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### NOISE EFFECTS ON AQUATIC INVERTEBRATES: BEHAVIOURAL AND PHYSIOLOGICAL RESPONSES

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## Abstract

Anthropogenic noise is recognised as a global pollutant. Not only in natural environments, but also in aquaculture ones, this pollutant is present and pervasive. In this doctoral study, the effects that anthropogenic noise can cause on behavioural, biochemical and molecular levels on invertebrates, were evaluated. Two case studies were considered which involved two species of the genus *Cherax spp*, *Cherax destructor* and *Cherax quadricarinatus* (commercially important species bred in different parts of the world), and of *Cyrtograpsus angulatus* (is an ecological important species in South America). Through the design and implementation of experimental plans in a controlled environment, a complete and integrated analysis was carried out to study the effects that anthropogenic noise could have on species, starting from visible changes such as behavioral ones, up to the molecular level, with the evaluation of changes in gene expression. From the results obtained it was highlighted that the two species emit acoustic signals, and in *Cherax destructor*, the sex and composition of the group influence the emission of these signals. These results were used to build a baseline for the evaluation of noise impact on behaviour of these species. In the subsequent experiments, it was demonstrated that exposure to a linear sweep from 1 to 20 kHz for 45 minutes, modified the behaviour and the hemolymphatic parameters that one used as indicators of stress. The impact of noise, at the molecular level, has led to alterations in the expression of genes that play a key role in the stress response and immunity of crustaceans.

For the second case study, the effects of anthropogenic noise and natural predator sound on foraging behaviour and biochemical stress parameters in the crab *Cyrtograpsus angulatus*, were evaluated. The species is an important component of the macrofauna of the Mar Chiquita reserve. The study highlighted that, while the sound produced by the





crab's natural predator has a greater influence on foraging behavior, anthropogenic noise acts more on the biochemical parameters associated with stress.

**Keywords: Acoustic emissions, behavior, biochemical responses, crustaceans, HSPs, noise**



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# 1. Introduction

## 1.1 Underwater Noise

Underwater noise is a component present in aquatic environments and includes both natural and artificial sounds (Hildebrand 2009). However, in recent decades, the frequency band previously dominated by natural sounds such as wind, waves, and biological sounds is now widely influenced by high maritime traffic (Andrew et al. 2011) and other sources of anthropogenic noise (Hildebrand 2009). When we use the term "noise", we mean a sound that causes disturbance. Noise from maritime transport has become a ubiquitous presence in almost all marine habitats (Duarte et al. 2021). Many human activities introduce various types of energy into aquatic environments, such as radioactive energy, electromagnetic fields, heat, but the most well-known and pervasive, is sound (Duarte et al. 2021). Most human-generated sounds are unintentional but are a byproduct of essential human activities, including maritime traffic, drilling, construction, and the operation of offshore wind farms, etc. However others are intentional, such as sonar or military exercises (Hawkins et al. 2015). Anthropogenic noise has been officially identified as a form of pollution endangering marine ecosystems and is recognised as one of the main causes of pollution in the 21st century. Both at low frequencies (less than 1000 Hz) and at medium frequencies (1-20 kHz), environmental noise has been increasing over the past 50 years (Halperin 2014, Duarte et al. 2021). This has elevated anthropogenic noise to international relevance and a leading role in international directives and programs. The Marine Strategy Framework Directive (MSFD 2008/56/EC) provides 11 descriptors of the state of the marine environment and requires Member States of the European Community to periodically assess these descriptors to evaluate short- and long-term changes. Of these, descriptor 11 concerns underwater noise,



recognised in two distinct components of impulsive noise (descriptor 11.1) and continuous low-frequency sounds (descriptor 11.2) (2008/56/EC - Marine Strategy Framework Directive (MSFD)).

When we talk about noise pollution, we are referring to the increase in environmental noise levels due to human activities that generate sounds. Understanding the physics of sound is important to understanding the propagation power of noise and how it is perceived by animals (Hawkins & Popper 2017). Sound is a form of acoustic energy created by a vibrating object traveling through a medium (such as water or air). In water, objects immersed in water generate sound. Sound propagates as a longitudinal elastic wave because the energy travels parallel to the direction of propagation of the wave. Acoustic waves can be described both by the speed with which a small part of the medium vibrates, called the velocity of the particles, and by the corresponding pressure associated with the vibration. If we want to study the variation of pressure in the medium (water or air), we can study it both as a function of frequencies and as a function of time. The variation in pressure over time is called an acoustic signal. The particles in the medium do not travel with the sound wave but oscillate, transmitting motion to nearby particles (Nedelec et al. 2016). There are three ways to characterise a sound wave amplitude, namely peak pressure, peak-to-peak pressure, and root mean square (RMS) pressure as defined in Equota 1. Peak pressure is the pressure range between zero and the maximum pressure of the signal. Peak-to-peak pressure is the pressure range between the most negative and the most positive pressure of the signal. Meanwhile, the root mean square (RMS) pressure, which is the most complex method to characterise a sound wave, is the square root of the average of the square of the pressure of the sound signal over a specific duration.

### **Equota 1**



$$x_{rms} = \sqrt{\frac{1}{n} \sum_{j=1}^n x_j^2} = \sqrt{\frac{x_1^2 + x_2^2 + \dots + x_n^2}{n}}$$

When we talk about environmental noise, we assume we are talking about fluctuations in sound pressure. Sounds produced by human activities in aquatic environments, can be divided into two classes of noise: impulsive noise and continuous noise. Impulsive noise is a short-duration sound that may or may not repeat over time and has broad frequency bands (Rako-Gospic & Picciulin 2019), while continuous noise is a long-lasting sound that persists over time and can extend over long distances; it may contribute to an increase in background noise (Dekeling et al. 2016). To characterise a noise source for the purpose of assessing potential impact, two types of measurements are considered: Sound Pressure Level (SPL) and Sound Exposure Level (SEL). Depending on the type of source, SEL is used to characterise a continuous source:

### Equota 2

$$SEL = 10 \log_{10} \int_0^t p^2 dt / t^2_{ref} p^2_{ref}$$

While SEL is used when the source is continuous, SPL is used when the source is impulsive (Southall et al. 2019).

Sound pressure is measured in pascals (Pa) in the International System of Units, but since a wide range of pressure values covers hearing and sound production in mammals, Sound Pressure Level (SPL) is typically measured in decibels (dB), on a logarithmic scale relative to a reference of 1  $\mu$ Pa ( $P_0$ ) for underwater sound as follows (Hildebrand 2009):

### Equota 3

$$SPL \text{ dB re } 1 \mu\text{Pa} = 10 \log_{10} (P/P_0)^2 = 20 \log_{10} (P/P_0)$$

The Sound Pressure Level (SPL) and Sound Exposure Level (SEL) are two important parameters used to quantify the intensity and duration of sound in acoustic measurements.



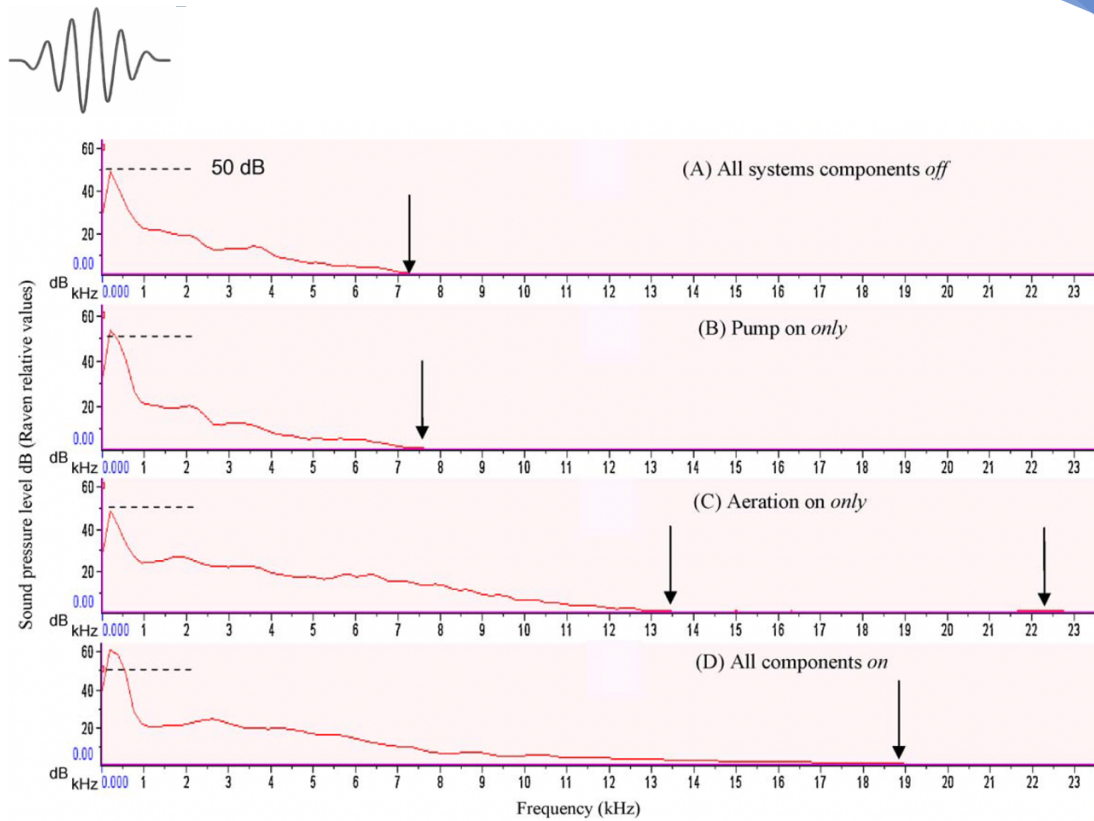
In summary, SPL quantifies the instantaneous intensity or volume of a sound wave, while SEL measures the cumulative energy exposure to sound over a specified period of time. Both parameters are essential for assessing and managing the impact of noise on the environment.

It has been found that noise is increasing in the oceans (Duarte et al. 2021), but this pollutant is also consistently present in aquaculture environments, although often overlooked (Wysocki et al. 2007, Zhang et al. 2023b). Few studies consider noise in aquaculture (Bart et al. 2001, Martins et al. 2010, Sigray & Andersson 2011). Aquaculture is the fastest-growing sector in the food industry globally, with enormous expansion potential. Total production of aquatic animals is expected to increase to 202 million tonnes by 2030 (FAO 2020). With the intensification of aquaculture production, stressors such as handling, inadequate rearing conditions, poor water quality, and pathogens in aquaculture systems, pose potential threats to animal welfare and consequently animal health (Huntingford 2008). The impact of noise on farmed species is also receiving increasing attention. Indeed, the welfare of fish or crustaceans farmed in aquaculture is an important issue for the sector in terms of production efficiency, quality, and quantity (Ashley 2007, Albalat et al. 2022, Wuertz et al. 2023). It has been observed that improved animal welfare presents a series of evident benefits, such as increased growth rates (Sneddon et al. 2016), reduced susceptibility to diseases (Lieke et al. 2020, Kulkarni et al. 2021), and the meat quality also seems to be better in unstressed animals (Barragán-Méndez et al. 2018). The first study on the measurement of underwater environmental noise in aquaculture systems was conducted by Bart et al. (2001). Their results showed how noise levels are influenced by tank materials. Furthermore, both low and high-frequency sounds are present in aquaculture environments. High-frequency noise is primarily generated by electric motors, oscillating air bubbles, aeration, and water pump



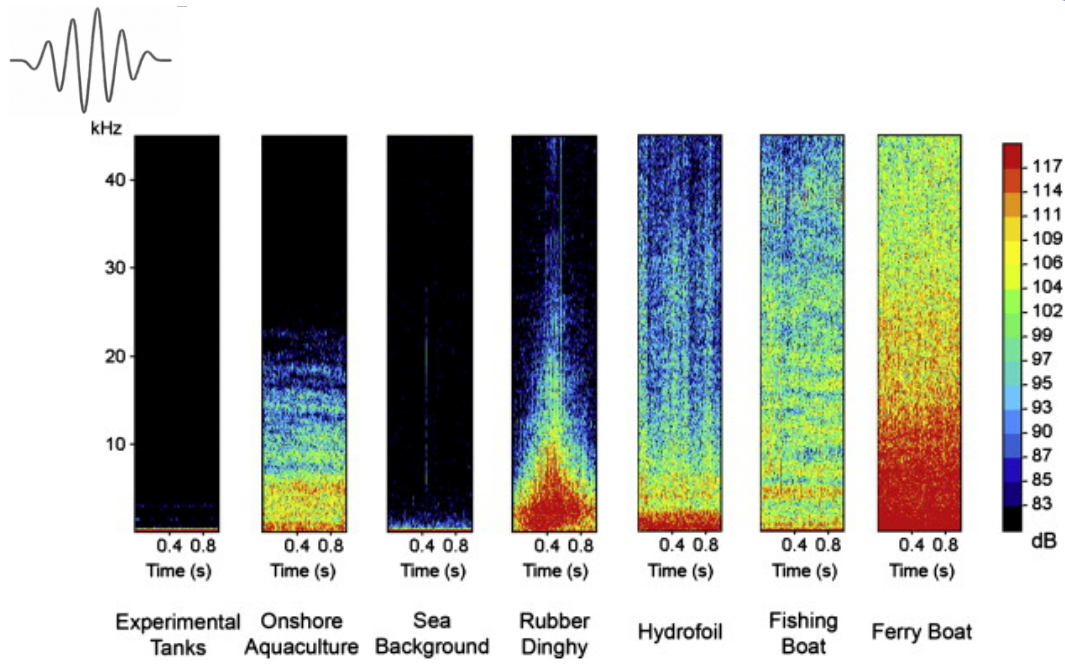


action, while low-frequency noise is generated by water flows and ground vibrations. In the high-frequency region (1-2 kHz), Sound Pressure Levels (SPL) ranged from 100 to 115 dB (re: 1  $\mu$ Pa). In the low-frequency region (25-1000 Hz), SPL ranged from 125 to 135 dB (reference: 1  $\mu$ Pa). Aquaculture systems continue to intensify, necessitating an increase in the use of aerators, air pumps, water circulation machines, feeding, etc. Bart et al. (2001) found that electric aerators significantly contributed to noise levels in outdoor ponds, with an increase from 65 to 127 dB when the aerator was active. Craven et al. (2009) in their study, attempted to measure and quantify the sound landscape in an aquaculture environment, confirming that equipment such as aerators, pumps, and filters make a dominant contribution to the acoustic environment of aquaculture facilities. Figure 1 shows the individual components of the system and their contribution and effect for each: (A) all system components off, (B) only the pump on, (C) only the aerator on, and (D) all system components on.



**Figure 1.** Taken by Craven et al. (2009) The individual components of the aquaculture system. The arrows indicate where the sound dissipates.

Another study that recorded aquaculture sounds both onshore and in open sea is Filiciotto et al. (2013), demonstrated that offshore aquaculture positively influences some stress response indices in *Sparus aurata* compared to land-based aquaculture. Furthermore, taking this work as a starting point, acoustic stimuli belonging to the range recorded by Filiciotto et al. (2013) under onshore aquaculture conditions were used in the thesis work (Figure 2).



**Figure 2.** Taken by Filiciotto et al. (2013). Spectrogram of different noise stimuli: frequency (kHz) vs. time (s). Intensity is reflected by the color scale (dB re 1  $\mu$ Parms)



## 1.2 Aquatic invertebrates

Aquatic invertebrates play a crucial role in marine and freshwater ecosystems, representing a significant part of aquatic ecosystem biodiversity and contributing to ecosystem function, nutrient cycling, and food webs (Collier et al. 2016). They include various taxa, such as mollusks and crustaceans. Their diversity provides resilience to ecosystems and ensures the stability of food webs. They are a vital food source for numerous aquatic and terrestrial organisms, forming the basis of aquatic food webs, providing sustenance for fish, birds, and mammals (Macadam & Stockan 2015).

Some aquatic invertebrates are sensitive to changes environmental conditions, such as water quality, temperature, and pollution (Xu et al. 2014, Bonacina et al. 2023) and as such, they can serve as bioindicators of ecosystem health (Gresens et al. 2009). For example, some invertebrates, such as freshwater mussels, are used in water filtration systems to improve water quality (Collier et al. 2016). Monitoring changes in the abundance and diversity of aquatic invertebrates can provide valuable information about the condition of aquatic habitats and the effectiveness of conservation efforts (Nkwoji et al. 2010, Leigh et al. 2013).

Freshwater shrimp and mussels, for example, are considered ecosystem engineers. They can modify habitats by burrowing, filtering water, or creating physical structures such as dams and burrows. These changes can have significant impacts on local hydrology, water quality and habitat availability for other species (Commito et al. 2005, Emery-Butcher et al. 2020).

Furthermore, many have economic importance. They constitute an important economic and food resource, given that many species of invertebrates, mainly mollusks and crustaceans, are farmed in various parts of the world.



### 1.2.1 Production of sounds

Acoustic signal production allows for very efficient communication, presenting species-specific advantages, regardless of light conditions or long-distance transmission capabilities, especially for species inhabiting extreme or turbid habitats, making other communication systems inefficient (Buscaino et al. 2021). Many species of invertebrates use acoustic signals in various contexts, such as mating (Salmon & Atsides 1968, Amorim & Vasconcelos 2008, Buscaino et al. 2015, Filiciotto et al. 2019), agonistic activities (Miguel Simões et al. 2008, Ceraulo et al. 2022). Sound travels about five times faster in water (about 1500 m/s) than in air (about 340 m/s) because water density is greater, and it also attenuates less over the same distance. This characteristic allows for long-distance communication in water but also implies a long-distance noise impact on aquatic animals (Slabbekoorn et al. 2018). Although visual signals are particularly used in aquatic environments, deep and turbid waters quickly reduce visibility and can negatively affect visual communication (Abrahams & Kattenfeld 1997, Frommen 2020).

Aquatic invertebrates utilise a wide range of mechanisms to produce sounds, which can vary significantly among species. Although they are not typically associated with vocal abilities like some vertebrates, they can generate sounds through physical movements or specialised structures. Among aquatic invertebrates, sound production is known in bivalves, echinoderms, and crustaceans.

