrium and posture. Celi et al. (2013) observed that red swamp crayfish showed altered aggressive behavioural patterns and changes in the components of the haemato-immunological system, such as serum glucose concentration; protein concentration; agglutinating activity; and THC, DHC and Hsp70 expression, when exposed to an acoustic stimulus in a frequency band of 0.1-25 kHz, clearly reflecting a stress condition.

The European spiny lobster *Palinurus elephas* (Fabricius, 1787) is a common crustacean species along the Mediterranean and northeastern Atlantic coasts (Hunter 1999). This species, which is primarily active at night for feeding and reproduction (Goni and Latrouite, 2005), represents one of the major targets of Mediterranean artisanal fisheries, and while catches are now reduced and sporadic, this fishery has a long history (Goni and Latrouite 2005; Groeneveld et al., 2006).

1.1 Objectives

The present technical report describe the experimental procedure adopted to investigate the behaviour (locomotor states and acoustic emissions) and biochemical (haemolymphatic parameters) responses of the European spiny lobster (*Palinurus elephas*) after the exposure to acoustic pollution

consisting of boats noises. The locomotor analysis automatically estimated the movement/position events of lobsters, offering a novel, high-throughput method of measuring the relationship of the lobster compared to traditional manual analyses. The biochemical effect on the lobsters was evaluated by estimating the serum glucose concentration; total protein concentration; and THC, DHC and Hsp70 expression as stress indexes.

2. EXPERIMENTAL PROTOCOL

2.1. Animal housing and experimental design

The present study was carried out at the Institute for the Marine and Coastal Environment of the National Research Council (CNR-IAMC) of Capo Granitola (SW Sicily, Italy).

After capture in the wild, 80 specimens of European spiny lobster (*Palinurus elephas*) were transferred to two indoor circular PVC tanks (2.35 m diameter and 1.5 m depth) for a month-long acclimation period and fed with frozen molluscs, shrimps and fish ad libitum. After the acclimation period, 36 lobsters (18 males and 18 females) of 301.96 ± 69.8 g in weight and 7.73 ± 0.69 cm in carapace length (mean \pm SD), individually or in groups of 4 individuals, were randomly collected from the holding tanks and released into the centre of an experimental tank. For the experimental procedure, we used only lobsters that had not recently molted. The lobsters were deprived of food for 5 days before the start of the experimental trials. All animals were kept under natural photoperiods.

The experimental treatments consisted of 30 min of sound exposure for only the animals treated with the boat noise condition. The sound exposure consisted of the projection of a random sequence of noises from the following motorboats: seven recreational boats, a hydrofoil, a fishing boat and a ferry boat. In total, 18 experimental trials were performed: six trials with single animal for each condition (the trials had males and females in equal ratio), and three trials with lobsters in groups of 4 animals for each condition (for each group, 2 males and 2 females were used), following the scheme reported in Figure 1:

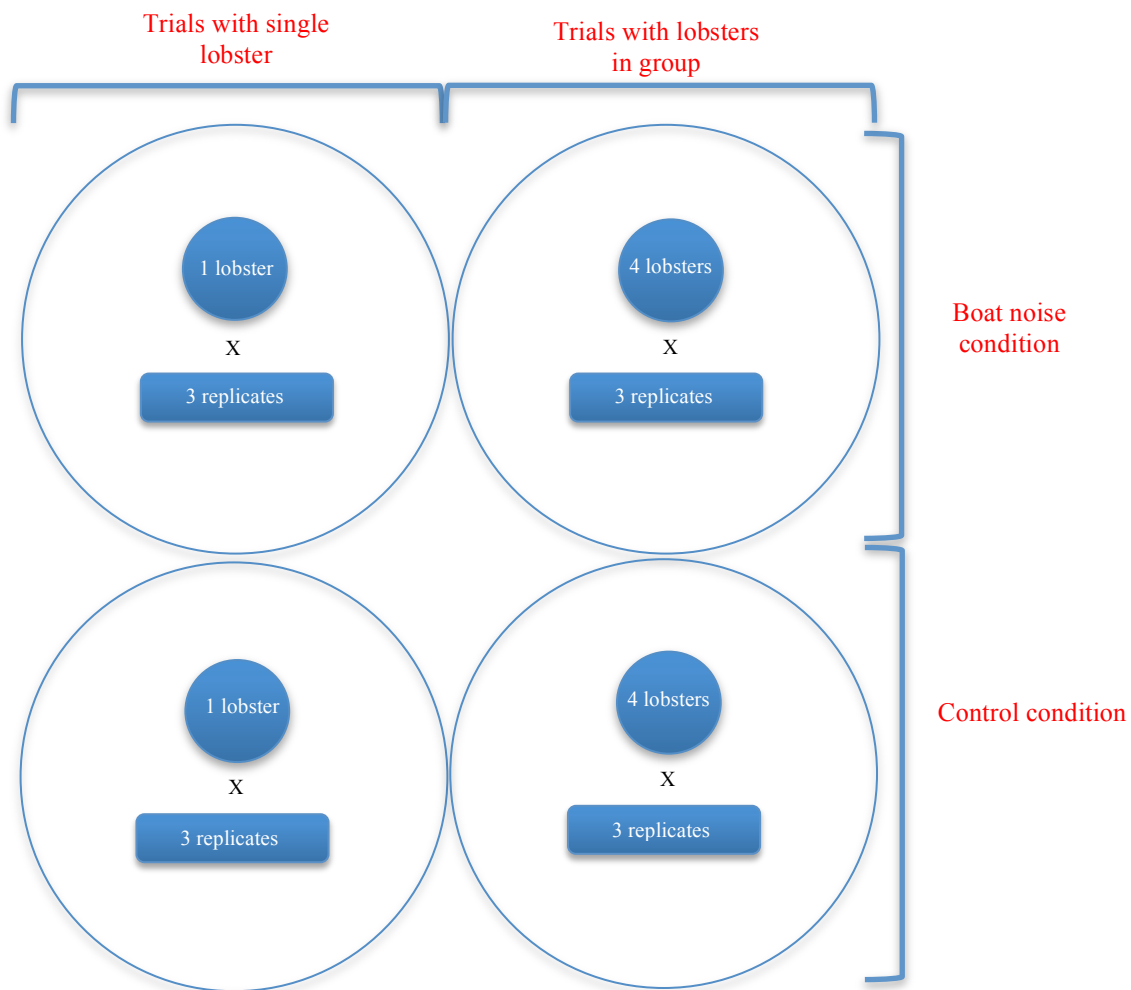


Figure 1. Schematic representation of the experimental protocol.

At the end of the experimental phase, both the control and exposed to boat noise animals were captured with a net and placed on crushed ice for 15 min to induce torpor or ‘cold anaesthesia’ to allow sampling of the haemolymph. The samples were collected from the experimental animals, and the lobsters then were transferred into a post-experimental tank and released after recovery. This experimental procedure was repeated for each specimen in both single and group conditions. The protocols for animal husbandry and experimentation were reviewed and approved in accordance with recommended standards (NRC 1996) and EEC Directive 86/609.

3. ACOUSTIC ACQUISITION AND PROJECTION SET-UP

To obtain acoustic recordings of the noises from boats and in the experimental tank, we used a calibrated hydrophone (model 8104, Bruel & Kjer) with a sensitivity of $-205.6 \text{ dB re } 1 \text{ V}/\mu\text{Pa} \pm 4.0 \text{ dB}$ in the 0.1-Hz to 80-kHz frequency band. 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). The signals were acquired at 300 kilosamples s^{-1} at 16 bits and were analysed by the Avisoft-SASLab Pro software (Avisoft Bioacoustics).

The marine traffic noise condition was obtained recording in an area near a harbour, characterised by the alternating passages of different types of boats. Moreover, the noise in the experimental tank was recorded to characterise the baseline noise of the study environment.