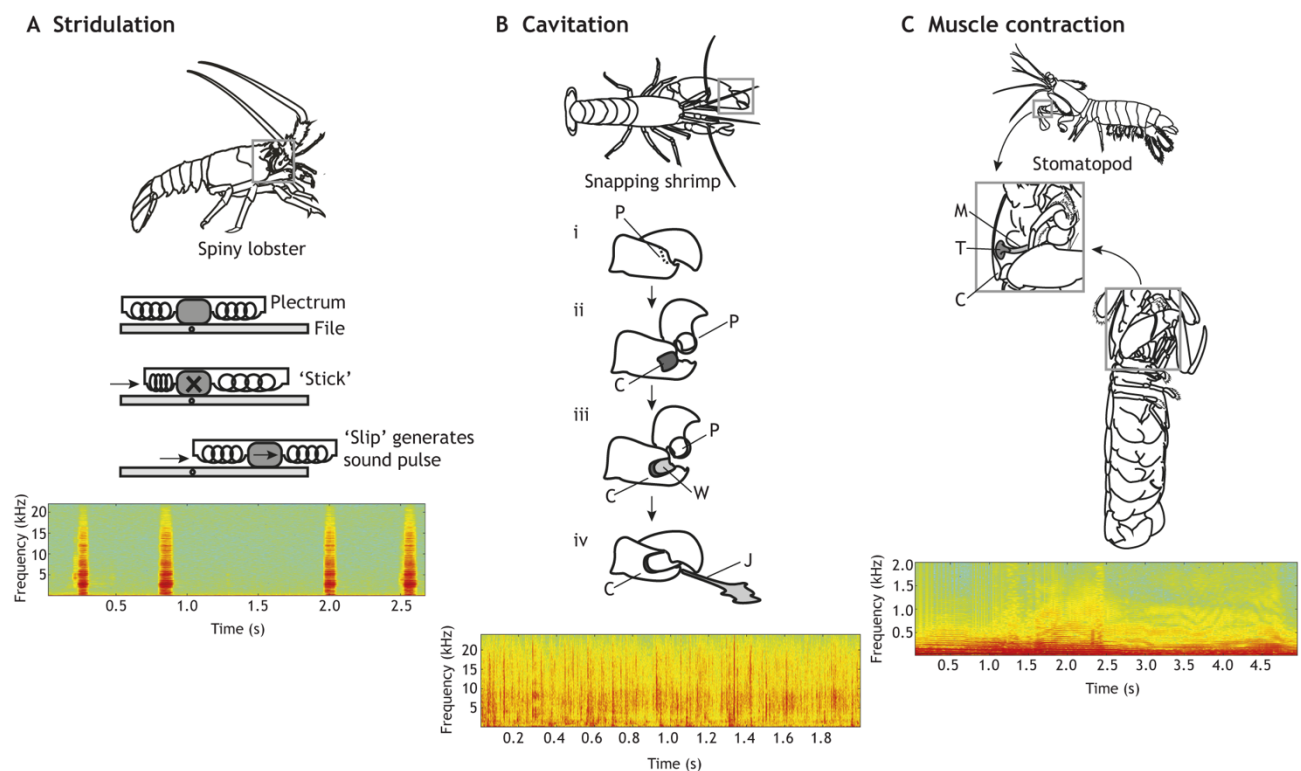
In bivalves, sound production has been studied in species such as *Perna perna* (Júnior et al. 2019) and *Pecten maximus* (Di Iorio et al. 2012), where valve movements are responsible for sound generation. Echinoderms like *Diadema antillarum* produce sounds during feeding, with frequencies ranging from 800 to 2800 Hz (Radford et al. 2008). Also, *Avechinus chronoticus* contributes to ambient sounds with an increase of 20-30 dB,



particularly during feeding at dawn/dusk (Radford et al. 2010). Communication through acoustic signals has been repeatedly hypothesized for crustaceans (Popper et al. 2001, Buscaino et al. 2011a). Various crustaceans are known for their ability to produce sounds, and in some decapod species, specific behaviours are associated with acoustic emission (Buscaino et al. 2011b). The presence of a hard exoskeleton enables crustaceans to use it to produce sounds through stridulation (Covich et al. 2010). This seems to be the most used form of production in crustaceans, but muscle vibration/contraction and snap/cavitation are also present (see Figure 3). Lobsters of the genus *Palinurus* produce rasps (Patek 2001, 2002, Goldman & Patek 2002), a sound produced by rubbing the antenna, used as a scraper, on a file on the plate beneath the eyes. The resulting sound is a series of impulses that have been shown to be species-specific; in *Palinurus interruptus* the peak frequency is 633 Hz (Patek et al. 2009) and in *Palinurus elephas* the peak frequency is 770 Hz (Jézéquel et al. 2021). Buscaino et al. 2011b demonstrate how these signals are emitted in anti-predator contexts. *Jasus edwardsii* produces a rasp-like sound during feeding, although its production mechanism is still unknown (Smith, 2021). Another type of sound production for crustaceans is muscle contraction that causes the carapace to vibrate. This signal production has been recorded in *Homarus americanus* and *Homarus gammarus* when animals were threatened or handled (Ward et al. 2011, Jézéquel et al. 2018). To date, the acoustic production of shrimp has been studied in *Procambarus clarkii*, *Euastacus armatus*, and *Faxonius limosus* (Breithaupt 2002). *P. clarkii* generates sounds through the movement of the scaphognathite (Favaro et al. 2011) and tends to emit signals during intraspecific interactions (Buscaino et al. 2012). *E. armatus* produces audible sounds through abdominal stridulatory organs (Sandeman & Wilkens 1982). Snapping shrimp produce the characteristic "snap" sound through the collapse of a cavitation bubble during rapid closure of the claw. The production of these



signals is associated with many behaviours, competitive or agonistic encounters (Herberholz & Schmitz 1998, Dinh & Radford 2021) and, reproduction (Heuring & Hughes 2020). It has been observed that body size and snap sound pressure levels are positively correlated (Au & Banks 1998). Dinh & Radford (2021) have hypothesized that this correlation allows understanding of combat abilities, animal size, or even the reproductive quality of the animal. Also, in *Ovalipes trimaculatus*, the peak frequency varies with the size of the animal (Buscaino et al. 2015). The genus *Ovalipes* produces distinct sounds through stridulation (Stephenson, 1969). *Ovalipes catharus* produces rasp, zip, and bass signals. Each sound is used in different behaviours. For example, the zip is used in courtship or post-copulatory behavior only by males (Flood et al. 2019). Rasps have been associated with feeding behaviour (Flood et al., 2019), and with reproduction (Buscaino et al., 2015).



**Figure 3.** Taken by Radford & Stanley (2023). Schematics of three of the most common mechanisms of sound production with signal spectrogram in crustaceans.





### 1.2.2 Sensitivity and detection of sounds

Aquatic invertebrates exhibit a variety of adaptations to perceive their environment, including sound. Although they lack ears like vertebrates, many marine invertebrates are remarkably sensitive to acoustic signals. However, our understanding the ability of aquatic invertebrates to perceive sound, and their sensitivity to it, is still fragmented. Some research indicates that bivalves, cephalopods, and crustaceans are sensitive to particle vibrations (Roberts et al. 2016, Dinh & Radford 2021). Wherever there is sound, there is movement of particles, and this is sensed by invertebrates.

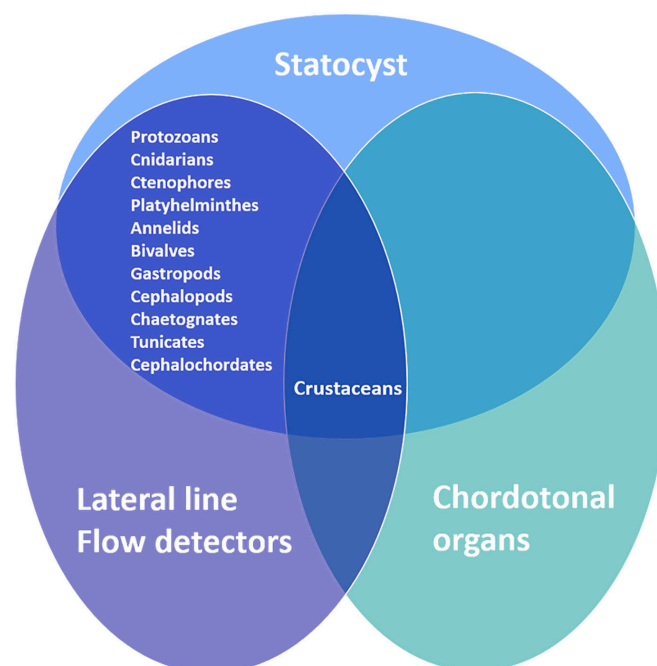
Aquatic invertebrates use three mechanisms to detect sound: internal statocysts, superficial mechanoreceptors, and chordotonal organs (Heinisch & Wiese 1987, Breithaupt & Tautz 1990, Budelmann 1992a) (see Figure 4). Invertebrate statocysts can be defined as internal receptor systems, and in crustaceans, they can be located on the basal segment of the antennule and uropod and are analogous to the inner ear in vertebrates. The statocyst is a chamber containing fluid and a gelatinous mass of sand grains, the statolith, and it has been shown that statocysts respond to particle movement (Lovell et al. 2005, Radford & Stanley 2023). Superficial mechanoreceptors are epidermal sensory cells that can be both chemosensitive and mechanosensitive (Cate & Derby 2002). Decapod crustaceans, for example, have single or grouped sensory hairs that bend upon contact with water, stimulating the sensory cells mechanically (Jézéquel et al. 2021). Only crustaceans have chordotonal organs, which are proprioceptive organs that control movement of joints (Whitear, 1960). *Carcinus maenas*, for instance, detects vibrations through chordotonal organs found in the legs (Whitear, 1962). Establishing an animal's sensitivity to acoustic or vibrational stimuli is not easy. To achieve this goal, measurements of electrophysiological responses, behavioural observations, or



evaluations of auditory evoked potentials (AEP) are needed (Tautz & Sandeman 1980, Breithaupt & Tautz 1990). Regarding invertebrates, this translates into studying species' sensitivity to particle movement rather than to sound pressure, as invertebrates are more responsive to the latter component of sound (Breithaupt & Tautz 1990, Popper et al. 2001). The two most recent studies on sensitivity in bivalves are attributed to Roberts et al. (2015) and Charifi et al. (2017), who quantified sensitivity in *Mytilus edulis* and *Crassostrea gigas*, respectively. The bivalve *Mytilus edulis* is capable of perceiving noise from vibrations transmitted by both the substrate and the surrounding water. The animal responded to sound with valve closure, used as a behavioral indicator of reception and response (Roberts et al., 2015). Another study demonstrating sound sensitivity through escape behavioral responses in *Sepia officinalis* was conducted by Samson et al. (2014), who observed greater escape behaviors between frequencies of 80 and 300 Hz and at sound levels exceeding 140 dB re 1  $\mu$ Pa rms. In cephalopods, particle movement causes particles inside the cephalopod to move with similar phase and amplitude (André et al. 2016). Although in cephalopods, the statocyst is considered the primary sound detection organ, its likely functions as an accelerometer in response to the vibratory particle movement of sound (Budelmann 1995, Mooney et al. 2010). Budelmann (1992b) and Solé et al. (2018) also show that cuttlefish and squid have "epidermal lines" of ciliated sensory cells sensitive to water oscillations from 0.5-400 Hz, thus being able to perceive hydrodynamic pressure. Using AEPs, Lovell et al. (2005, 2006) studied the auditory capabilities of *Palaemon serratus*, finding that the species is sensitive to frequencies ranging from 100 to 3000 Hz. Jézéquel et al. (2021) demonstrated that *H. americanus* is sensitive to a frequency range of 80-250 Hz. Dinh & Radford (2021) observed that *Alpheus richardsoni* could detect particle acceleration frequencies from 40 to 1200 Hz. Other crustaceans have shown sensitivity to sound, such as *Crangon crangon* (Heinisch



& Wiese 1987, Berghahn et al. 1995), freshwater shrimp like *Orconectes limosus* and *Procambarus clarkii* (Offutt 1970, Breithaupt & Tautz 1990, Goodall et al. 1990), *Cherax destructor* (Tautz & Sandeman 1980), for example, has shown postural changes in response to water vibrations (Goodall et al., 1990). *Pagurus bernhardus* has shown antenna movement in response to particle movement (Roberts et al. 2016).



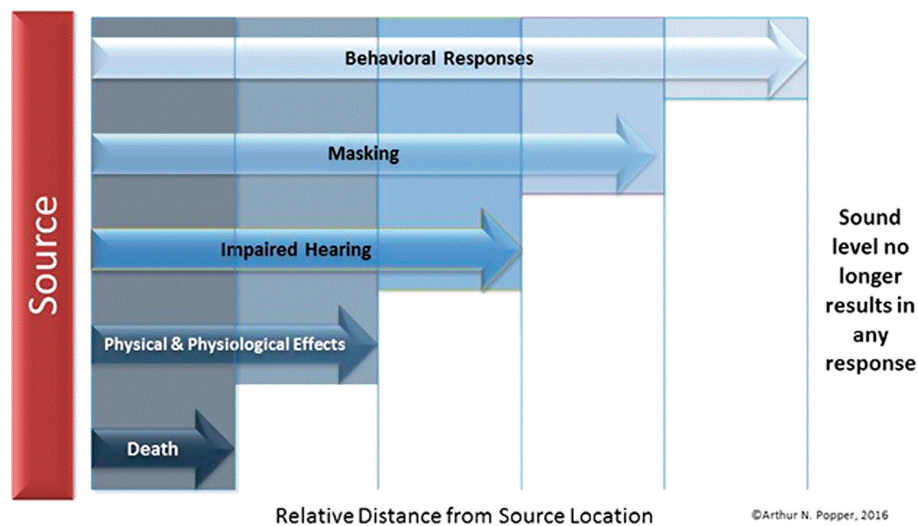
**Figure 4.** Taken by Solé et al. (2023). Marine Invertebrate sound sensory systems.

### 1.3 Effects of anthropogenic noise on invertebrates

Several studies highlighted how anthropogenic noise can impact aquatic animals (Buscaino et al. 2010, Papale et al. 2012, Popper & Hawkins 2019, Erbe et al. 2019, Mauro et al. 2020), but only recently have there been advances in research on invertebrates (Celi et al. 2012, Villalobos-Jiménez et al. 2017, Solé et al. 2023). The noise impact on invertebrates, and aquatic organisms in general, depends on various factors,



including the spectral characteristics of the noise source to which the animal is exposed, its proximity to it, or the duration of exposure. There are still too few studies evaluating the effects of noise on such a diverse taxon as invertebrates to understand the actual impact it may have on different species. It has been highlighted how exposure to intense noise or for prolonged periods can lead to physical damage (McCauley et al. 2003, André et al. 2011), physiological stress (Snitman 2022, Solé et al. 2023) and behavioral changes (Filiciotto et al. 2014, Cox et al. 2018, Sal Moyano et al. 2023). However, there is still little clarity on the frequencies that stimulate the majority of invertebrates. Hawkins & Popper (2017) illustrated in Figure 5 the likely effects that a noise source can have on species depending on the distance, emphasizing that the distance varies depending on the type of source and the type of species under consideration.

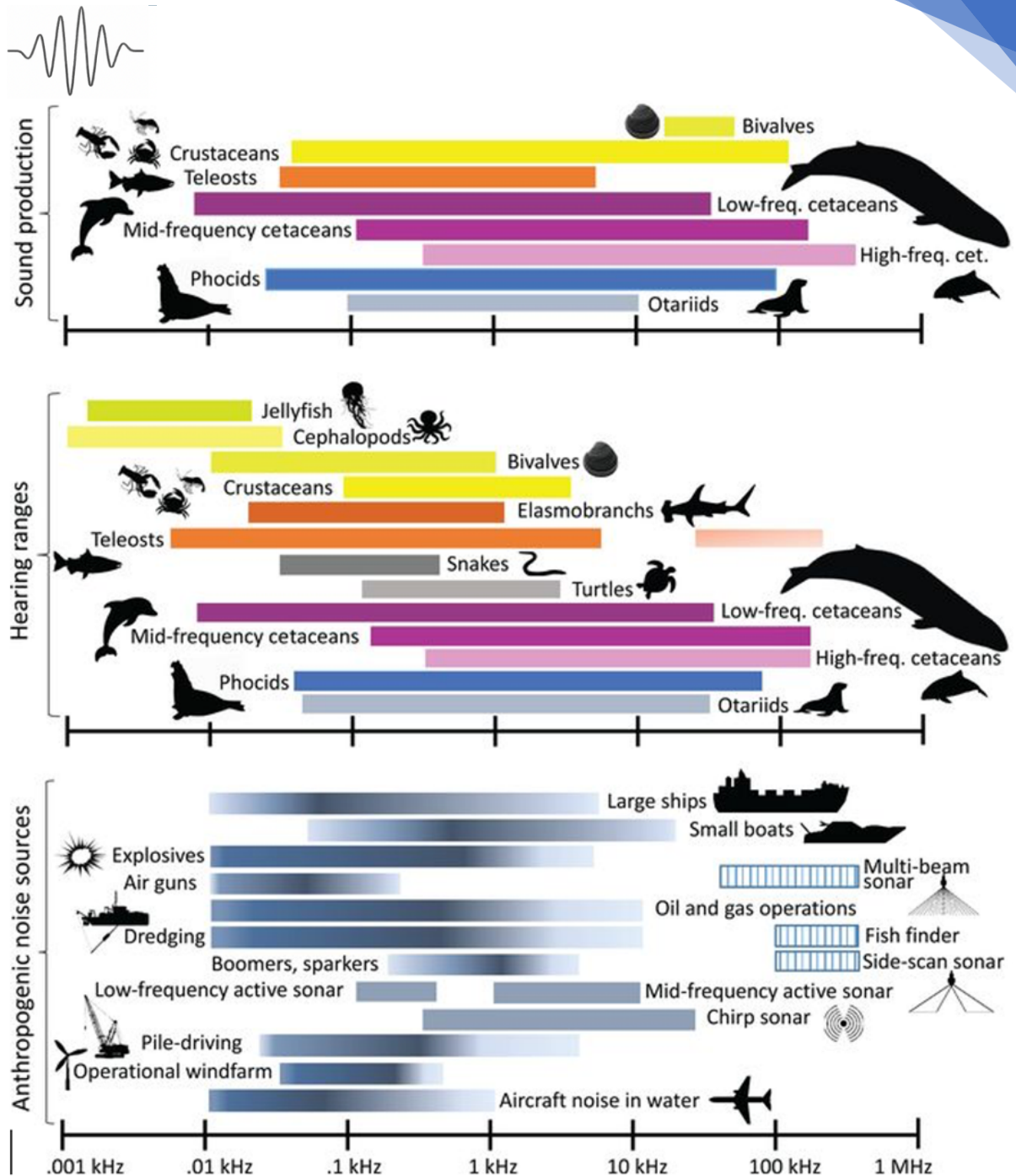


**Figure 5.** Taken by Hawkins & Popper (2017). Potential effects of a sound at different distances from a source.

As seen in the preceding paragraphs, many aquatic species emit acoustic signals and consequently it is reasonable to hypothesize they are also able to perceive such signals (Popper et al. 2001). The emitted signals are species-specific, presenting specific acoustic



parameters for each species. Many species use sound for information exchange and in various biological contexts (Buscaino et al. 2015, Ceraulo et al. 2022). These sounds often have their reception limited in the presence of a noise source, or the signals are partially received. Looking at Figure 6, we see how anthropogenic sounds often have frequency ranges that overlap with the frequency ranges of audibility and sound production of aquatic organisms, which try to adopt different strategies to counteract the effect of noise, but often this is not sufficient.



**Figure 6.** Taken by Duarte et al. (2021). Sound production and hearing ranges of marine taxa and frequency ranges of selected anthropogenic sound sources. These ranges represent the acoustic energy over the dominant frequency range of the sound source, and color shading roughly corresponds to the dominant energy band of each source.



### 1.3.1 Physical and physiological responses

Distinguishing between physical damages, which can also lead to organism death, and physiological ones is challenging; the literature, these one often used interchangeably. Physiological responses to stress can be classified into adaptive biochemical adjustments, which promote organism equilibrium recovery during stressful situations, or maladaptive ones, harmful behaviours that compromise individual performance (Stoner 2012). Exposure to noise sources can cause either the immediate death of animals or sub-lethal injuries. It has been seen in crustaceans, sublethal effects have been observed after noise exposure in *H. americanus*, weeks to months after exposure (Payne et al., 2007). On the spiny lobster, *Jasus edwardsii*, Fitzgibbon et al. (2017) suggest that exposure to seismic air guns has a chronic negative immune impact, as the noise suppressed total hemocyte counts for up to 120 days and showed elevated THC levels for up to 365 days after exposure. In *Cotylorhiza tuberculata* and *Rhizostoma pulmo*, scanning electron microscopy revealed lesions in the sensory epithelium of statocysts after exposure to low-frequency sound (Solé et al. 2018). In the study on *Callinectes sapidus*, it was seen how underwater explosions led to the death of the animals (Moriyasu et al., 2004).

Within the *Mytilus* genus, two species were used to evaluate noise effects physiologically, *M. edulis* and *M. galloprovincialis*. Both species are model species for ecotoxicological studies; in study of Wale et al. (2019), boat noise was shown to cause DNA damage and oxidative stress. In study of Vazzana et al. (2016), *M. galloprovincialis* was seen to significantly increase glucose, total protein, total haemocyte count (THC), heat shock protein 70 (HSP70) protein expression, and acetylcholinesterase (AChE) activity, measured in plasma and tissues, in the frequency range of 0.1-5 kHz. In the digestive gland of *Mytilus galloprovincialis*, high-frequency acoustic stimulations led to increased alkaline phosphatase, esterase, and peroxidase activity (Vazzana et al. 2020a). The





echinoderms showing signs of physiological stress after noise exposure include *Amphiura filiformis* (Solan et al. 2016) and *Arbacia lixula*, where a significant change in enzyme activity and HSP70 gene and protein expression in the cell-free coelomic fluid was found (Vazzana et al. 2020b). It is known that cephalopods show sensitivity to environmental changes and stress factors; among the examined cephalopods, including *Loligo vulgaris*, *Illex coindetii*, *Sepia Officinalis* and *Octopus vulgaris*, animals showed damage to sensory systems (Guerra et al. 2004, André et al. 2011, Solé et al. 2013, 2018) after exposure to various noise sources. In *Sepia officinalis*, exposure to low-frequency sounds affected the statocyst endolymph, resulting in immediate protein composition changes 24 hours after sound exposure (Solè et al., 2018). In *Architeuthis dux*, low-frequency noise caused tissue damage that subsequently led to animal death (Guerra et al., 2004). Among the early studies considering the effect of noise on crustaceans, the study of Lagardère (1982) showed a reduction in growth and reproduction rates in *Crangon crangon* after exposure to 30 dB sounds between 25 and 400 Hz frequency range. Filiciotto et al. (2014) and Celi et al. (2015) demonstrated the physiological responses of *Palinurus elephas* after boat noise exposure, proving the species' sensitivity to the pollutant.

Considering freshwater invertebrates, Celi et al. (2012) showed at the hemolymph level that *Procambarus clarkii* under high-frequency acoustic stimulation had a significant increase in HSP70, differential hemocyte count, and glucose levels. There are few works examining the effects of noise in aquaculture. Several studies in literature focus on acoustic impact in fish farming. For example, it has been demonstrated that growth rates of *Salmo salar* (Terhune et al. 1990) and *Oncorhynchus mykiss* (Davidson et al. 2009) decreased due to noise exposure. Additionally, Filiciotto et al. (2013) showed that soundscapes simulating land-based aquaculture significantly influenced immune



indicators (serum cortisol, glucose, red blood cell count, hematocrit value) in young *Sparus auratus*.

### 1.3.2 Behavioral response

Many animals exposed to various sources of noise exhibit behavioural changes that can persist after exposure ends or even after some time (Day et al. 2017). There are several behaviours particularly important in the lives of animals that can be examined, including foraging behaviour, reproductive behaviour, motility, agonistic behavioural, antipredator behaviors. Behavioural responses of aquatic invertebrates to acoustic stimuli vary depending on the species and characteristics of the emitted stimulus. Considering bivalves, *Mytilus edulis* after exposure to pile driving noise respond with valve closure increase (Spiga et al. 2016), while in *Magallana gigas*, exposure to boat noise causes a decrease in valve activity (Charifi et al. 2017). In *Sepia officinalis*, the intensity of the defensive behavioural response depended on the amplitude and frequency of the stimulus (Samson et al. 2014). In crustaceans like *Palinurus elephas*, locomotor behaviors increased after exposure to boat noise (Filiciotto et al. 2014). Conversely, in *Neohelice granulata*, the same stimulus resulted in a reduction in locomotor activity (Snitman 2022). Considering foraging behaviour, *Panulirus longipes* exposed to white noise takes longer to search for food (Meyer-Rochow et al. 1982). In freshwater shrimp, *Neocaridina davidi*, anthropogenic noise altered foraging behavior, with an increase in foraging time in stressed shrimp (Azarm-Karnagh et al. 2022). In *Carcinus maenas*, foraging behavior was disrupted after exposure to ship noise (148-155 dB RMS re 1  $\mu$ Pa) (Wale et al. 2013), and anthropogenic noise (129.5 to 142.0 dB re 1  $\mu$ Pa) altered crab feeding aggregation (Hubert et al. 2018). Additionally, it negatively affected crabs' ability to camouflage and predator escape responses (Carter et al. 2020).



In other species, noisy conditions increased predation risk, as observed in *Pagurus bernhardus* (Nousek-McGregor and Mei, 2016; Tidau and Briffa, 2019), or in *Palinurus elephas*, which spent more time outside of shelter after exposure to boat noise (Filiciotto, 2016). Changes in locomotion have been observed in many species of decapod crustaceans exposed to boat noise (Filiciotto et al., 2014; Filiciotto et al., 2016). *Procambarus clarkii* decreased agonistic behavior after exposure to sweeps with frequencies ranging from 100 to 25.000 Hz (Celi et al., 2013). In *Neohelice granulata*, boat noise decreased maternal care behaviors (Sal Moyano et al. 2023).

The noise can also alter the acoustic parameters of sounds produced by species; for example, the number and amplitude of acoustic signals produced by *Athanas nitescens*, *Alpheus macrocheles*, and *Alfeo Glabro* increased after exposure to pile driving (Spiga 2016).



## 2. Aim

This PhD project aims to increase information regarding the effects, *i.e.*, potentially measurable changes, due to the exposure of anthropogenic noise on some invertebrate species. The focus in the literature is almost exclusively dedicated to marine mammals and fish and little is known about the effects that noise can have on invertebrate species. Furthermore, the frequencies band of sounds considered in the experimental tests were chosen to including a multitude of sounds coming from anthropic activities, also from the aquaculture environment (Filiciotto et al., 2013). Understanding the behavioral and physiological effects of anthropogenic aquatic noise on invertebrates is essential for assessing the impact of human activities on aquatic animals and for developing management and conservation strategies that take these complex interactions into account.

In particular, a behavioural and acoustic study was carried out to distinguish between male and female emitted acoustic signals and to understand whether these signals were associated with particular states or behavioural events. Subsequently, the influence of acoustic stimuli on behaviour and on some biochemical and molecular parameters was investigated. By analysing changes in motility, acoustic signal emissions, total protein concentration, pH, osmolarity and enzyme activities we aim to gain an in-depth understanding of the impact of acoustic stimuli on crayfish behavior and stress-related physiological parameters. Furthermore, at the molecular level, changes in the expression of genes, such as HSP21, HSP70, HSP90, crustin and lysozyme provide a more complete analysis of the effects of such stress and provide a further approach for assessing welfare in aquaculture.



Additionally, considering the importance of foraging for animals and its importance for the survival of populations, it was analysed how anthropic noise and biological sound influenced the foraging behavior and some biochemical parameters of the crab *Cyrtograpsus angulatus*. A species forming part of the macrofauna of the Mar Chiquita lagoon reserve (Argentina).

This information constitutes important inputs for the implementation of mitigation measures both in the natural environment and in the farm environment, with the possibility of increasing productivity, quality and welfare of the species present in aquaculture.



### 3. First case study: *Cherax spp*

*Cherax* is the most common and species-rich of the nine genera of Australian freshwater crayfish. Among the species of the genus *Cherax*, two species are bred in Sicily, *Cherax quadricarinatus* (Von Martens, 1868) and *Cherax destructor* (Clark, 1836), commonly known respectively as red crayfish and yabby, are two members of the Parastacidae family. Crayfish exhibit morphological and behavioral characteristics that contribute to their ecological success. Thanks to their claws they are able to dig into substrates and build elaborate burrows for shelter and protection (Reynolds 1980). *Cherax spp.* it feeds on small arthropods or plants (Reynolds 1980, Basil & Sandeman 2000). They have a high tolerability to variations in temperature and salinity (Mills & Geddes 1980, Meade et al. 2002) which allows them a wide distribution in various freshwater habitats (Crandall & Buhay 2008) having a key role as an indicator of quality of water (Reynolds 2013). The species *Cherax destructor* and *Cherax quadricarinatus* have demonstrated some level of tolerance to environmental pollutants (Khan & Nugegoda 2007, Baudry et al. 2022). They have significant economic value (Jones & Ruscoe 2000) and their rapid growth and early maturity sexual (Beatty et al. 2005, Ghanawi & Saoud 2012) make them highly suitable for commercial aquaculture. Given all these characteristics, the species are highly commercialised worldwide and can be considered optimal experimental models (Nguyen et al. 2016). Shrimp with aquaculture potential have been the targets of translocations, raising concerns that it may become an invasive species and represent a threat to biodiversity (McNeely 2001, Lynas et al. 2004). Crayfish tend to form social groups and establish hierarchy through postural behaviors or aggressive competition. Once the hierarchy is established, crayfish continue to display specific behaviours based on their social status, such as occupying shelters or preferred territories, having priority



access to food resources, or engaging in social interactions with other individuals (Bergman & Moore 2003). In light of this, it is important to study the behaviour of *C. destructor* in order to implement effective monitoring actions. However, despite its wide distribution, few studies have been reported on this species and they mainly focus on dominance behaviours (Baird et al. 2006, Patullo et al. 2009). Some authors (King et al. 2022) have demonstrated that *C. destructor* prefers to share resources rather than fight, showing placid behavior in both inter- and intraspecific interactions. Sex and body mass are important factors in determining dominance between *C. destructor* and other *Cherax* species, proving that larger animals tend to dominate during agonistic interactions (Lynas et al. 2007).

### **3.1 Behavior and emission of sounds**

#### **3.1.1 Materials and methods**

##### *3.1.1.1 Collection and housing of animals*

The experiment was conducted at the Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF) of the University of Palermo. The animals were supplied by the yabby aquaculture facility located in 'Fiumefreddo di Sicilia' (eastern Sicily). For this study, 46 adult freshwater crayfish *Cherax destructor* (23 males and 23 females) aged 12 months were used. To determine the sex of each animal, the two female and male genital openings were checked in the ventral part, the female openings are located at the base of the third pair of legs and the male openings at the base of the fifth pair of legs. Carapace, claw and cephalothorax lengths were  $10.35 \pm 1.26$  cm,  $5.8 \pm 0.99$  cm,  $5.34 \pm 0.84$  cm (mean  $\pm$  SD), respectively. The animals were singularly housed in holding tanks (dimensions 35.6 x 23.4 x 22.8 cm) with continuous aeration  $O_2 > 5.0$  mg/L, constant temperature of  $21 \pm 1$  °C and under a controlled photoperiod of 12/12

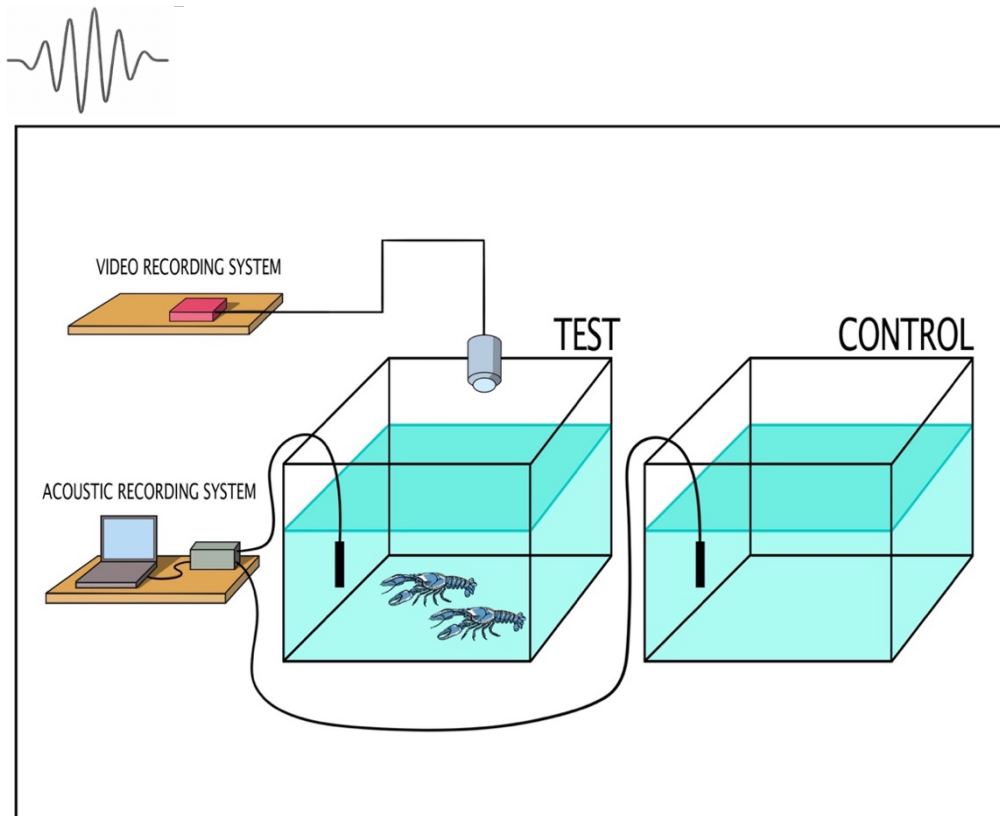




h of light and dark. They were fed daily with commercial diet (5% of body weight, Malta Cleyton, Mexico).

### *3.1.1.2 Experimental setup*

The study was divided into two different experimental settings: 'single experiment' and 'group experiment' (see Table 1). The single experiment consisted of two experimental layouts: 1 male (M) or 1 female (F). The group experiment consisted of five experimental layouts: 1) 1 female plus 1 female (FF); 2) 1 male plus 1 male (MM); 3) 1 male plus 1 female (MF); 4) 2 males plus 1 female (MMF); 5) 2 females plus 1 male (FFM). The set-up and experimental conditions of the study are shown in Table 1. The experiments were conducted from 8a.m to 4 p.m. using two rectangular tanks of the same dimensions (85 cm x 50 cm x 45 cm, water height 26 cm): one used for control trials (no animals in the tank) and one used for test trials. The crayfish were not fed for 1 day before the start of the experimental phase. For the experiments, crayfish were randomly selected from the holding tanks and placed in the test tank (Figure 7). No shelters were present in the tanks. After an acclimatisation period of 15 minutes, the behaviour and acoustic signals emitted were filmed and recorded for 45 minutes. Animals in each settings (Table 1) were monitored for a total of 18 hours and 45 minutes. Each crayfish was used only for one trial.



**Figure 7.** Schematic representation of experimental tanks equipped with hydrophone and video camera.

**Table 1.** Layout of the experimental design and number of acoustic and video monitoring analyzed. M= male; F=female.

Test setting	Test layout	Number of replicas	hours of acoustic and video monitoring, h: min
<b>single</b>	M	5	3:45
	F	5	3:45
<b>group</b>	F+F	3	2:15
	M+M	3	2:15
	M+F	3	2:15
	M+M+F	3	2:15
	F+F+M	3	2:15
<b>total</b>		25	18:45

### 3.1.1.3 Acoustic-video monitoring system and analysis

Behaviour was recorded with a video camera placed on top of only the test tank. The video acquisition system consisted of an analogue video camera (ScubaLight, Mantova, Italy) placed in the top of the test tank (Figure 7). The camera was linked to a digital video



recorder INCH H.264 LCD DVR (NTSC/PAL video system with H.264 video compression format, Motion detection area 16 x 12 grid/cm, hard disk storage Accommodates 1 SATA HDD). Video files were saved in .avi format in the recorder and later exported via USB. The acoustic system was composed by two calibrated hydrophones simultaneously monitoring both the experimental and control tanks in order to avoid attributing external environmental noise or internal acquisition system noise to the species under investigation (Figure 7)(Buscaino et al. 2021). The two calibrated hydrophones (model 8104, Bruel & Kjaer, Naerum, Denmark) had a sensitivity of  $-205.6 \pm 4.0$  dB re 1V  $\mu$ Pa in the 0.1Hz - 80 kHz frequency band (+4 dB and -12 dB in the frequency range of 0.1 Hz to 120 kHz). The hydrophones were connected to a two synchronised channels of an analogue/digital acquisition board (USGH416HB Avisoft Bioacoustics, Berlin, Germany, set with a preamplification of 40 dB), managed by a dedicated software (Avisoft Recorder). The signals of both channels were acquired at 300 k samples  $s^{-1}$  at 16 bit resolution. The acoustic and video systems clocks were synchronised at every test. Moreover, to avoid any delay in time among the two clocks, at the start and the end of every test, we produced an impulsive sound using two bars under the camera. The videos were analysed using the software EthoVision XT 9.0 (Noldus Information Technology, Netherlands) in a semi-automated mode. The Ethovision system is able to distinguish and follow the subjects from the background based on their greyscale/brightness. To do this, an experimental arena needs to be set. The dimensions of the experimental arena were calibrated by taking the actual dimensions of the pool as a reference, using two calibration axes, one vertical and one horizontal. Eleven behaviors (4 events and 7 states) were considered for this study:

Behavioral Events: Acoustic signals, Tail flip, Encounters, Fights.



Behavioral States: Fights duration, Velocity of movement, Distance moved, Angular velocity, Proximity.

The description of all behaviors is reported in the Table 2.

Velocity of movement, distance moved, angular velocity, proximity, walking and resting were measured by Ethovision with a temporal resolution of 2 seconds. Velocity of movement, distance moved, angular velocity was then summarised/averaged using a resolution of 120 seconds. Tail flip, encounters, fights and their duration were manually measured for each trial by an expert operator viewing the videos and noted the latter. To identify and quantify the sounds emitted by the yabby, all .wav files were visualised using the spectrograms (1024-sample Hanning window, sample rate 300 k) and oscillogram (SASLab Pro 5.3.2-16 software, Avisoft Bioacoustics, Germany) of both synchronised channels recorded from the test and the control tank. The signals were categorised in two groups based on their first peak of frequency (Buscaino et al. in prep.), assessed by visual inspection of spectrogram (frequency with maximum energy) (Table 2). The acoustic signals were considered as behavioral events and the number of signals emitted every 120 seconds was summarised and divided for the number of specimens of each replicate. To couple sound, behavioral events and status, the behavior which was recorded in the same time interval as the detection of a sound characterised by a high signal to noise ratio, was verified. Among the states, it was also verified if the specimens were walking or resting during sound emission and if the specimens were in proximity or not. The proximity was considered 1 if the distance among the animals was  $< 6$  cm. The proximity was 0 if the distance was  $> 6$  cm. This measurement was calculated based on the length of the carapace and claws so that animals are in physical contact when distance between their

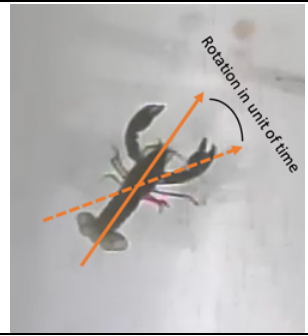
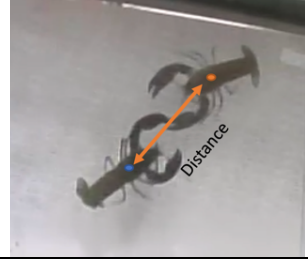


central points are less of 6 cm. For the proximity we consider all the trials in group (FF, MM, MF, FFM, MMF).



	Behaviour name	Behaviour description	Image
Events	Acoustic signal	Number of impulses per specimen per trial (45 minutes). Using the first frequency peak, sounds were divided in two categories: “high sound” with a peak frequency higher than 20 kHz and “low sound” with a peak frequency lower than 20 kHz	<p>High sound</p> <p>Low sound</p>
	Tail flip	The tail flip is a typical avoidance behaviour consisting of a rapid abdominal flexion leading to a new position away from the opponent (Buscaino et al. 2012)	
	Encounters	When one specimen approached touching another without showing any menace.	
	Fights	The approach between two or more specimens that continued in series of competitive activities (Bergman & Moore 2003)	
States	Fights duration (s)	The duration in seconds of the fight	
	Velocity of movement (cm/s)	Distance moved from the centre point of the subject in the unit of time (averaged every 2 s)	
	Distance moved (cm)	The cumulative distance travelled by the central point of the subject from the previous sample to the current one (assessed every 2 s)	



	Angular velocity (Deg/s)	Change in direction of the longitudinal axis in the unit of time.	
	Distance among specimens – proximity	Distance among the centre of the body of one crayfish and the other. The proximity was 1 this distance was $\leq 6$ cm. The proximity was 0 if the distance $> 6$ cm.	
	Walking	Yabby uses its legs to move itself to another location. This state was assessed only when there was a sound emission (2 sec before and 2 sec after sound emission)	
	Resting	Yabby maintains its positions. This state was assessed only when there was a sound emission (2 sec before and 2 sec after sound emission)	

**Table 2.** Description of the events and behavioural states of *Cherax destructor* measured during the different trials. See also videos in the supplementary materials.

#### 3.1.1.4 Statistical analysis

Using the chi-square test, it can be seen that the distribution of the data did not show a normal distribution. Acoustic emissions, behavioral events and states, the differences between the two sexes (males VS females), between test settings (singles VS group) and the seven different layouts (M, F, FF, MM; MF; MMF; FFM) were investigated using Mann-Whitney U-test (for two parameters comparison) and Kruskal-Wallis test (for multi-parameters comparisons). In the latter case, the post-hoc multiple comparisons test was applied. Moreover, differences on sound emission considering proximity condition



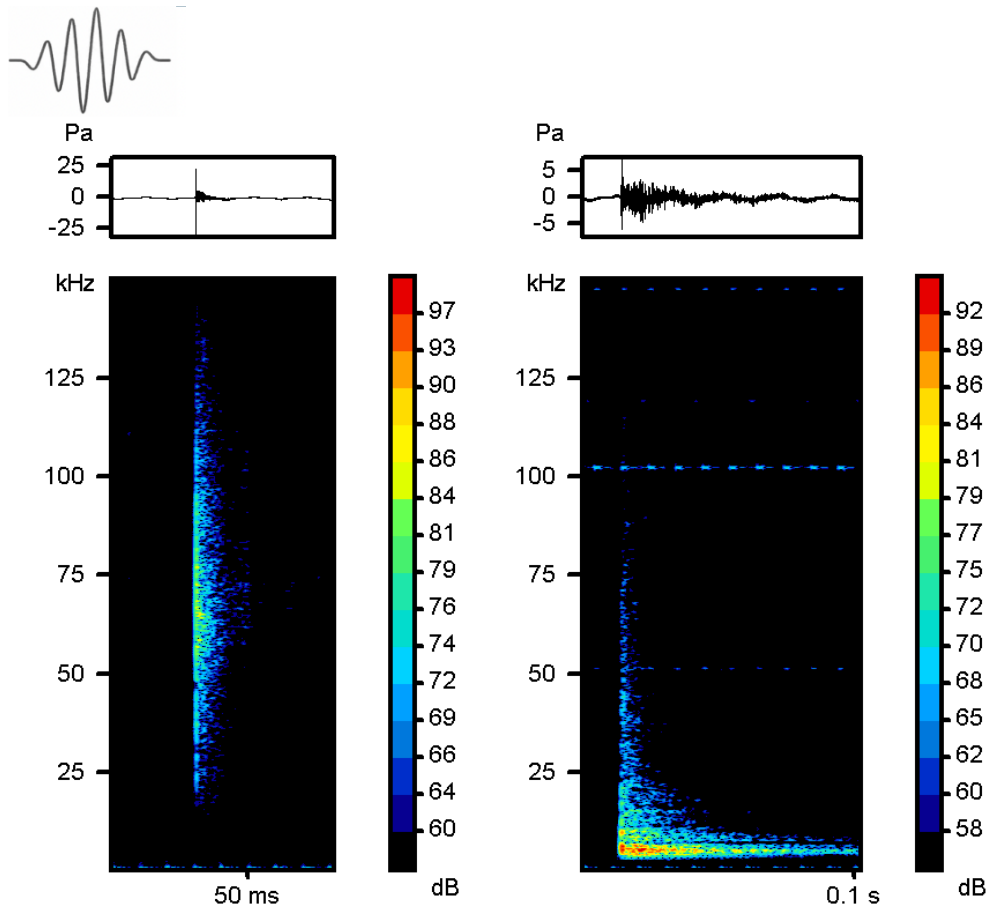
were evaluated using Mann-Whitney U-test. The results were considered statistically significant at  $p < 0.05$ . All statistical analyses were performed with STATISTICA 8.0.