To project the acoustic stimulus of different boats inside the experimental tank, a playlist with the acquired wave file was created. The playlist was projected using the “loop mode” function of the Avisoft-SASLab recorder software (Avisoft Bioacoustics) through the stereo output of the PC connected to a Power Amplifier (type 2713, Bruel & Kjer - Naerum, Denmark). The amplifier was, in turn, connected to the underwater loudspeaker (Model UW30, Lubell, Columbus, Ohio, USA).

The baseline noise of the tank and the acoustic stimulus projected inside the tank (see Figure 2) were recorded using the same acoustic equipment and set-up employed for the acquisition phase. The hydrophone was placed at a 0.5 m depth in the centre of the tank.

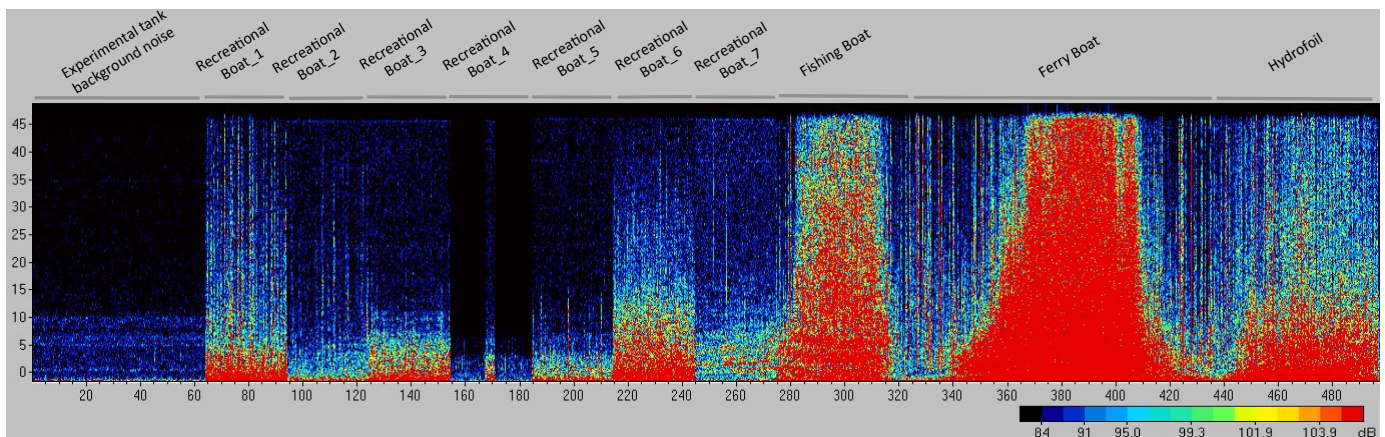


Figure 2. Spectrogram of the experimental tank background noise and of different boat noise stimuli: frequency (kHz) versus time (s). The intensity is reflected by the colour scale (dB re 1 μ Pa, root mean square, 1024-sample FlatDown window, sampling frequency 96 kHz).

4. VIDEO MONITORING AND ANALYSIS

This video system was used to monitor the lobsters' behaviour and was synchronised with the system used to record the acoustic signals.

Videos for the behavioural monitoring were recorded with an analogical camera (model 830, Skynet Italia s.r.l.) placed on the top of the experimental tank. The camera was linked to a PC and the file were managed by Nero Vision 12.0 (Nero Development & Services GmbH, Germany).

Recorded videos were also analysed with EthoVision XT 9.0 software (Noldus Information Technology, Wageningen, Netherlands). The experimental arena was calibrated across the bottom wall of the tanks, and the calibration axes were placed to designate the origin (0,0) at the tank center as showed in Figure 3.