### 3.1.2 Results

#### 3.1.2.1 Acoustic signals

A total of 3929 sounds were recorded during the tests and they were distinguished in two classes, high and low frequency sounds (Figure 8). Specifically, we recorded 653 high frequency sounds and 3276 low frequency sounds (Table 3). The yabby of both sexes produces both impulsive signals (Figure 8). Grouped animals produced fewer low frequency sounds than single animals (Grouped animals  $64.7 \pm 36$ ; single animals  $109.6 \pm 10$  Mean  $\pm$  SE; Mann-Whitney U Test:  $Z = 2.9$ ,  $p < 0.001$ ). At the contrary, high frequency sounds were mostly emitted by grouped animals than single animals (Mann-Whitney U Test:  $Z = -5.4$ ,  $p < 0.0001$ ). Singled males emitted significantly more low frequency sounds than singled females (Mann-Whitney U Test:  $Z = -5.06$ ,  $p < 0.001$ ; Figure 9, Table 3). No significant differences were found in higher frequency sounds between single males and females (Figure 9, Table 3). Comparing the low frequency sounds between the different layouts, results showed that the presence of two males with a female determined an increase of sounds emission compared to MF layout (Kruskal-Wallis multiple comparison test:  $df = 6$ ,  $N = 997$ ,  $Z = 4.9$ ,  $p < 0.0001$ ). In the FF groups, the specimens emitted higher number of signals than all the other layouts (Kruskal-Wallis multiple comparison test  $p < 0.05$ ) (Table 4). Considering the high frequency sounds, the number of signals emitted in FFM group were significantly higher than the ones emitted in single layouts and FF group (Kruskal-Wallis multiple comparison test  $p < 0.05$ ) (see Figure 9, Table 4).

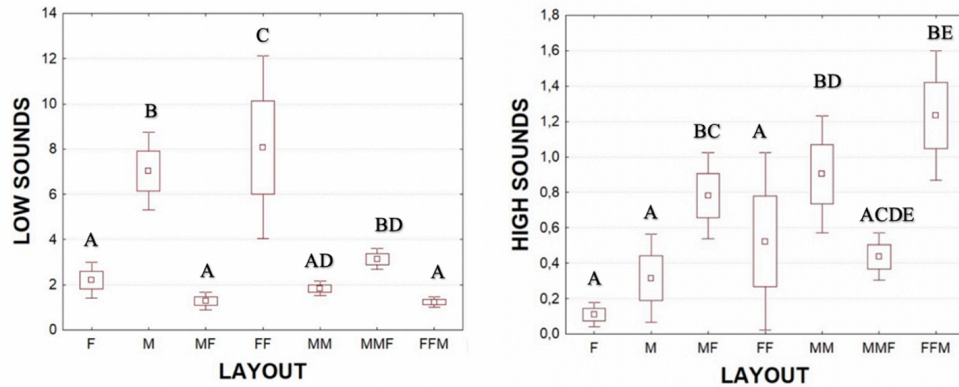




**Figure 8.** Spectrograms of a high sound and low sound from a male. Spectrograms show frequency (kHz) vs. time (s), with intensity in dB re 1  $\mu$ Pa on colour scale, 1024-sample Hanning window. The Oscillogram at the top. Wav. File of these sounds are available on supplementary materials.

**Table 3.** Total number of sounds and mean number ( $\pm$  standard error) per specimen and per replica recorded for test layout typology.

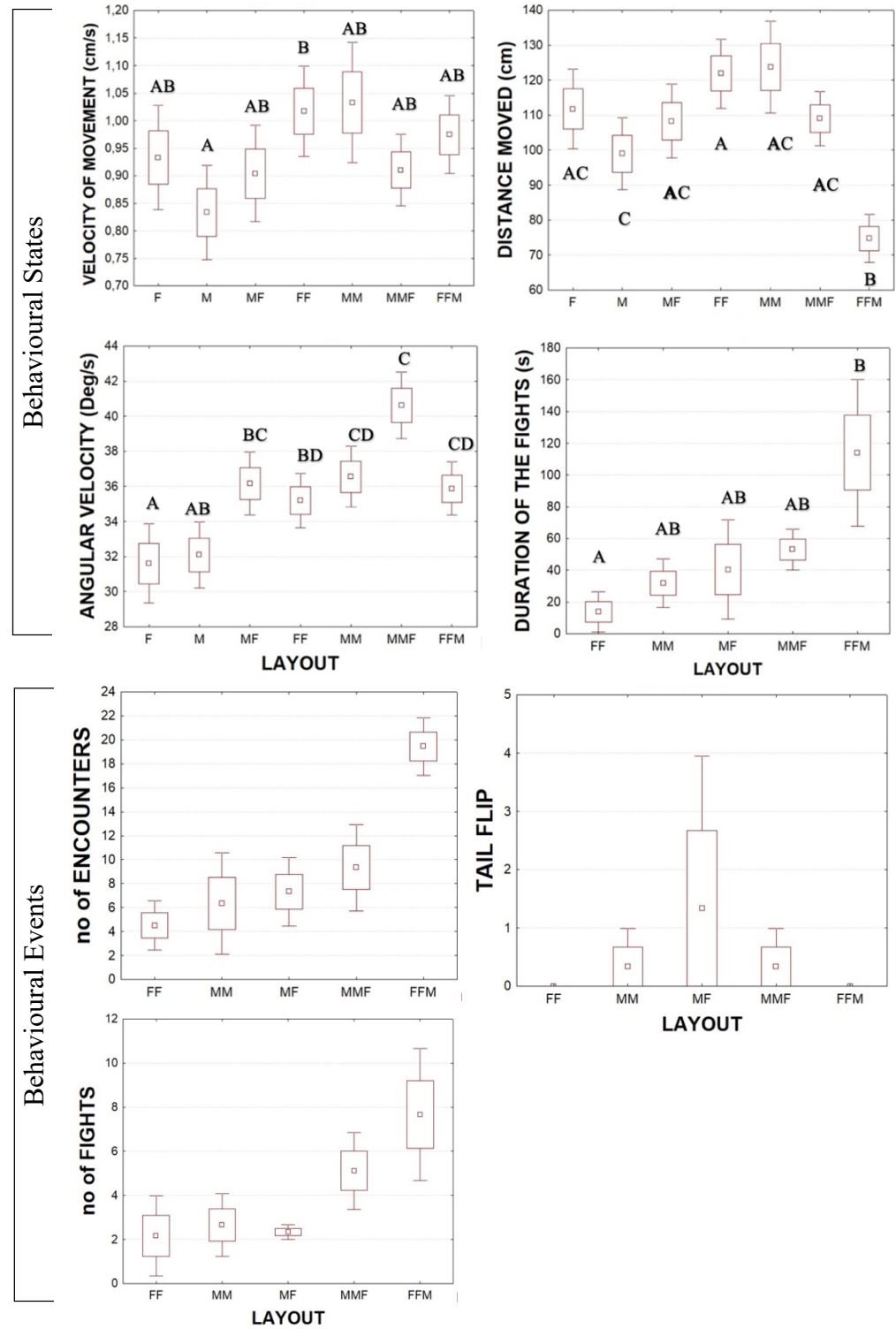
	Layout	High frequency sound, total no.	High frequency sound per specimen and per replica (Mean $\pm$ SE)	Low frequency sound, total no.	Low frequency sound per specimen and per replica (Mean $\pm$ SE)	Total Emission, high and low frequency sound
<b>Single</b>	F	13	2.6 $\pm$ 1.24	243	48.60 $\pm$ 18.69	256
	M	34	6.8 $\pm$ 5.58	806	161.2 $\pm$ 49.9	840
<b>Group</b>	MM	120	20 $\pm$ 4.75	249	41.5 $\pm$ 7.12	369
	FF	69	11.5 $\pm$ 4.43	1066	177.66 $\pm$ 37.02	1135
	MF	103	20.66 $\pm$ 6.91	169	29.16 $\pm$ 6.11	272
	MMF	86	9.55 $\pm$ 3.88	518	68.4 $\pm$ 7.75	604
	FFM	228	25.31 $\pm$ 9.10	225	25.10 $\pm$ 0.64	453



**Figure 9.** Number of (a) low sounds and (b) high sounds produced during different layouts per specimen in 120 seconds. Showing mean  $\pm$  25th to 75th percentiles; error bars:  $1.96^* \pm SE$ ; The different letters indicate significant differences ( $p < 0.05$ ) amongst the experimental layouts.

### 3.1.2.2 Behavioural events and states

Considering all states and event variables, only angular velocity showed significantly higher values in male than in female animals (Mann-Whitney U Test:  $Z = -2.2$ ,  $p < 0.002$ ) and in grouped animals compared to single animals (Mann-Whitney U Test:  $Z = -6.5$ ,  $p < 0.0001$ ) (Figure 10). Comparing the behavioural states and events between the different layouts, the results of multiple comparisons are reported in Figure 4. In particular, the group FFM showed lower values in the distance moved (Kruskal-Wallis multiple comparison test  $p < 0.05$ ), and higher duration of fights compared to other layouts (Kruskal-Wallis multiple comparison test  $p < 0.05$ ) (Figure 10, Table 4). The group FF showed higher values velocity of movement and distance moved compared to single male (Kruskal-Wallis multiple comparison test  $p < 0.05$ ; Table 4).

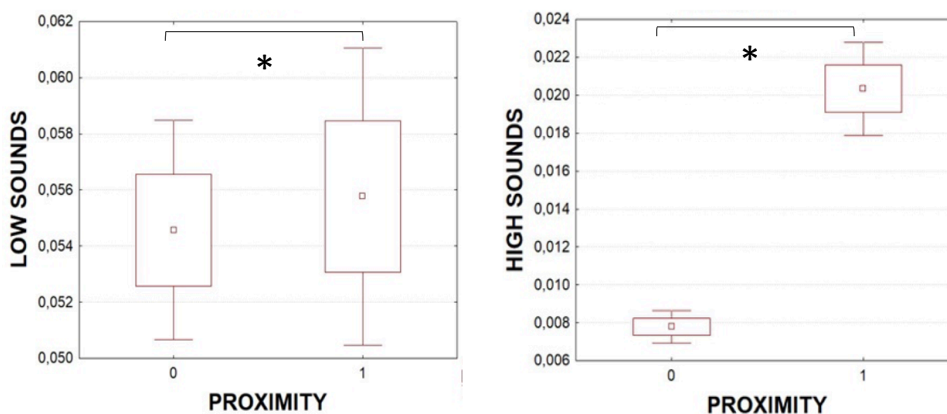


**Figure 10.** Behavioural States and Events in different layouts showing mean  $\pm$  25th to 75th percentiles (box) and error bars:  $1.96^* \pm SE$ ; The different letters indicate significant differences ( $p < 0.05$ ) amongst the experimental layouts.



### 3.1.2.3 Acoustic and behaviour association

The analysis of synchronised acoustic and video data did not show any particular behavioral event/state associated to sound emission. Individuals of both sexes in single layout emitted signals both while walking and in a resting state, and both moving their claws and not. Animals in the group emitted significantly more low frequency sounds (Figure 5, Mann-Whitney U Test  $p < 0.05$   $Z = -4.2$ ) and high frequency sounds (Figure 11, Mann-Whitney U Test  $p < 0.05$   $Z = -3.8$ ) when the distance between the individuals was less than 6 cm (proximity=1). This result is stronger if we consider that 75% of our observations had proximity equal to 0.



**Figure 11.** Mean number of low (left) and high (right) sound per specimen, (averaged on 2 second) in the groups, considering the proximity (proximity = 1 if distance  $\leq$  6 cm; proximity = 0 if distance is  $>$  6 cm). Boxplot: mean  $\pm$  25th to 75th percentiles (box) and error bars:  $1.96 \times \pm$  SE. Mann-Whitney U Test (\* $p < .05$ )



**Table 4.** K-W Multiple Comparisons z' values, in red the values with  $p < 0.05$ .

		F	M	MF	FF	MM	MMF	FFM
<b>Low sounds</b> K-W test: H=138.8 $p=0.0001$	F		5,629	0,103	3,342	2,523	4,946	0,922
	M	5,629		5,993	9,224	3,384	1,452	5,398
	MF	0,103	5,993		3,406	2,761	5,367	1,095
	FF	3,342	9,224	3,406		6,173	9,095	4,778
	MM	2,523	3,384	2,761	6,173		2,357	1,890
	MMF	4,946	1,452	5,367	9,095	2,357		4,687
	FFM	0,922	5,398	1,095	4,778	1,890	4,687	
<b>High sounds</b> K-W test: H=110.1. $p=0.0001$	F		0,680	5,153	0,144	4,850	2,869	5,277
	M	0,680		4,428	0,568	4,125	2,090	4,499
	MF	5,153	4,428		5,266	0,328	2,884	0,266
	FF	0,144	0,568	5,266		4,948	2,879	5,430
	MM	4,850	4,125	0,328	4,948		2,532	0,088
	MMF	2,869	2,090	2,884	2,879	2,532		2,876
	FFM	5,277	4,499	0,266	5,430	0,088	2,876	
<b>Angular velocity</b> K-W test: H= 55.2 $p=0.0001$	F		0,597	3,627	3,057	3,954	6,252	4,140
	M	0,597		2,993	2,425	3,319	5,556	3,464
	MF	3,627	2,993		0,599	0,338	2,462	0,301
	FF	3,057	2,425	0,599		0,938	3,117	0,942
	MM	3,954	3,319	0,338	0,938		2,097	0,060
	MMF	6,252	5,556	2,462	3,117	2,097		2,339
	FFM	4,140	3,464	0,301	0,942	0,060	2,339	
<b>Distance moved</b> K-W test: H= 78.9 $p=0.0001$	F		1,841	0,805	1,409	0,788	0,457	5,562
	M	1,841		1,124	3,332	2,716	1,631	3,480
	MF	0,805	1,124		2,327	1,676	0,440	4,980
	FF	1,409	3,332	2,327		0,656	2,107	7,498
	MM	0,788	2,716	1,676	0,656		1,394	6,805
	MMF	0,457	1,631	0,440	2,107	1,394		6,028
	FFM	5,562	3,480	4,980	7,498	6,805	6,028	
<b>Velocity of movement</b> K-W test: H=15.5 $p=0.016$	F		1,804	0,841	1,381	0,801	0,592	0,588
	M	1,804		1,050	3,266	2,690	1,456	2,599
	MF	0,841	1,050		2,336	1,727	0,339	1,572
	FF	1,381	3,266	2,336		0,613	2,217	0,937
	MM	0,801	2,690	1,727	0,613		1,551	0,281
	MMF	0,592	1,456	0,339	2,217	1,551		1,377
	FFM	0,588	2,599	1,572	0,937	0,281	1,377	
<b>Duration of the Fights</b> K-W test: H=9.4 $p=0.05$	MF	-	-		1,187	0,274	0,639	1,734
	FF	-	-	1,187		0,913	1,826	2,921
	MM	-	-	0,274	0,913		0,913	2,008
	MMF	-	-	0,639	1,826	0,913		1,095
	FFM	0,588	2,599	1,572	0,937	0,281	1,377	



<b>no of Encounters</b> K-W test: H=8.7 p=0.06	<b>FFM</b>	-	-	1,734	2,921	2,008	1,095	
	<b>MF</b>	-	-		1,004	0,411	0,320	1,780
	<b>FF</b>	-	-	1,004		0,593	1,324	2,784
	<b>MM</b>	-	-	0,411	0,593		0,730	2,191
	<b>MMF</b>	-	-	0,320	1,324	0,730		1,461
	<b>FFM</b>	-	-	1,780	2,784	2,191	1,461	
<b>no of Fights</b> K-W test: H=9.4 p=0.05	<b>MF</b>	-	-		0,274	0,183	1,552	2,191
	<b>FF</b>	-	-	0,274		0,456	1,826	2,465
	<b>MM</b>	-	-	0,183	0,456		1,369	2,008
	<b>MMF</b>	-	-	1,552	1,826	1,369		0,639
	<b>FFM</b>	-	-	2,191	2,465	2,008	0,639	
	<b>MF</b>	-	-		0,776	0,137	0,137	0,776
<b>Tail Flip</b> K-W test: H=2.3 p=0.6	<b>FF</b>	-	-	0,776		0,639	0,639	0,000
	<b>MM</b>	-	-	0,137	0,639		0,000	0,639
	<b>MMF</b>	-	-	0,137	0,639	0,000		0,639
	<b>FFM</b>	-	-	0,776	0,000	0,639	0,639	



### 3.1.3 Discussion

This study shows that *Cherax destructor* produces acoustic signals of two different types, high and low peak frequency impulses. *C. destructor* have a highly developed sense of sight, but in the wild they often live in murky waters where the visibility is reduced very quickly and this can adversely affect visual communication (Abrahams & Kattenfeld 1997), necessitating the use of non-visual techniques (Basil & Sandeman 2000), such as chemical (Moore 2005) or, likely, acoustic signals. When the crayfish are burrowed into mud or on land, they resort to chemical communication (Bergman 2005), but thus may not be fully effective. As a consequence, visual and chemical communication might be complemented with the use of acoustic signals (Bradbury & Vehrencamp 2000). Moreover, *C. destructor* emitting more low frequency sounds compared to the high frequency sounds, could make the transmission of any information in the aquatic environment more effective, especially considering that sounds at lower frequency can travel for longer distance than the higher frequency sounds (Medwinn & Clay 1998). However, in decapod crustaceans little is known about their sensitivity to sound (Popper et al. 2001), and sound-based communication in *C. destructor* needs still be investigated with further studies.

We found that the males from single M experiment emitted more low sounds than both the female from single F experiment and groups, with exception of FF. Even if a possible communication role of sounds in this species has yet to be proven, in males they could serve in maintaining contact with other not nearby conspecifics. *Cherax destructor* prefers to stay close to a familiar animal by using chemical signals or visual identification (Crook † et al. 2004, Van Der Velden et al. 2008). This hypothesis was previously proposed in lobsters (Buscaino et al. 2011a), where their stridulation could be seen not only as an anti-predator strategy, but also as signals to warn conspecifics of the danger of



predation or to potentially recruit other conspecifics to help them. In females, low-frequency signals were emitted more when they are in pairs, the FF group. In this layout, they showed also the lowest values of encounters, fights, duration of fights, and no Tail Flip. Mostly studies have been focused on the agonistic activities of males in crustaceans but also females tend to fight. Has been demonstrated that crab species males tend to fight to determine male dominance in the group, while females fight for food and shelter (Dalosto et al. 2019). In *C. destructor*, the different behaviors of males and females were investigated by Walter et al. 2011, finding that they showed different behaviors during social interactions: females mainly relied on body signals (“unreliable signals”) to resolve disputes, as opposed to males of the same species who prefer to opt for fighting. The emission of acoustic signals in *C. destructor* could be used by females as an indirect way of establishing dominance avoiding injuring interaction. It has been seen how thanks to indirect signals exchange between crayfish conspecifics, dominant or subordinate animals can avoid injury by reducing the number of competitive interactions or the intensity of conflicts (Moore 2005). There are no external changes that indicate receptivity in *Cherax* females (Barki & Karplus 1999), but the presence of the eggs in the weeks following the experiments indicates that they could be receptive and suggests the role of the high number of sounds produced.

High frequency sounds did not tend to be emitted differently depending on sex, but were emitted more if the animals were in groups and in close proximity. These signals were fewer compared to the low frequency sound and could be emitted accidentally; however further studies could help to explain their possible role in the ecology of this species.

Considering the behavioral events in different layout, no significant differences was found. The duration of fight was longer in the FFM than FF groups. Moreover, in the FFM groups, the number of encounters and fights was higher compared to other layout





(although there is no statistical significance a difference could emerge by increasing replicas), in addition, comparisons indicate that there are differences between the FF and FFM groups (Table 4), and the marginal  $p$  values (e.g.,  $p = 0.06$  in the overall test) show differences. In the behavioural states, the angular velocity was higher in the grouped animals than singles. In the crayfish the interactions by a third crayfish has implications for hierarchies and social behaviour (Zulandt et al. 2008). In this arrangement the animals also emitted more high-frequency sounds. In our data the agonistic approaches were stronger if two females and a male were present, no tail flips were performed during the fights, probably because no clear hierarchy was formed (Graham & Herberholz 2009).

The groups MMF did not show any particular differences compared to the others layouts, no increase of the fights number or the duration of the fights, neither in sound emissions. These results suggest that these males did not compete for female. On the contrary, in other decapods species, previous research demonstrated an increase of agonistic interactions when two males are in presence of a female. For example, when the crabs *Ovalipes trimaculatus* and *Neohelice granulata* were put into groups consisting of two males and one female, an increase in motility parameters were observed depending on receptivity state of female (Buscaino et al. 2015, Filiciotto et al. 2019).

The comparison among the low sound's emission events with the synchronised behaviours event and states did not reveal a specific movement/mechanisms of sound emission. *C. destructor* sound emission probably involves structures not monitored by the camera such as the mouth, missiles, organs imputed for this purpose or other mechanisms. The low sounds are emitted both when the animals are moving and when they are resting, suggesting that the production of sounds is not, or to a reduced extent, determined by the organs imputed to movement. In the freshwater decapod *P. clarkii*, sound is generated by the movement of the scaphognathite up and down within the



chamber formed by the efferent gill channel (Favaro et al. 2011). Also the freshwater *Euastacus armatus* produces audible sounds through abdominal stridulatory organs (Sandeman & Wilkens 1982). In our study the video camera on top of the tank and the resolution of the videos could not be high enough to detect a specific movement suggesting that the sound emission should be internal or localised in the ventral part of the body. Moreover, the sounds are emitted both when the animals are moving and when they are resting, suggesting that the production of sounds is not determined by the organs imputed to movement or if it was to a reduced extent. In other decapod crustaceans such as lobsters and *P. clarkii* (Buscaino et al. 2011a, 2012), tail flip is associated with the production of sounds. However, in this work, none correlation was found between tail flip events and acoustic emission.

**Data from this study published:** De Vita C, Mauro M, Vazzana M, Arculeo M, Arizza V, Ceraulo M, Buscaino G (2023) Acoustic Signals and Behavior of the Invasive Freshwater Crayfish *Cherax destructor* (Clark, 1936). JMSE 11:1147.

## 3.2 Impact of noise on behavior level

### 3.2.1 Materials and Methods

#### 3.2.1.1 Collection and housing of animals

The study took place at the Department of Biological, Chemical, and Pharmaceutical Sciences and Technologies (STEBICEF) of the University of Palermo, Italy. The crayfish specimens were sourced from an aquaculture facility located in Fiumefreddo di Sicilia, Sicily.

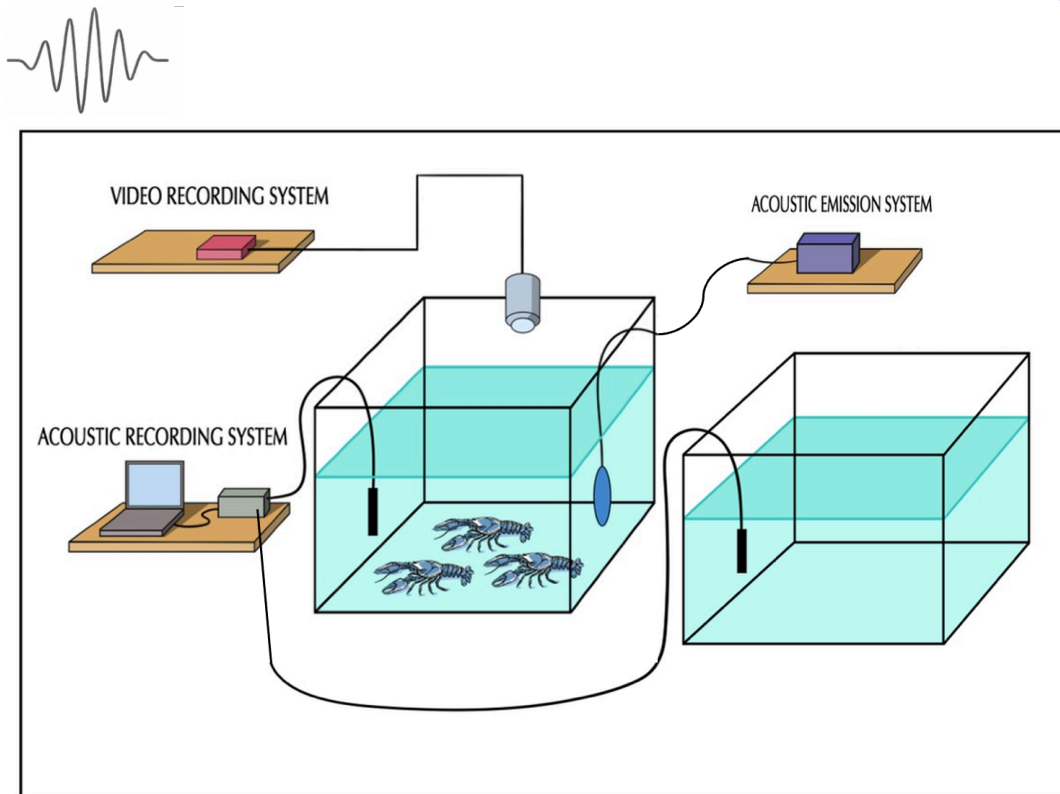


The experiment utilised a total of 60 adults 12 months old (30 *Cherax destructor* and 30 adults *Cherax quadricarinatus*, all acclimatized for a week). The crayfish were similar in size, as body mass is an important factor influencing aggressive interactions in crayfish (Pavey & Fielder 1996, Lynas et al. 2007). Individuals of *C. quadricarinatus* weighed  $56.43 \pm 0.28$  g and had a carapace length of  $11.6 \pm 0.6$  cm, while individuals of *C. destructor* weighed  $55.39 \pm 0.79$  g and were  $10.67 \pm 0.54$  cm long (mean  $\pm$  standard deviation). Each crayfish was housed individually in holding tanks measuring 35.6 x 23.4 x 22.8 cm, ensuring continuous aeration with oxygen levels  $O_2 > 5.0$  mg/L.

The tanks maintained a constant temperature of  $21^{\circ} \pm 1^{\circ}C$  and followed a controlled photoperiod of 12 hours of light and 12 hours of darkness. The crayfish were fed a daily diet consisting of commercial food (5% of their body weight) from Malta Cleyton (Mexico) up to 24 h before the sampling.

### 3.2.1.2 Rationale and experimental procedures

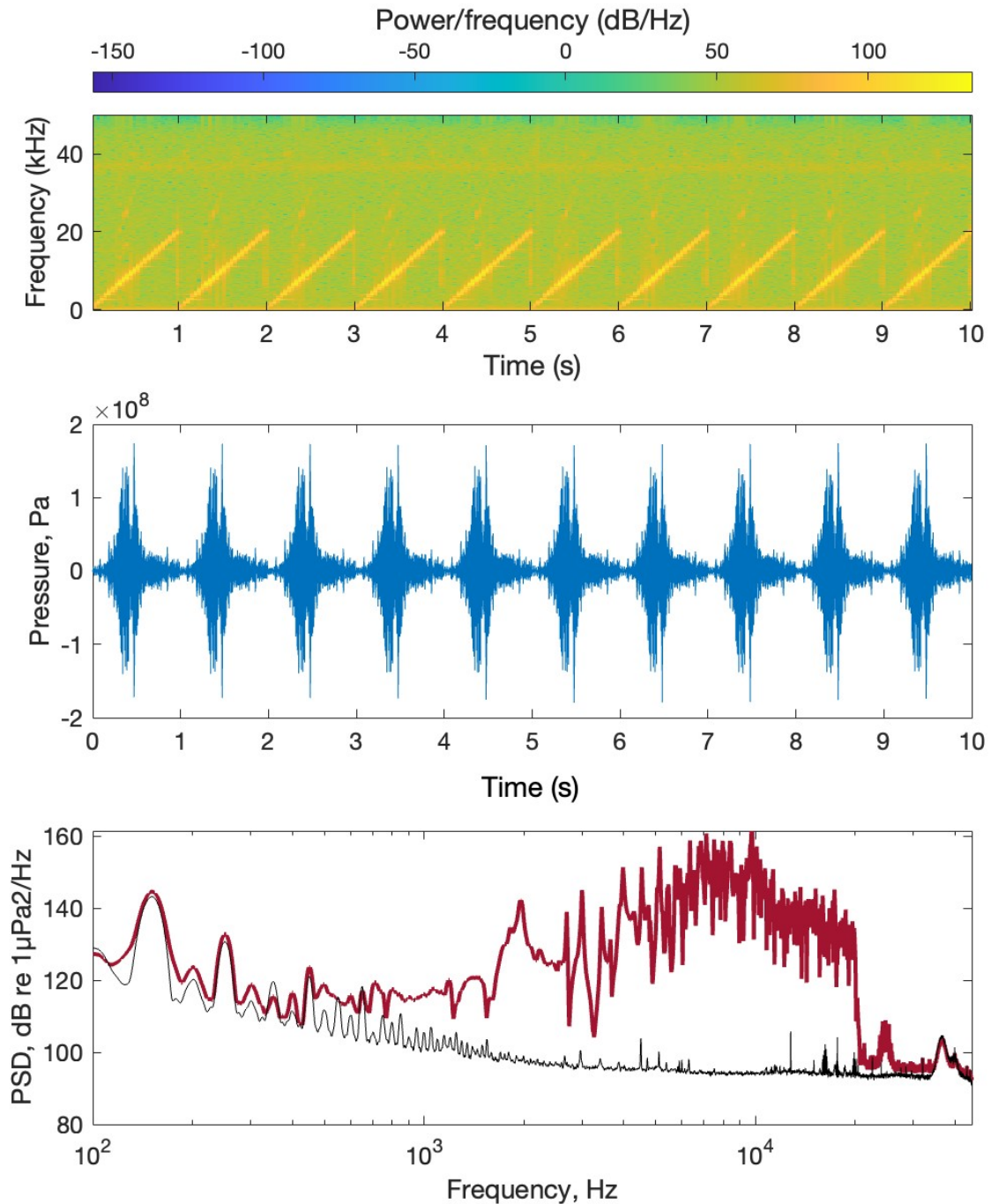
The crayfish were randomly collected from the holding tanks in groups of three individuals, assigned to either the control or test group and used for a single experiment. For each species a total of 10 experimental trials (five for control and five for test in random sequence) were conducted. To prevent other factors from influencing the crayfish's behavior, the experimental tank had not any refuges since Capelli & Hamilton (1984) have shown that the presence of refuges could increase competitive struggles among crayfish. After a period of acclimatation, the experimental phase start and the behavior of the crayfish was monitored and recorded for 45 minutes (Figure 12). During the experimental phase, only the test groups were exposed to 45 minutes of acoustic stimulus; whereas, control groups did not receive any stimulus.



**Figure 12.** Schematic representation of the experimental tank equipped with an underwater speaker, a video camera placed over the center of the tank and acoustic recording system. In the control tank, acoustic stimuli were not emitted from the speaker.

### 3.2.1.3 Acoustic stimulus

The acoustic stimulation used in the study was a linear sweep from 1 to 20 kHz, lasting 1 second and repeated for 45 minutes (Figure 13). This frequency band corresponds approximately to the frequency range of the noise recorded in onshore aquaculture (unpublished data by BioacousticsLab IAS-CNR and Figure 2 in Filiciotto et al. 2013). An underwater loudspeaker (Model UW30, Lubell, Columbus, Ohio, USA) driven by a signal generator (Agilent 33210A, USA) was used to generate the acoustic stimuli. Acoustic stimulation was monitored during the duration of experimental trials using an acquisition system described in the section ‘video and audio monitoring system and analysis’. The sound pressure level inside the test tank during acoustic stimulation varied between 134 and 153 dB (Lp re 1  $\mu$ Pa rms). Figure 1 shows the spectrogram, the oscillogram and the power spectral density (PSD) of ten consecutive sweep. In the PSD the peak frequency was around 7-10 kHz with an amplitude of 160 dB (re  $1\mu\text{Pa}^2\text{Hz}^{-1}$ ).



**Figure 13.** Spectrogram (top) and oscillogram (middle) of ten consecutive sweeps (FFT length of 8192, time segments overlap 50%, x axis: time; y axis: frequency on a linear scale; sampling frequency 100 kHz). Below: PSD, power spectrum density (dB re 1  $\mu\text{Pa}^2/\text{Hz}$ ) of a ten consecutive sweeps (10 s) and tank background noise (black line).



#### 3.2.1.4 Video and audio monitoring system and analysis

The behaviour of the crayfish was recorded using a video camera positioned on the top of the test tank (Figure 12). To discriminate the acoustic signals emitted by the animals from the surrounding environmental sounds, a control tank without animals was acoustically monitored (De Vita et al. 2023). The video capture system employed an analogic video camera (ScubaLight, Mantova, Italy) situated on the tank's upper surface. The video camera was connected to a digital INCH H.264 LCD DVR (compatible with NTSC/PAL video systems and utilising the H.264 video compression format)–Video files were saved in .avi format within the recorder and subsequently exported via USB. The acoustic stimulus, the tanks background and the crayfish sounds were recorded with two calibrated hydrophones (model 8104, Brüel & Kjær, Nærum, Denmark) with a sensitivity of  $-205.6 \pm 4.0$  dB re  $1\text{V}/\mu\text{Pa}$  within the frequency range of 0.1 Hz to 80 kHz. The hydrophones were connected to two synchronised channels of an analog-to-digital acquisition card (USGH416HB Avisoft Bioacoustics, Berlin, Germany), which featured a pre-amplification of 40 dB. Data acquisition was facilitated by dedicated software (Avisoft Recorder), with both channels capturing signals at a sampling rate of 300 k samples per second and a resolution of 16 bits.

Videos were analysed both manually and using the software EthoVision XT 9.0 (Noldus Information Technology, The Netherlands) in semi-automatic mode. The EthoVision system utilises grayscale/brightness distinctions to distinguish and track subjects against the background. The experimental arena was set up, which required calibration using two axes - one vertical and one horizontal, referencing the actual tank size.

Four events (Tail flip, Encounters, Fights and Acoustic signals) and three motility behavioural states (Velocity, Distance moved, Angular velocity) were considered (Table 2, De Vita et al. 2023).



Through the screening of the video of an experienced operator, the number of Tail flip, Encounters and Fights were manually detected and summarised for each trial.

To quantify the acoustic signals produced by crayfish, all .wav files were visualised using spectrograms and oscillograms and the number of emitted signals were summarised every 1 minutes (SASLab software, Avisoft Bioacoustics, Germany) (De Vita et al. 2023).

The motility states were automatically measured by Ethovision with a temporal resolution of 2 seconds.

**Table 5.** Description of the states and behavioral events considered during the experiments

	<b>Behaviour</b>	<b>Description</b>
<b>EVENTS</b>	Acoustic signals	Number of impulses every 1 minute.
	Tail flip	Rapid flexion of the extended abdomen leading to a new position away from the opponent.
	Encounters	When one specimen approached another without showing any signs of aggression or menace.
	Fights	Interaction between two or more specimens leads to a range of competitive and aggressive activities.
<b>STATES</b>	Velocity (cm/s)	Distance moved from the center point of the subject in the unit of time.
	Distance moved (cm)	The cumulative distance travelled by the central point of the subject from the previous sample to the current one.
	Angular velocity (Deg/s)	Change in direction of the longitudinal axis in the unit of time.

### 3.2.1.5 Statistical analysis

The data were tested for goodness of fit of the normal distribution using the Shapiro-Wilk normality test. The data did not show a normal distribution. Mann-Whitney U test was used to compare the events number of encounters, fights, and tail flip between control



and test trials. To evaluate how behavioural state (velocity, angular velocity, and distance moved) and acoustic signals changed between stressed and control animals, generalized linear mixed models (GLMM) were run in R (glmmTMB package) including velocity, angular velocity, distance moved and the number of acoustic signals as dependent variables and test-control as independent factors. In order to avoid self-replication, the factor “time” was included as a random factor. Validation graphs (*e.g.*, residuals versus fitted values, Q–Q plots, and residuals versus the original explanatory variables) were analysed to check the possible model misspecification and the presence of outliers.

### 3.2.2 Results

A total of 138 encounters, 112 fights and 19 tail flips (control group: 57 encounters, 34 fights, 4 tail flips; test group: 81 encounters, 78 fights, 15 tail flips) were recorded for the species *C. quadricarinatus*. A total of 163 encounters, 58 fights and 112 tail flips (control group: 81 encounters, 18 fights, 43 tail flips; test group: 82 encounters, 40 fights, 69 tail flips) were recorded for the species *C. destructor*.

Both species exposed to acoustic disturbance showed significantly more fights than control group (Figure 14, Table 6). The number of encounters and tail flips did not show significant differences between the tested and control animals (Figure 14, Table 6). Both species showed significant alterations in the emission of acoustic signals, with an increase in emissions in *C. quadricarinatus* and a decrease in *C. destructor* (Figure 14, Table 7). Distance moved and velocity showed a significant increase in tested animals in both species. At the contrary, angular velocity significantly decreased in tested *Cherax destructor* (Table 7, Figure 14).





**Table 6.** Means and standard error of the values of behavioral states and events of *Cherax quadricarinatus* and *Cherax destructor* exposed (test) and not exposed (control) to an acoustic stimulus and results of Mann-Whitney U Test.

	<i>Behaviour</i>	<i>Control</i>	<i>Test</i>	<i>U-Mann Test</i>
<b><i>Cherax destructor</i></b>	<i>No. of encounters</i>	11.4±1.66	16.2±3.89	<i>W</i> = 14; <i>p</i> =0.83
	<i>No. of fights</i>	6.8±1.62	15.6±1.93	<i>W</i> = 1; <i>p</i> <0.05
	<i>Tail flip</i>	0.80±0.37	3.00±2.07	<i>W</i> =9; <i>p</i> =0.52
<b><i>Cherax quadricarinatus</i></b>	<i>No. of encounters</i>	16.2±3.62	16.4±2.99	<i>W</i> = 12; <i>p</i> =1
	<i>No. of fights</i>	3.60±1.81	8.00±2.23	<i>W</i> = 2; <i>p</i> <0.05
	<i>Tail flip</i>	8.60±6.43	13.8±7.81	<i>W</i> = 10; <i>p</i> =0.66

**Table 7.** Result of Generalised Linear Mixed Model on distance moved, velocity and angular velocity and acoustic signal between test and control, including time as a random effect divided by species (a) *Cherax destructor* and (b) *Cherax quadricarinatus*.