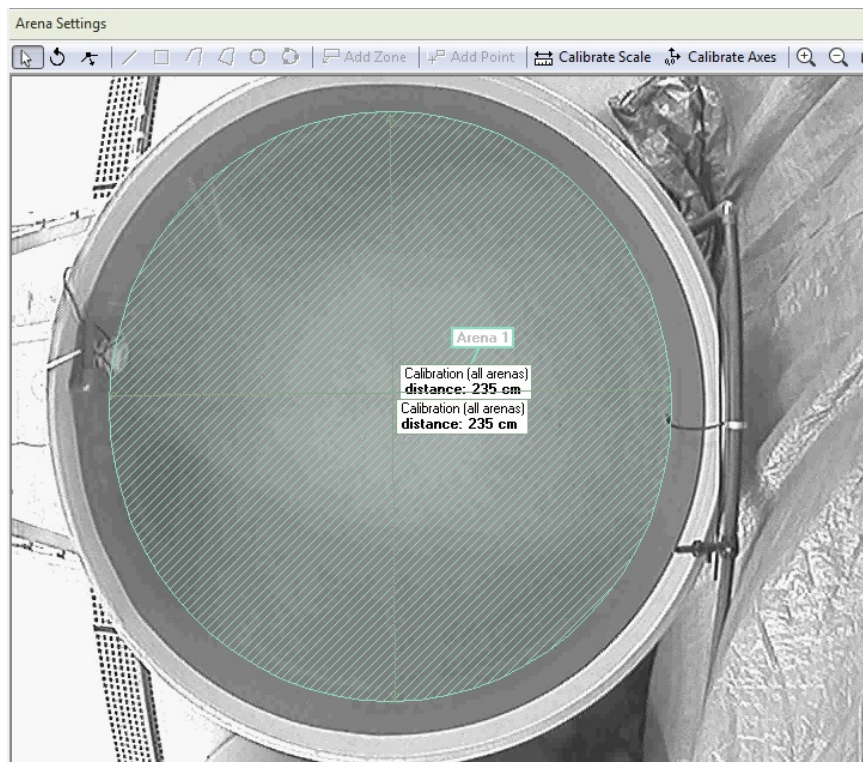


Figure 3. Arena calibration phase with EthoVision XT 9.0 software.

The trials with animal in group were analysed adopting the SIM (Noldus, 2011) is an add-on to the EthoVision XT program that enables detecting multiple unmarked animals in a social context, capable of assessing lobsters shoaling behaviour by simultaneously tracking all specimens and recording dynamic changes in social behaviour between the subjects. Since marking lobsters with unique colors is methodologically difficult, center-point detection of unmarked animals (using the

same algorithms as the marker-assisted tracking) was chosen as the default setting for the experimental group trials, followed by averaging data for each group (Figure 4). EthoVision XT software analyses each frame and distinguishes the object(s) from the background on the basis of their greyscale/brightness values, extracting the coordinates of the geometric center and surface area for each object per frame.



Figure 4. Example of the lobsters Identification phase using the center-point detection of unmarked animals method of EthoVision XT 9.0 software.

EthoVision acquired data at 25 frames per second, and the chosen variables were dependent to the different trials. In the trials with single animal were evaluated the following behaviours: Distance Moved (the distance travelled by the center point of the subject from the previous sample to the current on, expressed in centimetres), Mobility Continuous (the percentage changed pixels of the detected subject between current sample and previous sample), Mobility State (immobile/mobile/high mobile, calculates the duration for which the complete area detected as animal is changing, even if the center point remains the same. The duration is calculate on the threshold of 10%), Velocity (distance moved by the center point of the subject per unit time, centimetres/seconds), Movement (Moving/Not Moving State; the state is moving if the subject running average velocity exceeds the start velocity of 1.80 cm/s, the state then becomes Not Moving if the subject running average velocity reach the stop velocity set below of 1.50 cm/s); for

trials with animals in group the calculated variables were the above described for trials in single with the addition of Inter-lobster Distance (the distance between a body point of a subject and a body point of another subject. The distance is calculated for each subject, actor, relative to the other subjects, receivers), Proximity (with two possible states, in Proximity and Not in Proximity. The state is In Proximity when the distance between the selected body points of the focal subjects and the body points of another subject is lower than a threshold of 5 cm. The state is Not in Proximity when the distance is greater than the threshold).