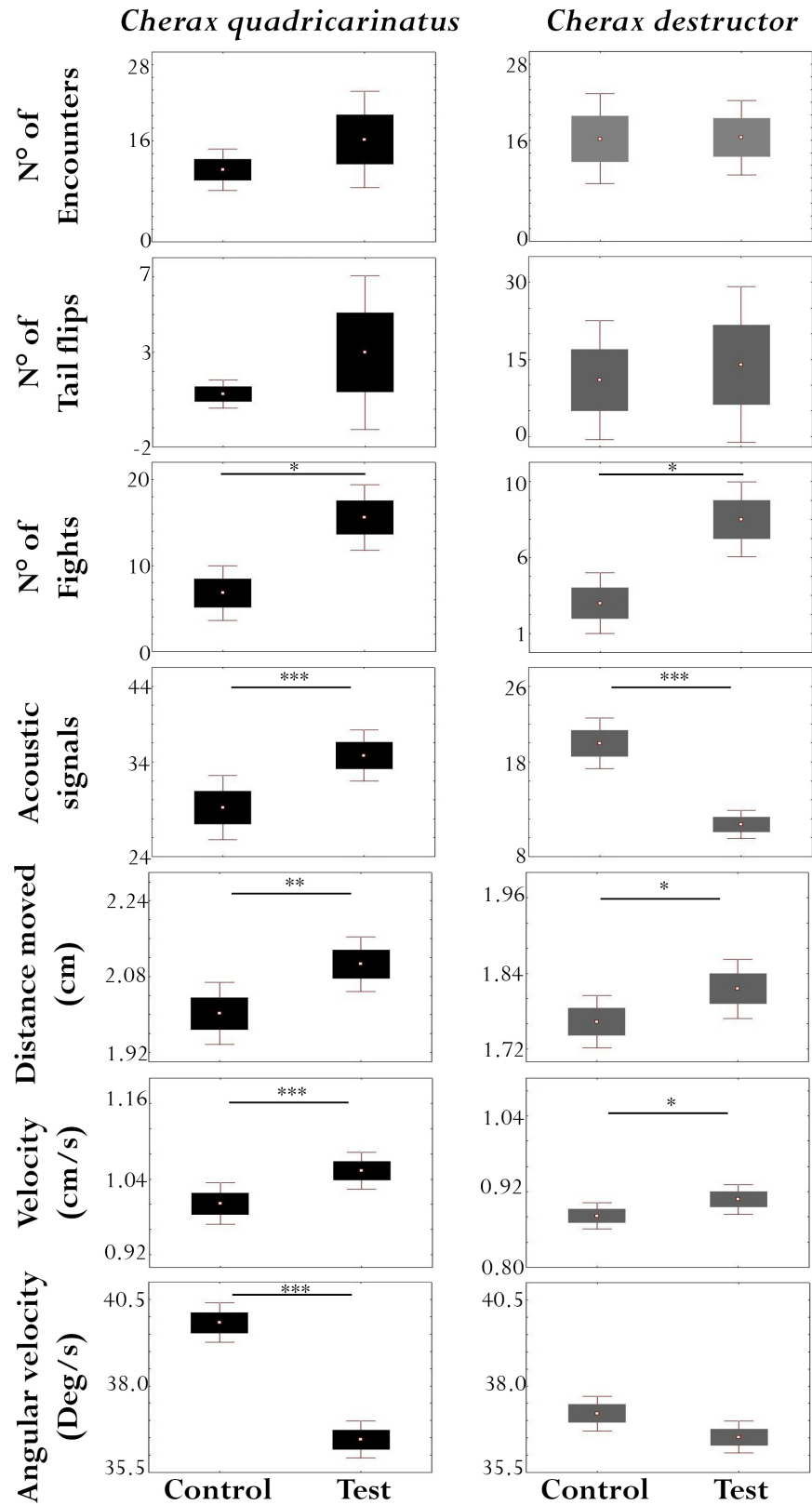
a

<b><i>Generalised Linear Mixed Models (GLMM) species Cherax destructor</i></b>					
<i>Variables</i>		<i>Estimate</i>	<i>SE</i>	<i>t-Value</i>	<i>p-Value</i>
<b><i>Distance moved</i></b>	<i>Intercept</i>	0.566	0.006	92.43	<0.001
	<i>TESTv CONTROL</i>	-0.016	0.008	-2.007	<0.05
<b><i>Velocity</i></b>	<i>Intercept</i>	1.17	0.013	87.61	<0.001
	<i>TESTv CONTROL</i>	-0.031	0.015	-2.055	<0.05
<b><i>Angular velocity</i></b>	<i>Intercept</i>	0.026	0.0002	108.5	<0.001
	<i>TESTv CONTROL</i>	0.0005	0.0003	1.56	0.11
<b><i>Acoustic signal</i></b>	<i>Intercept</i>	2.93	0.05	57.80	<0.001
	<i>TESTv CONTROL</i>	0.564	0.024	22.84	<0.001



b

<b>Generalized Linear Mixed Models (GLMM) species <i>Cherax quadricarinatus</i></b>					
<b>Variables</b>		<i>Estimate</i>	<i>SE</i>	<i>t-Value</i>	<i>p-Value</i>
<b>Distance moved</b>	<i>Intercept</i>	0.499	0.005	85.05	<0.001
	<i>TESTv CONTROL</i>	-0.024	0.0079	-3.10	<0.01
<b>Velocity</b>	<i>Intercept</i>	1.06	0.013	79.4	<0.001
	<i>TESTv CONTROL</i>	-0.049	0.014	-3.39	<0.001
<b>Angular velocity</b>	<i>Intercept</i>	0.025	0.0002	104.7	<0.001
	<i>TESTv CONTROL</i>	0.002	0.0003	6.7	<0.001
<b>Acoustic signal</b>	<i>Intercept</i>	3.34	0.036	91.06	<0.001
	<i>TESTv CONTROL</i>	-0.16	0.017	-9.76	<0.001



**Figure 14.** Behavioural states and events resulting significantly different between the control and test groups in *Cherax destructor* and *Cherax quadricarinatus*. Box shows



mean  $\pm$  25th to 75th percentile; error bars:  $1.96^* \pm \text{SE}$ . Statistical differences between control and acoustic-exposed animals are shown as  $***p < 0.001$ ;  $**p < 0.01$ ;  $*p < 0.05$ .

### 3.2.3 Discussion

In this study two species (*C. quadricarinatus* and *C. destructor*) of freshwater crayfish of the genus *Cherax* were used to study the impact of noise on behavioural parameters. The major literature is focused on the effects of the anthropogenic noise on marine species, but despite the freshwater soundscape is also heavily impacted by anthropogenic noise (Rountree et al. 2020) and freshwater species are also negatively affected (Celi et al. 2012), there is a critical lack of data on the effect of noise on freshwater species. Both species studied here have considerable commercial value and they are farmed in many parts of the world (Jones & Ruscoe 2000, Coughran et al. 2009, Ghanawi & Saoud 2012). In recent years there has been an attempt to give increasing importance to animal welfare in farming conditions. The stress in aquaculture facilities could be caused by multiple potential sources, the effects of which can lead to reduce welfare and compromised health conditions of farmed animals, which ultimately affects the profitability of the aquaculture industry. Among them the noise impact has been poor studied (Bart et al. 2001, Filiciotto et al. 2013). Given the high resilience, tolerance to a wide range of conditions (Meade et al. 2002, Khan & Nuggeoda 2007, Baudry et al. 2022) and ease of maintenance in the laboratory of *C. quadricarinatus* and *C. destructor*, these could actually serve as good models to better understand the impact of acoustic stimuli on behavioural dynamics and biochemical parameters that reflect stress conditions.

Our study showed as, when exposed to acoustic stress, both species showed a significant increase in the number of fights, while no significant changes were found in the number of encounters and tail flips. This result suggests an increase of agonistic approaches under



noise condition. In crayfish, dominance relationships is mediated by aggressions behaviours allowing them to acquire higher quality resources (Zulandt et al. 2008). A similar response to our results was found in *Crangon crangon* exposed to noise with an increase level of aggression rate (Lagardare 1982). Other similar responses was found in *Carcinus maenas* exposed to stressful conditions (Dissanayake et al. 2009). The authors highlighted as a reduced physiological condition resulted in an increased competitive ability. The chemical-physical environment, including environmental noise, is particularly relevant for aquaculture and our results highlighted as it could influence the aggression of crayfish. An increase in fighting could result in physical damages to the animals and consequently to a decrease in the market value of the product and the risk of mortality.

In our study the number of acoustic signals produced by *C. destructor* and *C. quadricarinatus* resulted affected by acoustic stress, but while in *C. quadricarinatus* the sounds increase significantly, in *C. destructor* the opposite occurs. Noise can cause different responses in the communication mechanism of animals. Some animals attempted to compensate for the noise increasing their vocal effort for successful communication (Lombard effect) (Picciulin et al. 2012, Holt & Johnston 2014), other reduced the emission (De Jong et al. 2018; Ceraulo et al. 2021). Few studies have investigated the impact of noise on the emission rate of crustacean's acoustic signals. In snapping shrimp (*Athanas nitescens*, *Alpheus macrocheles*, *Alpheus glaber*) the exposition to impulsive acoustic stimuli caused an increase of number and amplitude of snaps with potential effects on the energy balance of the animals and on the probability of detection by predators (Spiga 2016). *C. quadricarinatus* e *C. destructor* respond differently to acoustic stress, trying to compensate for the disturbance by increasing or decreasing the emission of signals.



Although acoustic communication has not yet been demonstrated in these species, a recent behavioural study by De Vita et al. (2023) identified the signals emitted by *Cherax destructor*, hypothesising a sensitivity to these signals. Crustaceans are known to be sensitive to the velocity component of water vibrations and possess sense organs (Breithaupt & Tautz 1990, Popper et al. 2001). While, it was demonstrated that *C. destructor* have specialised sensory hairs on their claws (chelipeds) that respond to water vibrations (Tautz & Sandeman 1980), no-studies about acoustic perception of *C. quadricarinatus* are available. The different responses found for these species suggest a different role of signals emission, but further studies are needed to investigate this aspect. Behavioural states resulted affected by noise in both species. Animals exposed to acoustic stress showed an increase in velocity and distance moved. The angular velocity significantly decreases only in *C. quadricarinatus*, even if the same trend can be found also in *C. destructor*. Many studies have been focused on the effects of noise on crustacean's motility parameters, showing different responses. In the other crayfish *Procambarus clarkii* the exposition to an acoustic stimulus similar to our (Linear sweep: 0.1-25 kHz with a peak amplitude of 148 dB re 1  $\mu$ Pa rms at 12 kHz frequency) did not reveal significant differences in motility (Celi et al. 2012). In other crustacean species, the exposure to boat noise increased locomotor activity (velocity, distance moved) of *Palinurus elephas* (Filiciotto et al. 2014); whereas the *Palaemon serratus* spent more time resting (Filiciotto et al. 2016). Ship Noise (135-140 dB re 1  $\mu$ Pa, frequency range: 100Hz-2kHz) in *Nephrops norvegicus* reduced locomotion activity (Solan et al. 2016). The increasing of motility behaviours could determine an increased consumption of the energy balance. This, combined with the increase of agonistic interactions and aggressive behaviours, could be particularly relevant considering the ecological aspects of these species, indeed changes on locomotor behavioural states can have implications for the



reproduction, survival, and growth of this species, as demonstrated for other decapod crustaceans (Herrnkind et al. 1975, Spanier et al. 1988, Lawton 1995). As previously described for fish (Boisclair & Sirois 1993), the associated metabolic costs may impair various activities, including reproductive functions, food acquisition, and regulation in response to environmental disturbances. The combination of acoustic signals variation and behavioural indicators (such as posture, isolation, lack of appetite or others) can be a useful tool to identify animal malaise (Ginovart-Panisello et al. 2020).



### 3.3 Impact of noise at biochemical level

The species *Cherax destructor* and *Cherax quadricarinatus* have demonstrated a certain level of tolerance to environmental pollutants (Baudry et al. 2022, Khan & Nugegoda 2007). A recent study established a baseline of cellular and biochemical parameters for both *Cherax spp.* species to identify the welfare status of these animals (Mauro et al. 2022). To date, there are no published studies on the potential effects of underwater noise on these species.

This objective of this study is to investigate the influence of acoustic stimuli on the biochemical parameters of the freshwater crayfish species *Cherax quadricarinatus* and *Cherax destructor*. By analysing changes in total protein concentration, pH, osmolarity, and enzyme activities, we aimed to gain a thorough understanding of the impact of acoustic stimuli on crayfish stress-related physiological parameters.

#### 3.3.1 Materials and Methods

##### 3.3.1.1 Collection and housing of animals

The details of this paragraph are explained in the materials and method section 3.2.1.1

##### 3.3.1.2 Rationale and experimental procedures

The experimental procedures are explained in the previous paragraphs (see section 3.2.1.2 materials and methods). At the conclusion of the experimental phase, the animals were captured using a net and placed on ice for ten minutes to induce "cold anesthesia" (reducing metabolic activity levels) and allowing the successive sampling (Figure 15). Then, samples were immediately collected from the animals and, after their recovering, they were transferred to a tank.

##### 3.3.1.3 Acoustic stimulus

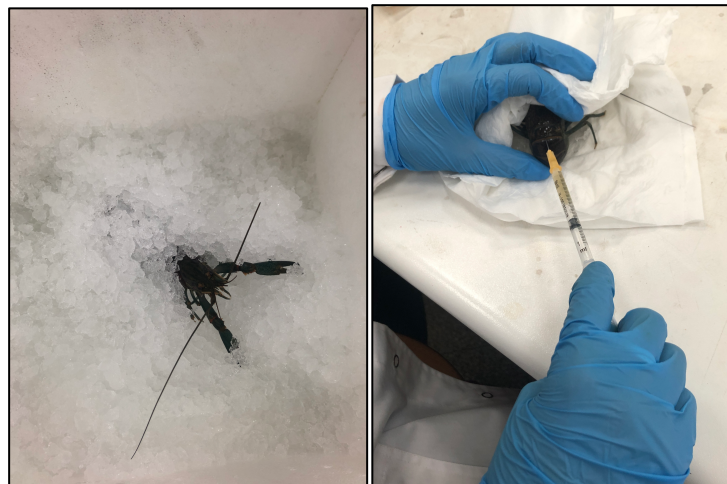




The acoustic stimulation used in the study was a linear sweep from 1 to 20 kHz, lasting 1 second and repeated for 45 minutes (Figure 13). The characteristics are described in the previous paragraphs (see 3.2.1.3 Acoustic stimulus)

#### 3.3.1.4 Haemolymph collection

One millilitre of haemolymph was collected from each animal by inserting a 21-gauge needle into the pericardial sinus at the base of the first abdominal segment using anticoagulant solution (0.2 M sodium cacodylate with 1% glutaraldehyde) (Figure 15). The sample, kept on ice, was immediately centrifuged at 800×g for 10 minutes at 4 °C to separate the cells from the cell-free. After centrifugation, a cell pellet was obtained and used for enzymatic assays while cell-free samples were used to assess total protein, osmolality and pH values.



**Figure15.** Cold anesthesia and haemolymph collection.

#### 3.3.1.5 Enzymatic Assay



The cells were crushed on ice through the potter for 5' using RIPA 1X added with antiprotease 1:200. Then the samples were sonicated for 3', centrifuged at 4°C for 20 min at 15500g. For enzymatic assay the supernatant of cellular lysate obtained were used.

Alkaline phosphatase and esterase activity were measured using 96-well plate by incubating equal volumes of sample and buffer. For alkaline phosphatase the buffer used was 4mM p-nitrophenyl phosphate in 100mM ammonium bicarbonate containing 1mM MgCl<sub>2</sub>, pH 7.8 at 30°C, while for esterase the buffer used was 0.4 mM p- nitrophenyl myristate substrate in 100 mM buffer ammonium bicarbonate containing 0.5% Triton X-100, pH 7.8, 30 °C. Enzyme activities were evaluated at 405 nm every five minutes for one hours and was expressed in U/μg and calculated as:

$$(\text{Abs/ min}) \times (1000/\text{Eb}) \times (V_f/V_i)$$

where Abs/min is the absorbance value measured for each sample divided by 60 min,  $V_f$  and  $V_i$  indicates the final and initial volume of the plate well and Eb is an experimental constant (18.4 for alkaline phosphatase activities and 16.4 for the esterase). One unit of activity was defined as the amount of enzyme required to release 1 μmol of p-nitrophenol produced in one min.

Peroxidase activity was measured using 96-well plate incubated for 30 min 50 μl of sample with 100 μl of TMB (3,3', 5,5' tetramethylbenzidine). The reaction was stopped with 50 μl of 2 M sulphuric acid (H<sub>2</sub>SO<sub>4</sub>). 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.

### *3.3.1.6 pH, osmolality and total protein evaluation*

pH was evaluated on cell-free samples, using a pH-meter with a microelectrode (pH 8 bench meter from XS Instrument). Osmolality was estimated using an osmometer (Roebing, Messtechnik Berlin, Germany) on cell-free samples and total proteins were



calculated on the cell pellet and on cell-free samples. The protein concentration values were evaluated by Bradford assay (Bradford, 1976).

#### 3.3.1.7 Statistical analysis

The data were tested for goodness of fit of the normal distribution using the Shapiro-Wilk normality test. The data did not show a normal distribution. Mann-Whitney U test was used to compare the biochemical parameters (total protein levels, osmolarity, pH, and enzyme activities) between control and test trials.

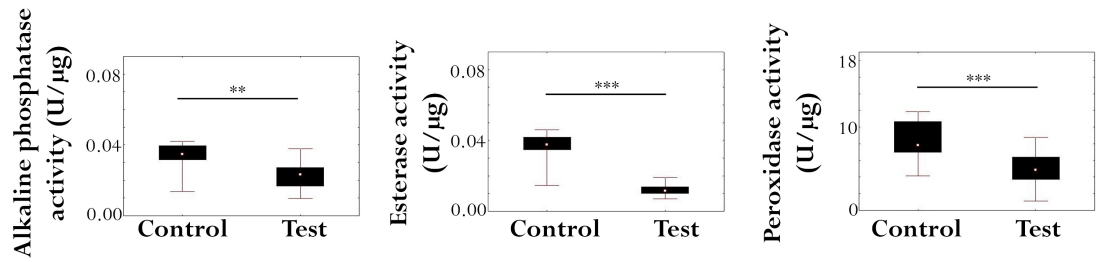
### 3.3.2 Results

#### 3.3.2.1 Enzymatic Assay

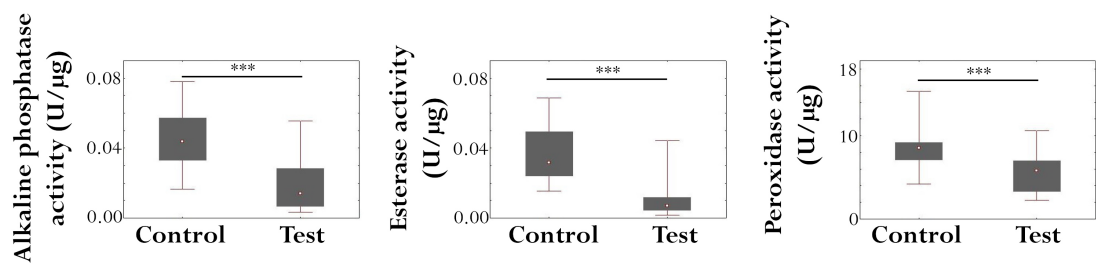
Enzyme activities of both *Cherax spp* respond to noise similarly. Alkaline phosphatase and esterase activity significant decrease in tested *C. quadricarinatus* and in *C. destructor* respect to the control. Regarding antioxidant enzyme, also peroxidase activity significant decreased in tested crayfish respect to the control in both *Cherax spp* (Table 8, Figure 16).



### *Cherax quadricarinatus*



### *Cherax destructor*



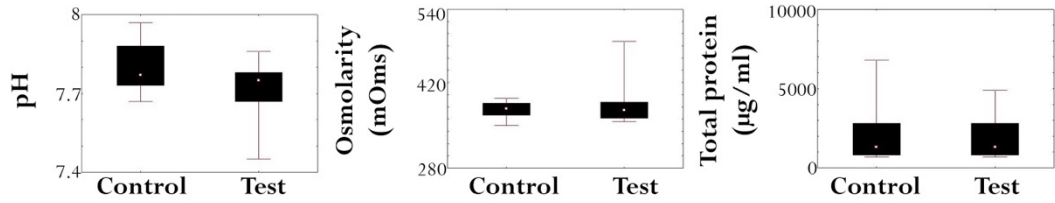
**Figure 16.** Alkaline phosphatase, esterase and peroxidase activity of *Cherax quadricarinatus* and *Cherax destructor*. Statistical differences between control and acoustic-exposed animals are shown as \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ . Show mean  $\pm$  25th to 75th percentile; error bars:  $1.96^* \pm SE$ .

#### 3.3.2.2 pH, Osmolarity and Total Protein evaluation

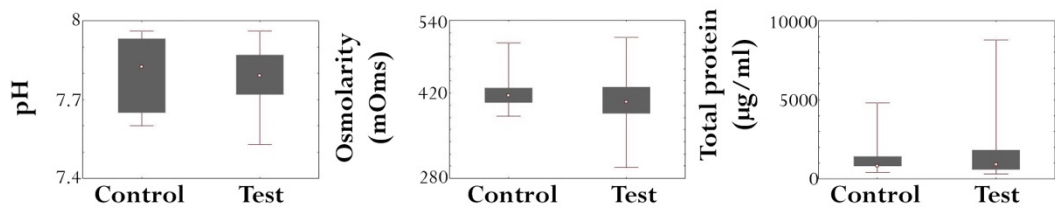
pH, osmolarity and total protein level showed similar results comparing experimental animals with control animals, showing no significant differences (Table 8, Figure 17).



### *Cherax quadricarinatus*



### *Cherax destructor*



**Figure 17.** pH, osmolality and total protein of *Cherax quadricarinatus* and *Cherax destructor*. Show mean  $\pm$  25th to 75th percentile; error bars:  $1.96^* \pm SE$ .



**Table 8.** Means and standard deviations of enzyme activity values on the significant cell lysate and values of pH, Total protein, Osmolarity levels in cell-free haemolymph of *Cherax quadricarinatus* and *Cherax destructor* exposed to an acoustic stimulus and results of Mann-Whitney U Test.