Average inter-lobster distance was calculated by averaging inter-lobster distances between all members of the shoal. Inter-lobster distance was defined as the distance between two subjects as measured from the center point of each lobster. Proximity duration (s) was defined here as the average amount of time a subject spent close (within 0.5 cm) to another subject. An example of the real time analysis of lobster using EthoVision XT 9.0 software is showed in Figure 5.

In all experiments, the subject loss due to misdetection by video-tracking software was <2%. Behavioural data were exported to Excel to generate total and per-minute plots for each endpoint.

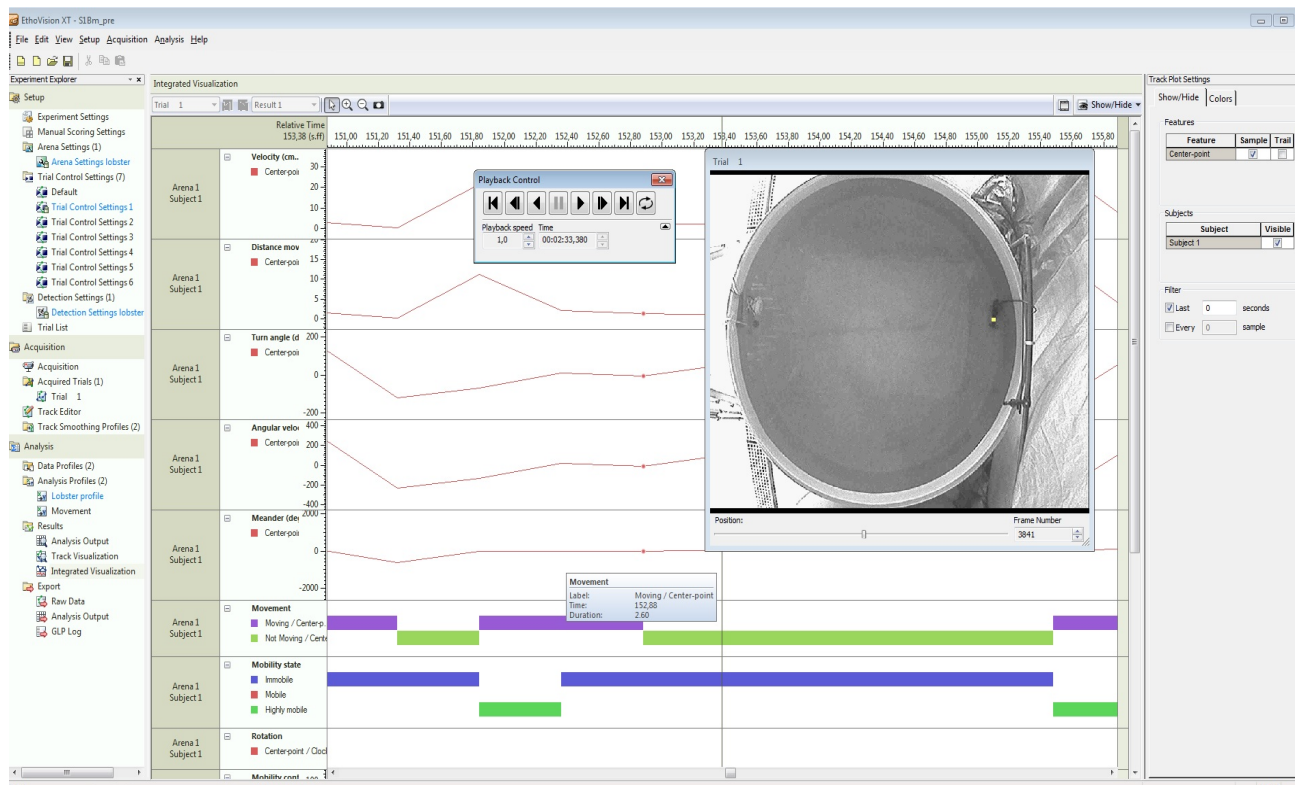


Figure 5. Real time analytical phase of the behaviours selected using EthoVision XT 9.0 software.

5. BIOCHEMICAL STRESS ANALYSIS

2.4. Animal haemolymph sampling

Lobster haemolymph was collected into a syringe containing an equal volume of anticoagulant (0.45 M NaCl, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA) and immediately placed on ice to avoid clotting. Freshly collected haemocytes were examined by light microscopy to perform THC and DHC measurements.

After the cell counts, the samples were centrifuged at 800 g for 10 min at 4°C to obtain the plasma fraction and haemocyte pellets, which were quickly frozen at –20°C and stored for biochemical analysis.

2.4.1. Characterisation of *Palinurus elephas* haemocytes

The total haemocyte count (THC) was performed to determine the total number of haemocytes per millilitre of lobster haemolymph using a Neubauer haemocytometer chamber. Haemocytes were classified according to Li and Shields (2007) using the presence or absence of cytoplasmic granules as simple criteria. To perform the differential haemocyte count (DHC; %), a small drop of haemolymph was smeared on a slide, fixed in 1% glutaraldehyde in sea water and stained with May–Grünwald–Giemsa, dehydrated with absolute ethanol and xylene and then mounted in Eukitt medium (Fluka). Cells were counted in random areas on each slide, and the relative proportions of various classes were computed (Mahmood and Yousaf, 1985). A total of 200 cells were counted on each slide.

2.4.2. Glucose level in the haemolymph

Haemolymph glucose was determined by using a glucose assay kit (Sigma, St. Louis, MO). Before measuring glucose, the haemolymph was mixed with 300 µl of 95% ethanol and centrifuged at 12,000 g for 10 min at 4°C. A free sample of 100 µl of protein was used following the kit's protocol.

2.4.3. Total protein analysis of haemolymph

The total protein concentration (PC) of the spiny lobster haemolymph was estimated using a Quibit® 2.0 Fluorometer (Invitrogen). The data were quantified with standards.

2.4.4. SDS-PAGE and Western Blot

Haemocytes pellets were crushed on ice for 1 h in 1 ml and lysed in 500 µl of RIPA buffer, pH 7.5, supplemented with a cocktail of protease inhibitors using a glass daunce homogeniser. Lysates were centrifuged at 15000 g for 30 min at 4°C. The supernatants were collected and dialysed against 50 mM Tris-HCl (pH 7.5), and the protein content was estimated with the Qubit® 2.0 Fluorometer (Invitrogen).

The equivalent of 25 µg of serum protein and 25 µg of total tissues lysates were separated on 7.5% SDS-PAGE under reducing conditions (Laemmli et al., 1970). SDS-polyacrylamide minigels were transferred to nitrocellulose membranes using a semidry transfer apparatus (BioRad) and blocked with 5% bovine serum albumin (BSA) in TBS-T (20 mM Trizma base, pH 7.5, 300 mM NaCl, 0.1% (v/v) Tween-20 with 0.02% sodium azide) for 1 h at room temperature (r.t.). The membranes were incubated over night at 4°C with a mouse monoclonal anti-heat shock protein 70 antibody (Sigma, 1:2500 dilution), washed with TBS-T (three times for 5 min each), and incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG (1:10000 for 1 h at r.t.). After washing with TBS-T (three times for 5 min each), the membranes were incubated with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (BCIP/NBT). Alpha Imager software was used for densitometric analysis of the immunoblotted bands. 36 lobsters (18 males and 18 females) were examined, and each test was repeated in triplicate.