	<i>Variables</i>	<i>Control</i>	<i>Test</i>	<i>U-Mann Test</i>
<b><i>Cherax quadricarinatus</i></b>	<i>Alkaline phosphatase activity (U/μg)</i>	0.033±0.007	0.022±0.007	<i>W</i> = 183; <i>p</i> <0.01
	<i>Esterase activity (U/μg)</i>	0.036±0.007	0.012±0.003	<i>W</i> = 223; <i>p</i> <0.001
	<i>Peroxidase activity (U/μg)</i>	8.4±2.3	5.1±1.9	<i>W</i> = 194; <i>p</i> <0.001
	<i>pH</i>	7.79±0.02	7.71±0.02	<i>W</i> =157; <i>p</i> =0.066
	<i>Total Protein (μg/ml)</i>	1980±438	2006±377	<i>W</i> = 109; <i>p</i> =0.90
	<i>Osmolarity (mOsm/kg<sup>-1</sup>)</i>	375±3.25	382±7.4	<i>W</i> = 108; <i>p</i> =0.86
	<b><i>Cherax destructor</i></b>	<i>Alkalne phosphatase activity (U/μg)</i>	0.04±0.01	0.018±0.014
<i>Esterase activity (U/μg)</i>		0.03±0.01	0.011±0.011	<i>W</i> =207; <i>p</i> <0.001
<i>Peroxidase activity (U/μg)</i>		8.39±2.8	5.1±3.0	<i>W</i> = 181; <i>p</i> <0.001
<i>pH</i>		7.78±0.03	7.78±0.02	<i>W</i> = 119.5; <i>p</i> =1
<i>Total Protein (μg/ml)</i>		1289±304	2033±667	<i>W</i> = 102.5; <i>p</i> =0.93
<i>Osmolarity (mOsm/kg<sup>-1</sup>)</i>		426±10	409±12	<i>W</i> =118.5; <i>p</i> =0.34



### 3.3.3 Discussion

The modulation of biochemical parameters is an important indicator of stress condition. In fact, the biological response of aquatic organisms to different types of stresses for example hypoxia (Le Moullac et al. 1998, Zheng et al. 2022), viral infection (Dolar et al. 2020), environmental or anthropogenic stressors (Perazzolo et al. 2002, Filiciotto et al. 2014) involves changes at biochemical and cellular levels which can affect changes in behaviour of organisms. The mechanisms through which these stressors can mediate the effects on hemolymphatic parameters are not fully understood in decapods even if it is known that crustaceans lack of adaptive immunity and they are totally dependent on their innate immune (Mengal et al. 2023).

In our study, for the first time we evaluated enzymatic activities (alkaline phosphatase, esterase and peroxidase), osmolarity, pH, and protein concentration on *Cherax spp* to understand the effects of noise.

Alkaline phosphatase, esterase and peroxidase are enzymes which represent a part of the first line of defense in nonspecific immune responses in crustaceans (Duan et al. 2015). They have been used as biomarkers to understand, for example, the effect of environmental conditions (Perazzolo et al. 2002) and contaminants on many aquatic invertebrate species (Marenkov et al. 2018, Han et al. 2022). Alkaline phosphatase is a metalloenzyme, its role is to catalyze nonspecific hydrolysis of phosphate monoesters and, under stress conditions, this is involved in the degradation of carbohydrates, foreign proteins, and lipids to protect the individual from pathogen infection (Xue & Renault 2000).

On the other hand esterase is an enzyme present in different forms for different substrates and perform the hydrolysis of the ester bond (Hannam et al. 2008). Several scientific studies have shown that changes in these enzymatic activities can be related to the



performance and survival of invertebrate organisms (Yang et al. 2012, Wala et al. 2014). Despite this, no one in the literature has so far evaluated the effects of acoustic stress on alkaline phosphatase and esterase activity at the haemolymphatic level in *C. quadricarinatus* and *C. destructor*. Our results showed that in both species these enzymes significantly decreased in animals exposed to acoustic stress compared to controls. The decrease in enzymatic activities in crustaceans under stress could be the result of a combination of complex physiological effects that impair the synthesis, regulation and activity of the enzyme. Alkaline phosphatase for example, is known to be an enzyme involved in various physiological processes in crustaceans such as shell mineralization, digestion and detoxification (Sekaran et al. 2021). The decrease found in conditions of acoustic stress in these enzymes activities could be due to various factors such as metabolic (Guppy & Withers 1999, Anestis et al. 2007, McElroy et al. 2012) which negatively influence the production and activity of enzymes; impaired immune system as the body's energy and resources are redirected to deal with stress and repair any cellular damage (Segerstrom 2010); damage to the tissues or cells of the organs where the enzyme is produced (Wen et al. 2017)(e.g., the hepatopancreas) reducing its synthesis and secretion capacity (Zhang et al. 2024); induction of the production of reactive oxygen species (ROS)(Lushchak 2011), which can damage proteins, lipids and DNA, and enzymes, being proteins, are susceptible to oxidative damage (Qiu et al. 2011, Sahlmann et al. 2017); gene expression with alteration in the transcription and translation of enzymes, *i.e.* a reduction in the quantity of mRNA available for enzymatic synthesis with a consequent reduction in the enzymes released into circulation (Horst et al. 2007, Spriggs et al. 2010). To confirm this, in the past other authors have evaluated alkaline phosphatase to study the effects of other stressful conditions such as chemical contaminants in *Cherax spp.* For example, in *C. destructor* an increase in enzymatic activity was observed in the





hepatopancreas and gills in animals exposed to atrazine (Stara et al. 2018) while in *C. quadricarinatus* a decrease in animals exposed to cypermethrin after 72 hours and 96 hours, highlighting how the levels of contaminant and therefore toxicity could influence enzyme expression (Yuan et al. 2019). As regards mollusks, several authors have observed different trends in esterase activity in animals exposed to pesticides, showing how the enzymatic response can be species-specific (Galloway et al. 2002, Solé et al. 2010). Vioque-Fernández et al. (2007), evaluated esterase activity in the digestive gland, muscle tissue and nervous system of *Procambarus clarkii* when exposed to pesticides, highlighting a decrease in activity. Regarding acoustic stress, alkaline phosphatase and esterase activity has been evaluated in other invertebrate species and in particular in the cell-free coelomic fluid of *Arbacia lixula* (Vazzana et al. 2020b) and in the digestive gland of *Mytilus galloprovincialis*. In these two studies the responses were different although the acoustic stimuli were the same. In the first case the enzyme levels increased significantly compared to the controls while in the second case they decreased, highlighting how the enzyme levels found may depend on the species analyzed, the type of stress and the sample used (Vazzana et al. 2020a). In fact, in one case the enzyme levels were evaluated in the cell free and then released into circulation following stress, while in the second they were evaluated at the site of production probably immediately after release into circulation. In light of all this, it is possible to assume that the decrease found in these enzymatic activities is related to metabolic changes due to the energy involved in the observed behavioral responses. However, it should not be excluded that at the experimental time in which the parameters were evaluated the enzymes released into circulation were consumed to respond to stress and re-establish homeostasis.

In addition to enzymes of the hydrolase class, antioxidant enzymes play an important role in maintaining physiological homeostasis (Frías-Espéricueta et al. 2022). It is known that



many sources of stress can increase ROS levels in organisms by triggering the antioxidant response (Stara et al. 2018). Peroxidase is an enzyme that is part of the first line of defense against reactive oxygen species (ROS) that can cause damage to DNA, proteins, and lipids (Wang et al. 2012). It is involved in protection against oxidative stress, catalyzes the decomposition of hydrogen peroxide ( $H_2O_2$ ) into water and oxygen, thus contributing to the protection of cells from oxidative damage. However, under stress conditions, several mechanisms can negatively influence the activity of this enzyme, in fact in our study the peroxidase activity significantly decreased in both shrimp species subjected to acoustic stimulation. This decrease could be the result of a complex set of factors that include the increase in oxidative load, *i.e.* an excessive production of reactive oxygen species which can overwhelm the ability of peroxidase to neutralize them, leading to a decrease in enzymatic activity (Neves et al. 2000, Krishnan & Ramasamy 2006); depression of the immune system and therefore a lower level of enzyme production due to chronic stress; the redistribution of energy resources in stressful situations towards immediate vital functions such as escape (Adamo 2012, Robles-Romo et al. 2016). In crustaceans, especially aquaculture crustaceans, antioxidant activities have often been studied to evaluate the effects of different diets or maintenance conditions (Chen et al. 2021, Shehata et al. 2023). For example, in *Cherax cainii* and *Litopenaeus vannamei*, peroxidase activity increased in the hepatopancreas and haemolymph after addition of dietary selenium improving antioxidant capacity (Nugroho & Fotedar 2014). In *C. quadricarinatus*, however, peroxidase activity increased in the gills after spotting syndrome virus, but then decreased sharply during longer infection times (Wang et al. 2012). In *Ruditapes decussatus*, peroxidase activity in the gills significantly decreases after exposure to metals, indicating them as biomarkers for monitoring environmental pollution (Geret et al. 2003). Regarding acoustic stress, peroxidase was evaluated only in *Mytilus*



*galloprovincialis* and *Arbacia lixula*, showing, in agreement with esterase and alkaline phosphatase, an increase in the free cells of *A. lixula* and a decrease in the digestive glands of *M. galloprovincialis* (Vazzana et al. 2020b) highlighting again a probable high release into circulation in one case and the use of the peroxidase enzyme to re-establish homeostasis in another case. In light of all, could be possible that the decrease in enzymatic activity observed in our study may be due to oxidative stress caused by the acoustic stimulus and therefore, to an excessive production of reactive oxygen species compared to the ability of the peroxidase to neutralise them. Furthermore, this could also depend on a different redistribution of energy resources to implement the behavioral responses previously described.

Finally, exposure of both *Cherax* species to an acoustic stimulus produced no significant effects on total protein, pH, and osmolarity. It is known that a change in pH can prove harmful since a stable internal pH is essential for many physiological and biochemical processes (Hans et al. 2014). Indeed, the ionic composition of the hemolymph is of fundamental importance because it determines the transmembrane ionic gradients that provide the driving force for the flux of sodium, calcium and potassium. The pH of the hemolymph in crustaceans is a crucial parameter for: the maintenance of homeostasis (Wheatly & Henry 1992) and for the correct functioning of numerous physiological processes that concern enzymatic activities as many enzymes function in a very specific pH range (Wang et al. 2024); the balance of various ions that are essential for nerve transmission (Serova et al. 2020), muscle contraction (Lamb & Stephenson 1994); the acid-base balance of the body (Wheatly & Henry 1992); the transport of respiratory gases as it can affect the ability of hemocyanin, the respiratory protein in crustaceans, to bind and release oxygen and carbon dioxide (Mangum 1980).



An abnormal pH can compromise cellular respiration (Wilson et al. 1959). Several authors confirm this, for example in *Homarus americanus*, Qadri et al. (2007) suggest that changing hemolymphatic pH alters both the frequency and strength of the heartbeat; while Rose et al. (1998) demonstrated that exercise at any speed can cause a decrease in pH level. Knowing these values is important because exposure to stressful conditions can negatively influence ionic balance, causing extracellular acid-base changes, as observed in *Carcinus maenas* by Boitel & Truchot (1989). In conclusion, since the pH of the hemolymph is essential for the health and survival of crustaceans (Boitel & Truchot 1989) and is a critical parameter for their correct physiological functioning, not having found significant alterations following acoustic stress suggests that this type of acoustic frequency did not affect this biochemical parameter, but also that the organism at this time of exposure manages to implement a series of biochemical responses to re-establish homeostasis without compromising numerous vital processes. Total proteins are another widely used biomarker to evaluate health conditions in crustaceans and play a crucial role in many physiological functions: defense against pathogens, transporters of lipids, metals and other essential molecules, osmotic regulation, respiration (Lorenzon et al. 2011, Stara et al. 2018). Our result showed no significant change in total protein level in *Cherax spp.* under acoustic stress highlighting that this frequencies and time of emission did not negatively affect total protein levels. However, analyzing the literature, this parameter has shown to be very variable and dependent on physiological and environmental variables that must always be considered (e.g. type of acoustic frequencies, amplitude, duration of exposure), on the species and on the sample considered. In fact, following acoustic stimuli, this parameter in other crustacean species (Filiciotto et al. 2014) significantly increased at the haemolymphatic level in stressed animals, while in



agreement with our results they did not show significant changes in individuals of *P. clarkii* (Celi et al. 2012).

Regarding osmolarity, freshwater crustaceans must maintain a higher haemolymph osmolarity and ionic content than marine crustaceans (Bianchini & Wood 2008) and the gills are the main sites of osmoregulation (Pequeux 2023). In terms of osmoregulation,  $\text{Na}^+$  and  $\text{Cl}^-$  ions are known to constitute 90% or more of the hemolymph-osmotic pressure in most crustaceans, and exposure to environmental stressors and pathological agents could result in decreased  $\text{Na}^+$  regulation and  $\text{Cl}^-$  in crustaceans. This issue is important because in aquatic crustaceans the acid-base balance between the animal and its environment is regulated by cation/ $\text{H}^+$  and anion exchangers (Lucu 1990, Onken et al. 1991). Partial or complete loss of osmoregulatory capacity is generally linked to disruptions in osmotic and ionic regulation. In our study, the exposure of the two *Cherax* species to an acoustic stimulus did not lead to significant effects on the osmoregulatory capacity and, in agreement with other authors, the effect of stress on osmotic and ionic metabolism could be time- and dose-dependent (Lignot et al. 2000). The osmolarity levels observed in our animals were very similar compared to other crustacean species (Van Mai & Fotedar 2018). Furthermore, the values obtained were similar to what was reported by Mauro et al. (2022) who analyzed the physiological levels of osmolarity in the hemolymph of the same *Cherax* species, allowing to conclude that the acoustic stimulus used here did not influence the osmolarity of these animals. On the other hand, this cannot be generalized to the entire acoustic frequency/amplitude/exposure time band. The behavioural and physiological responses of both species suggest an ability to perceive sound in the frequency range of 1 to 20 kHz, but further studies are needed to understand the ecological roles of sound in these species.



### 3.4 Impact of noise at the molecular level

Information regarding changes in gene expression after noise exposure in crustaceans is limited (Zhou et al. 2018, Hall et al. 2023), and no one has considered gene expression change on *C. quadricarinatus*. Lu et al. 2021, in their study, evaluated the change in the expression of genes important from the immune and stress point of view after exposure to air in *C. quadricarinatus*.

Based on the work of Lu et al. (2021), in this study we aim to fully understand the impact of noise on this species by analyzing how noise may affect the freshwater crayfish *Cherax quadricarinatus* at the molecular levels. Changes in the expression of genes, such as HSP21, HSP70, HSP90, crustin, and lysozyme could provide a more comprehensive analysis of the effects of such stress.

#### 3.4.1 Materials and Methods

##### 3.4.1.1 Collection and housing of animals

The present study was carried out at the Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF) of the University of Palermo, Italy. The crayfish specimens came from an aquaculture facility in Fiumefreddo di Sicilia, Italy. The carapace, claw and cephalothorax lengths of the male crayfish were  $10.99 \pm 0.92$  cm,  $6.61 \pm 0.83$  cm and  $5.54 \pm 0.87$  cm (mean  $\pm$  SD) and weighed  $58.73 \pm 0.31$  g, and those of the females were  $9.98 \pm 0.80$  cm,  $5.52 \pm 0.37$  cm,  $5.15 \pm 0.61$  cm (mean  $\pm$  SD) and weighed  $56.62 \pm 0.43$  g. Prior to the experiment, all crayfish were acclimatized for one week in tanks (35.6 x 23.4 x 22.8), in dissolved oxygen levels  $> 5.0$  mg/L, a constant temperature of  $21 \pm 1^\circ\text{C}$  and a controlled photoperiod of 12 hours of light and 12 hours of darkness. The crayfish were fed daily with commercial feed (5% of body weight) from Malta Cleyton (Mexico), for up to 24 h prior to sampling.



#### *3.4.1.2 Rationale and experimental procedures*

The crayfish were randomly collected from the individual holding tanks and assigned to the control or test group. A total of 16 animals (8 males and 8 females) were used for the experiment. Specifically, 8 animals were used for the control group (4 males and 4 females) and 8 animals for the experiment (4 males and 4 females). For the experimental trials, each animal was taken from the acclimatization tank and placed alone in the experimental tank, where it was exposed to the acoustic stimulus for 45 minutes. The experimental trials were conducted in a rectangular experimental tank (85 × 50 × 45 cm, water height 26 cm) without any shelter. Like the experimental animals, the control animals were taken from the acclimatization tank and placed alone in the experimental tank, but did not receive any stimuli. At the end of each experiment, both the control and stimulated animals were caught with a net and placed on ice for 10 minutes to induce 'cold anesthesia', which allowed the collection of hemolymph. In detail, the hemolymph was withdrawn from the pericardial sinus of each crayfish using a 1 mL syringe containing an equal volume of ice-cold anticoagulant solution (0.2 M sodium cocodylate + 1% glutaraldehyde, filtered through a 0.22 µm filter). The haemolymph samples were centrifuged at 800 g at 4 °C for 10 min and pellets and supernatant were separated and stored at -80°C.

#### *3.4.1.3 Acoustic stimulus*



The acoustic stimulation used in the study was a linear sweep from 1 to 20 kHz, lasting 1 second and repeated for 45 minutes (Figure 13). The characteristics are described in the previous paragraphs (see 3.2.1.3 Acoustic stimulus).

#### *3.4.1.4 Real-time quantitative PCR*

Total RNA was extracted from the cellular pellet using the RNAqueous Phenol-free total RNA Isolation-Invitrogen Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations (Figure 18). Prior to retrotranscription, the quantity and quality of RNA was measuring using a spectrophotometer (Nanodrop, Thermo Scientific, Delaware USA). First-strand cDNA was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisherScientific) according to the manufacturer's guidelines.

Gene expression was assessed by RT-PCR using the Sybr-Green method with the primers listed in Table 9 (Lu et al. 2021). In our study, the  $\beta$ -actin gene was used as a reference marker for normalisation gene expression. RT-PCR analysis was performed using the Applied Biosystems 7500 real-time system. The reaction mixtures had a volume of 25  $\mu$ L and contained 12.5  $\mu$ L of Power SYBR-Green PCR MasterMix (Applied Biosystems, Waltham, MA, USA), 2  $\mu$ L cDNA reverse and 300 nM primers (forward and reverse). The specificity of amplification was tested by real-time PCR melting analysis. Each reaction was performed in triplicate. The  $2^{-\Delta\Delta C_t}$  method was used to quantify samples (Livak & Schmittgen 2001); transcript levels were normalised to those of  $\beta$ -actin to compensate for variations in the amount of RNA input. Relative expression was assessed as the ratio between the normalized value of the target gene in each treated sample and the normalized value obtained from samples under control conditions





**Figure 18.** Kit RNAqueous Phenol-free total RNA Isolation-Invitrogen by Thermo Fisher Scientific)

**Table 9.** Primers used for Real-time quantitative PCR.

Gene name	Sequence (5' -3')
Heat shock protein 21 (HSP21)	F: ACCACCAGAGTGATGGAGAACA R: ATAGGCGGAAGTGAAGGACC
Heat shock protein 70 (HSP70)	F: AAGCAGCAGCAAGTCTTCACC R: GGGATGCCACTCAACTCAAAC
Heat shock protein 90 (HSP90)	F: GCCGATAAAAAGCACCGTAAA R: CACAGTGGCTTGGTCTTATTGAG
Crustin 2 (CRU-2)	F: CTTCGTCTTGTGGTATTGGCTAT R: TTCTGTTCTGGAAGCAGTGGA
c-type lysozyme (C-LZM)	F: GCAACAGGAACGGTAGCAAG R: GCCACCCAAGCGGAATAT
$\beta$ -actin	F: ATCACTGCTCTGGCTCCTGCTACC R: CGGACTCGTCGTA CTCTCCTTGG

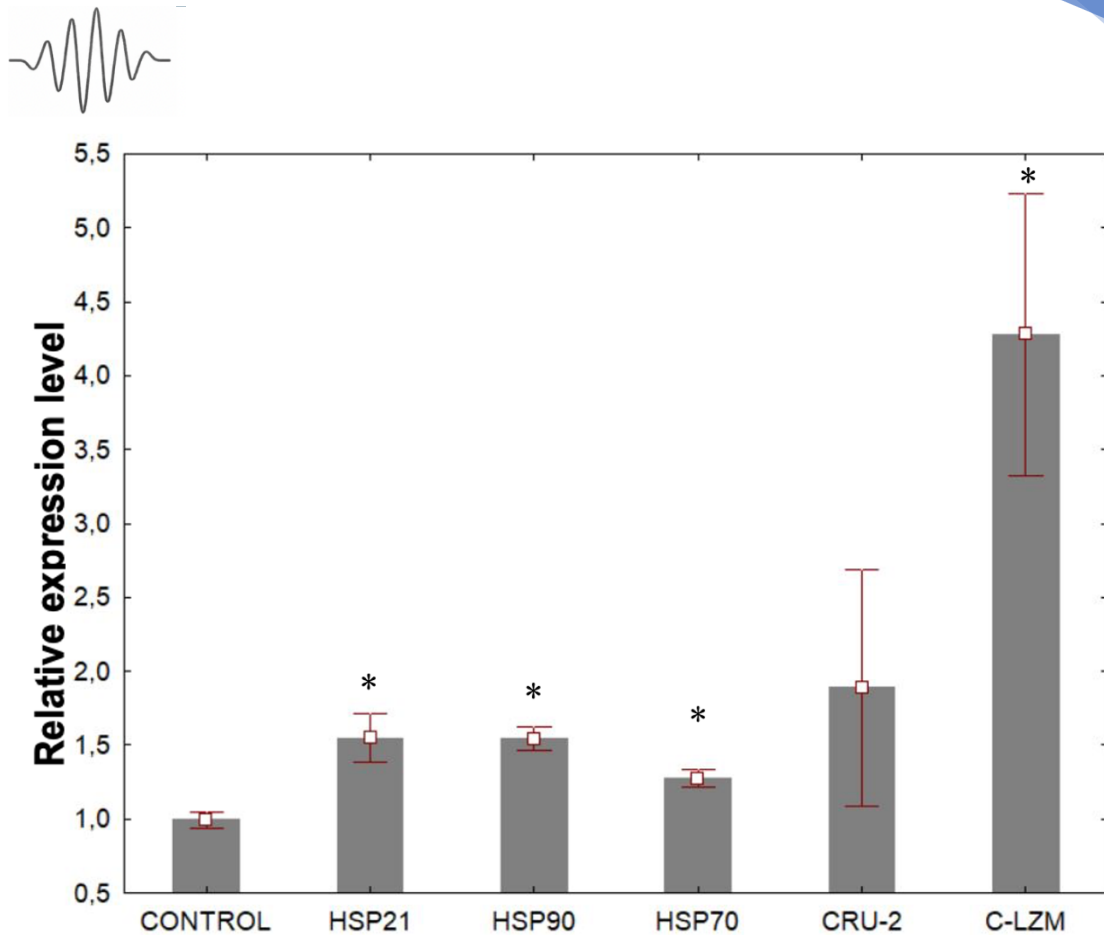


#### 3.4.1.5 Statistical analysis

To evaluate the distribution of the data, the Shapiro-Wilk test was used. For molecular data, Student t-test was used to analyze differences between control-test. A  $p$  value  $< 0.05$  was considered as the level of significance.