6. REFERENCES

- Andriquetto-Filho, J.M., Ostrensky, A., Pie, M.R., Silva, U.A., Boeger, W.A., 2005. Evaluating the impact of seismic prospecting on artisanalshrimp fisheries. *Cont. Shelf Res.* 25, 1720–1727.
- Bridges, C.M., 1997 Tadpole swimming performance and activity affected by acute exposure to sublethal levels of carbaryl. *Environ. Toxicol. Chem.* 16, 1935-1939.
- Celi M., Filiciotto F., Parrinello D., Buscaino G., Damiano M.A., Cuttitta A., D'Angelo S., Mazzola S., Vazzana M. 2013. Physiological and agonistic behavioural response of *Procambarus clarkii* to an acoustic stimulus *J. Exp. Biol.* 216, 709-18.
- Celi M., Filiciotto F., Vazzana M., Arizza V., Maccarrone V., Ceraulo M., Mazzola S., Buscaino G. 2015. Shipping noise affecting immune responses of European spiny lobster *Palinurus elephas* (Fabricius, 1787). *Can J Zool.* 93, 113–121.
- Christian, J., 2003. White's Cove Quarry Blasting: Potential Impacts on American Lobster. LGL Limited. St. John's, NL.
- Goni, R., Latrouite, D., 2005. Review of the biology, ecology and fisheries of *Palinurus* spp. species of European waters: *Palinurus elephas* (Fabricius, 1787) and *Palinurus mauritanicus* (Gruvel, 1911). *Cah. Biol. Mar.* 46, 127-142.
- Groeneveld, J.C., Goñi, R., Latrouite, D., 2006. *Palinurus* species, In: Phillips, B.F. (Ed.), *Lobsters: biology, management, aquaculture and fisheries*. Chap 12. Blackwell Scientific Publications, Oxford.
- Henry, M.G., Atchinson G.J., 1986. Behavioral changes in social groups of bluegills exposed to copper. *T. Am. Fish. Soc.* 115, 590-595.
- Hildebrand J.A., 2009 Anthropogenic and natural sources of ambient noise in the ocean. *Mar. Ecol. Prog. Ser.* 395, 5-20.
- Hunter, E., 1999. Biology of the European spiny lobster *Palinurus elephas* (Fabricius, 1787) (Decapoda: Palinuridea). *Crustaceana* 72, 545-565.
- Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

- Li, C., Shields, J.D., 2007. Primary culture of hemocytes from the Caribbean spiny lobster, *Panulirus argus*, and their susceptibility to *Panulirus argus* Virus 1 (PaV1) J. Invertebr. Pathol. 94, 48–55.
- Mahmood, A., Yousaf, M., 1985. Effect of some insecticides on the haemocytes of *Gryllus bimaculatus* de Geer. Pak. J. Zool. 17, 71-84.
- Payne, J.F., Andrews, C.A., Fancey, L.L., Cook, A.L., Christian, J.R. 2007. Pilot study on the effect of seismic air gun noise on lobster (*Homarus americanus*). Can. Tech. Rep. Fish. Aquat. Sci. No. 171. St. John's, NL. 34 p.
- Ross, D., 2005. Ship sources of ambient noise. IEEE J. Ocean. Eng. 30, 257–261
- Saglio, P., Trijasse, S., 1998. Behavioral responses to atrazine and diuron in goldfish. Arch. Environ. Con. Tox. 35, 484-491.
- Santulli, A. Modica, A., Messina, C., Ceffa, L., Curatolo, A., Rivas, G., Fabi, G., D'Amelio, V., 1999. Biochemical responses of European sea bass (*Dicentrarchus labrax* L.) to the stress induced by offshore experimental seismic prospecting in the Mediterranean Sea. Mar. Pollut. Bull. 38, 1105–1114.
- Sarà, G., Dean, J.M., D'Amato, D., Buscaino, G., Oliveri, A., Genovese, S., Ferro, S., Buffa, G., Lo Martire, M., Mazzola, S., 2007. Effect of shipping traffic on behaviour of bluefin tuna *Thunnus thynnus*. Mar. Ecol. Prog. Ser. 331, 243–253.
- Scholik, A.R., Yan, H.Y., 2001. Effects of underwater noise on auditory sensitivity of a cyprinid fish. Hearing Res. 152, 17–24.
- Wale, M.A., Simpson, S.D., Radford, A.N., 2013. Noise negatively affects foraging and antipredator behaviour in shore crabs. Anim. Behav. 86, 111-118.