#### 3.4.2 Results

Analysis of expression in haemocytes of C-LZM, CRU-2, HSP21, 90, 70 gene of adult of *Cherax quadricarinatus* after acoustic stimuli (sweep linear 1-20kHz) were examined by qRT-PCR (Figure 19). The figure shows that the transcripts was significantly modulated in response to stimuli ( $p$ -value  $< 0.05$ ). In particular HSP21, 90,70 showed a moderate increase after stimuli while C-LZM showed a significant increase and finally CRU-2 was not modulated.



**Figure 19.** Effect of noise on the relative expression of genes in *Cherax quadricarinatus*. Values are given as mean  $\pm$  SD for each gene. In pink females' crayfish and in blue males' crayfish. Significant difference: \* $p < 0.05$

### 3.4.3 Discussion

Noise is a very pervasive and often underestimated form of pollution. It is a pollutant present in both natural and cultivated contexts (Bart et al. 2001, Filiciotto et al. 2013, Rountree et al. 2020). Focusing on farmed animals, it has been shown that in noisy environments the growth and fitness of animals decreases (Lagardère 1982, Ginovart-Panisello et al. 2020). Furthermore, species may show different responses when subjected to pollutants or stressful conditions (Kadiene et al. 2020). For this reason, in this study, we evaluated the impact of a linear scan (1 to 20 kHz) on a decapod crustacean, *Cherax*



*quadricarinatus*, a commercially important species farmed in different parts of the world (Jones & Ruscoe 2000). Among the responses implemented by aquatic organisms, it is known that important biomarkers for understanding the effects of stress conditions are changes in the molecular expression of heat shock proteins (HSPs), crustin and lysozyme. Heat shock proteins (HSPs) represent the most important superfamily of multigens and play a crucial role in environmental adaptation and stress resistance (Cimino et al. 2002, Roberts et al. 2010, Wang et al. 2018). They also play an important role when crustaceans are subjected to acute environmental stress. Among these, the HSP70 genes are the most studied. Previous studies have shown that HSP70, HSP90 and HSP21 play crucial roles in protecting cells from environmental stress (Gao et al. 2014, Junprung et al. 2017). In our study, we investigated changes in the expression levels of two families of HSPs, one low molecular weight (HSP21) and two high molecular weight (HSP70 and HSP90) in hemocytes that play an important role in the primary immune response. Our results show an increase in HSPs gene expressions in *C. quadricarinatus* hemocytes. In agreement with our findings, HSP21 gene expression after pathogen contamination, temperature changes, and increased osmolarity increases in several crustacean species (Rungrassamee et al. 2010, Clark et al. 2013, Gao et al. 2014, Rao et al. 2016, Dai et al. 2020), in *C. quadricarinatus* hemocytes the expression of the HSP21 gene (Wu et al. 2018) increases when the animals are exposed to low temperatures with a cryoprotective role. As for HSP70, the most conserved family and the one most studied to evaluate the effects of environmental stress (Mukhopadhyay et al. 2003). We see increased HSP70 expression in haemocytes of *Eriocheir sinensis*, *Penaeus monodon*, *Procambarus clarkii* after exposure to the pathogen (Wongpanya et al. 2007, Gai et al. 2009, Zeng & Lu 2009). In agreement with our results, it was seen that in *Scylla paramamosain* the expression of HSP70 increases in hemocytes after osmotic and thermal stress (Yang et al. 2013)



hypothesizing that increased expression of Hsp70 could improve the resistance of *S. paramamosain* to salinity and temperature changes. This was also confirmed by Zhou et al. (2018), who demonstrated that in *S. paramamosain* exposed to different linear movements leads to an increase in HSP70 expression (Zhou et al. 2018). Similarly, our study indicated that exposure to acoustic stimuli induces the relative mRNA expression of HSP70, HSP21 and HSP90, indicating the crucial role of heat shock proteins (HSPs) in response to acoustic stimuli.

Antimicrobial peptides (AMPs) are indeed crucial components of the immune system, particularly within the innate immune response and play a significant role in the first line of defense against microbial infections (Bachère et al. 2000). Lysozyme (C-LZM) and crustin (CRU-2) are indeed significant antimicrobial peptides (AMPs) in crustaceans, playing crucial roles in their immune defense systems (Hikima et al. 2003, Yu et al. 2016). Crustaceans do not exhibit adaptive immunity, and antimicrobial peptides represent the first line of defense for these animals. Our results indicated that, compared to control, CRU-2 expression is not modulated. In contrast, C-LZM expression was significantly increased. Our results are in agreement with those of Zhang et al. (2023a), showing upregulation of lysozyme genes in *P.clarkii* after aluminum intoxication. C-type lysozyme, an enzyme that acts as a nonspecific innate immune molecule against bacterial pathogens, is expressed and stored primarily in shrimp hemocytes (Hikima et al. 2003). The observation of increased lysozyme expression in response to stress suggests that their regulation provides further evidence supporting the close link between the environment and immune capacity in shrimp.

Lysozyme certainly plays an important role in achieving homeostasis under conditions of acoustic stress but this aspect requires further investigation.





#### **4. Second Case Study: *Cyrtograpsus angulatus* (Dana, 1851)**

The experiments were conducted during the overseas period in Argentina, in collaboration with the research group of the University of Mar del Plata, Humedales y Ambientes Costeros - Biodiversidad de Invertebrados Marinos group, supervised by Dr. Sal Moyano María Paz. The effect of boat noise, hence anthropogenic sound, and biological sound produced by the fish *Pogonias courbina* (natural predator of the crab *Cyrtograpsus angulatus*) was evaluated on the foraging behavior and biochemical parameters of *Cyrtograpsus angulatus*.

*Cyrtograpsus angulatus* is an important predator in its natural habitats, contributing to regulating invertebrate populations and maintaining ecological balance (Spivak et al. 1996). Also, due to its high abundance is considered a key species (Spivak et al. 1994). Furthermore, this species is sensitive to environmental variations and disturbances in coastal ecosystems; its presence and abundance can be used as indicators of marine habitat health and integrity. Monitoring crab populations can provide valuable information on the health status of coastal ecosystems and the impact of human activities on the marine environment. The species lives in muddy tidal flats, but in the early life stage, to avoid predation, young crabs prefer refuges such as the reefs constructed by the polychaete *Ficopomatus enigmaticus* (Luppi et al. 2001). The species is part of the macrofauna of the Mar Chiquita coastal lagoon (37°44'28.138" S, 57°24'58.549" W, Province of Buenos Aires, Argentina). The lagoon is part of UNESCO's World Network of Biosphere Reserves, with a brackish water body connected to the sea (Reta et al. 2001). Coastal lagoons play a key role in coastal ecosystems and serve as feeding areas for many species (Pérez-Ruzafa et al. 2011). Despite various legal entities for management, both provincial and national, anthropogenic noise is present in the lagoon; a recent study



performed by Ceraulo et al., (2020) described the temporal and spatial patterns of biophony, geophony, and anthrophony characterising the soundscape in the Mar Chiquita lagoon. Foraging behavior is essential for survival and reproductive success, and it has been demonstrated that noise can influence such behavior in various species (Purser & Radford 2011, Evans et al. 2019, Yantén et al. 2022), including crustaceans (Wale et al. 2013). Crustaceans are capable of perceiving the presence of a predatory fish through the detection of their emitted sounds, and increased predation risk can lead to significant behavioural changes (Hughes et al. 2014). Regarding anthropogenic noise, it has been observed that it directly affects various behavioral and physiological parameters in crustaceans (Celi et al. 2012, 2015, Filiciotto et al. 2014, 2016). Foraging behaviour can be directly or indirectly influenced by noise; it can cause stress in affected animals, leading to a decrease in fitness and consequently causing a reduction in appetite or indirectly reducing locomotion (Mendl 1999). Noise can also act as a distraction, leading to alterations in cognitive processes involved in foraging behavior, such as food detection (Lupien & McEwen 1997). It has been demonstrated that *C. angulatus* produces impulsive acoustic signals (Ceraulo et al. 2022). However, there are no studies that consider the effect of noise and vocalisations of the fish predator *Pogonias courbina* on foraging behaviour of this species.

## 4.1 Materials and Methods

### 4.1.1 Collection and housing of animals

The study was conducted at the University of Mar del Plata, Argentina. Sixty adult crabs were collected in the coastal lagoon of Mar Chiquita (Figure 21 and 22). Only adult males with carapace sizes between 27 and 33 mm were considered, with a mean  $\pm$  standard deviation of  $31 \pm 0.65$  mm. The collected crabs were transported to the laboratory and





placed in aquariums ( $30 \times 35 \times 25$  cm, 26 L capacity, filled with 3 L of natural filtered seawater), with a density of four crabs/aquarium, under conditions of a controlled photoperiod of 12 hours of light and 12 hours of darkness, salinity of 23 g/l, continuous aeration and temperature  $24 \pm 2$  °C.



**Figure 21.** *Cyrtograpsus angulatus* in its natural environment: Mar Chiquita coastal lagoon.



**Figure 22.** Map of Mar Chiquita (Buenos Aires, Argentina). The yellow dot indicates where the animals were collected.

#### 4.1.2 Rationale and experimental procedures

Before each experiment, the animals were deprived of food for 72 hours. Each test involved the use of a single animal selected at random from the aquariums and placed in the center of circular experimental tanks (1.2 m diameter and 1.5 m depth) filled with seawater to a depth of 1.2 m. The animal, after an acclimatisation period of five minutes (during which no sound was emitted), was subjected to the treatment for 30 minutes (Table 10). In particular, 20 treatments were conducted where the animals were subjected to the sound of a fish predator of the species (*Pogonias courbina*), 20 treatments where the animals were subjected to noise from motorboat passages and 20 controls, where the animal was not subjected to any sound. After the five minutes of acclimatisation, a piece of squid of 1 cm<sup>2</sup> was placed in the center of the experimental tank and at the same time



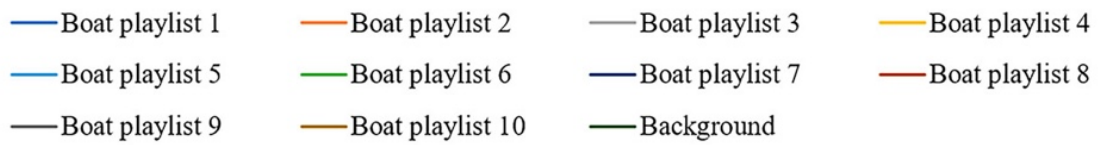
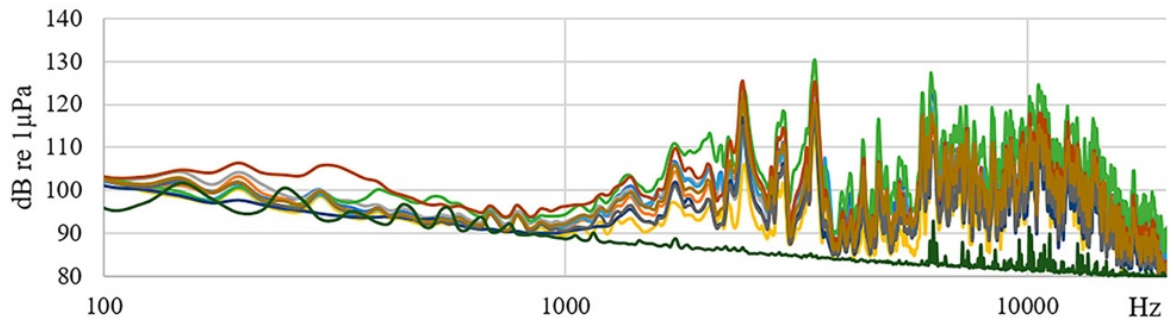
the video recording and the emission of the acoustic stimulus were started only in the case of the fish and boat sound treatments. At the end of the experiment, individuals were caught with a net and placed in a  $-20^{\circ}\text{C}$  freezer for ten minutes for anesthesia and euthanasia. The animals were then dissected and samples of hepatopancreas and gills were obtained and stored at  $-80^{\circ}\text{C}$  until subsequent biochemical analyses.

#### 4.1.3 Acoustic stimuli

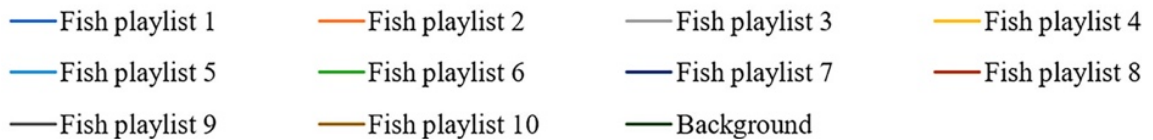
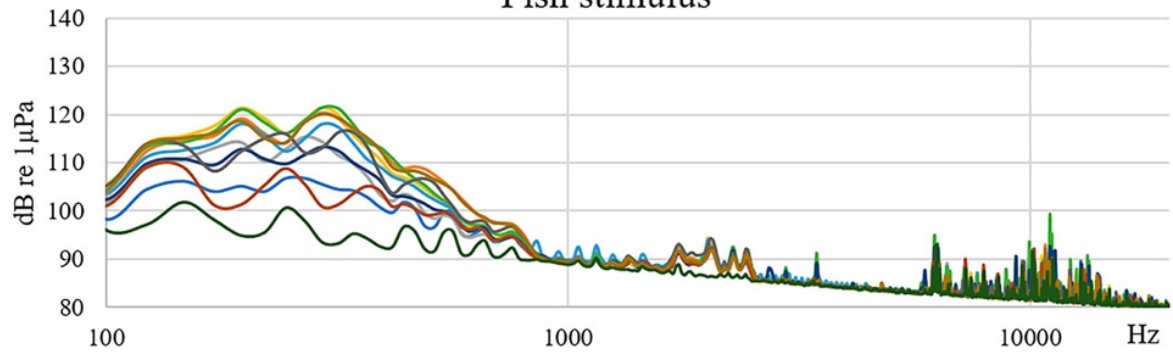
The acoustic recordings of the stimuli used in the experiments were obtained from recordings in the natural environment, Mar Chiquita coastal lagoon, made by Ceraulo et al. (2020) and subsequently used by Sal Moyano et al. (2023) which isolated the sound of the fish from the soundscape of the lagoon by applying a 1kHz low-pass filter while no filter was applied for the sound of the motorboat. Specifically, ten different 7-second playlists were played in loop both for the fish stimulus and the boat one (Figure 23). An underwater loudspeaker (Model UW30, Lubell, Columbus, Ohio, USA) driven by a signal generator (type APXII-300, American Pro, 230 V, 50 Hz, China) was used to generate the acoustic stimuli in tank.



### Motorboat stimulus



### Fish stimulus



**Figure 23.** Power spectrum of all playlists of the boat and fish stimuli (FFT size 4096, hamming window, resolution 24.4 Hz). Credit: Sal Moyano et al. (2023).



**Table 10.** Summary of experimental trials, sounds administered, number of animals, and total duration of trials.

<b>STIMULUS ADMINISTERED</b>	<b>N° ANIMALS</b>	<b>N° REPLICATION</b>	<b>TRIAL DURATION</b>
No sounds (Control)	1	20	30 min
Biological sound (Fish predator)	1	20	30 min
Anthropogenic sound (Motorboat)	1	20	30 min
<b>Total</b>	<b>60</b>	<b>60</b>	<b>30 h</b>

#### 4.1.4 Video monitoring system and analysis

The behaviour of the crabs was recorded using a video camera (AVTech, model KPD136ZALTP) located on the top the tank. The video camera was connected to a generic digital recorder and the video files were saved in .avi format and subsequently exported via USB.

Five behavioural parameters were considered: time taken to locate food, No. of interrupted feeding, velocity, angular velocity, animal/food distance. The first two parameters were calculated manually by an operator viewing the videos; in particular, for the parameter time taken to locate the food, the time taken by the animal to grab the food was considered, for the number of feedings interrupted it was counted if the animal leaves the food for 2 min and continues to move in the tank.

The velocity, angular velocity, animal/food distance were analysed using EthoVision XT 9.0 software (Noldus Information Technology, The Netherlands) in semi-automatic mode. The Ethovision system can distinguish and track subjects from the background based on their greyscale/brightness. To do this it is necessary to set up an experimental arena. The dimensions of the experimental arena were calibrated taking the actual



dimensions of the pool as reference, using two calibration axes, one vertical and one horizontal. The software provides as output the average values for each parameter of the extract considered. All behavioural parameters assessed are described in Table 11.

**Table 11.** Description and analysis of the behavioral parameters considered during the experiments.

<b>BEHAVIOURS</b>	<b>DESCRIPTION</b>	<b>ANALYSIS METHOD</b>
<b>TIME TAKEN TO LOCATE FOOD (MIN)</b>	Time taken to locate food considered as first time grasping food	Operator
<b>N° FEEDING INTERRUPTED</b>	It is considered interrupted whenever the animal leaves the food within 2 min and continues to move in the tank	Operator
<b>VELOCITY (CM/S)</b>	Distance moved from the center point of the subject in the unit of time.	Noldus Ethovision
<b>ANGULAR VELOCITY (DEG/S)</b>	Change in direction of the longitudinal axis in the unit of time.	Noldus Ethovision
<b>DISTANCE BETWEEN ANIMAL/FOOD (CM)</b>	The distances between the center of the animal and the food, calculated up to the detection of the food	Noldus Ethovision





#### 4.1.5 Samples collection

The extractions from the tissues considered, such as the hepatopancreas and gills (Figure 24), were carried out following the indications of Kwon et al. (2010) and modifications by Mitton et al. (2020). The two tissues considered were homogenised (1:10 w/v) in extraction buffer (0.1 M Tris-HCl, pH 7.8) and centrifuged at 10000 g for 15 minutes at 4 °C. After centrifugation, the supernatant was obtained and used for enzymatic assays glutathione-S-transferase (GST) and catalase (CAT) activity and to assess total protein.



**Figure 24.** Extractions from the gills and hepatopancreas of *Cyrtograpsus angulatus*.

#### 4.1.6 Total protein

Total protein was determined on the supernatants by the Bradford, (1976) method with bovine serum albumin (BSA) as standard protein, using a microplate spectrophotometer (Epoch Bio Tek).



#### 4.1.7 Enzymatic Assay

Glutathione-S-Transferase (GST) activity was measured in the supernatants following the Habig & Jakoby (1981) methodology. The absorbance at 340 nm generated by the conjugation of 1 mM glutathione (GSH) with 1 mM of 1-chloro-2,4-dinitrobenzene (CDNB) was monitored for 6 min at 25 °C (pH 7) using a microplate spectrophotometer (Epoch Bio Tek).

Catalase activity (CAT) activity was analysed in the supernatants following Rao et al. (1997) determining the initial of H<sub>2</sub>O<sub>2</sub> decomposition at 240 nm at 25 °C for 5 min using a microplate spectrophotometer (Epoch Bio Tek). The reaction buffer contained EDTA (5 mM) and H<sub>2</sub>O<sub>2</sub> (10 mM) in Tris-HCl (1 M, pH 8.0).

#### 4.1.8 Statistical analyses

Behavioral and biochemical data did not show a normal distribution. Both for behavioural (time spent on local food, velocity, angular velocity, n° feeding interrupted, distance animals/food) and biochemical (protein content and enzyme activity in gills and hepatopancreas) parameters the Kruskal-Wallis tests and hoc multiple comparison tests were used to explore differences between treatment (control, Motorboat, fish).

## 4.2 Results

### 4.2.1 Behavioural parameters

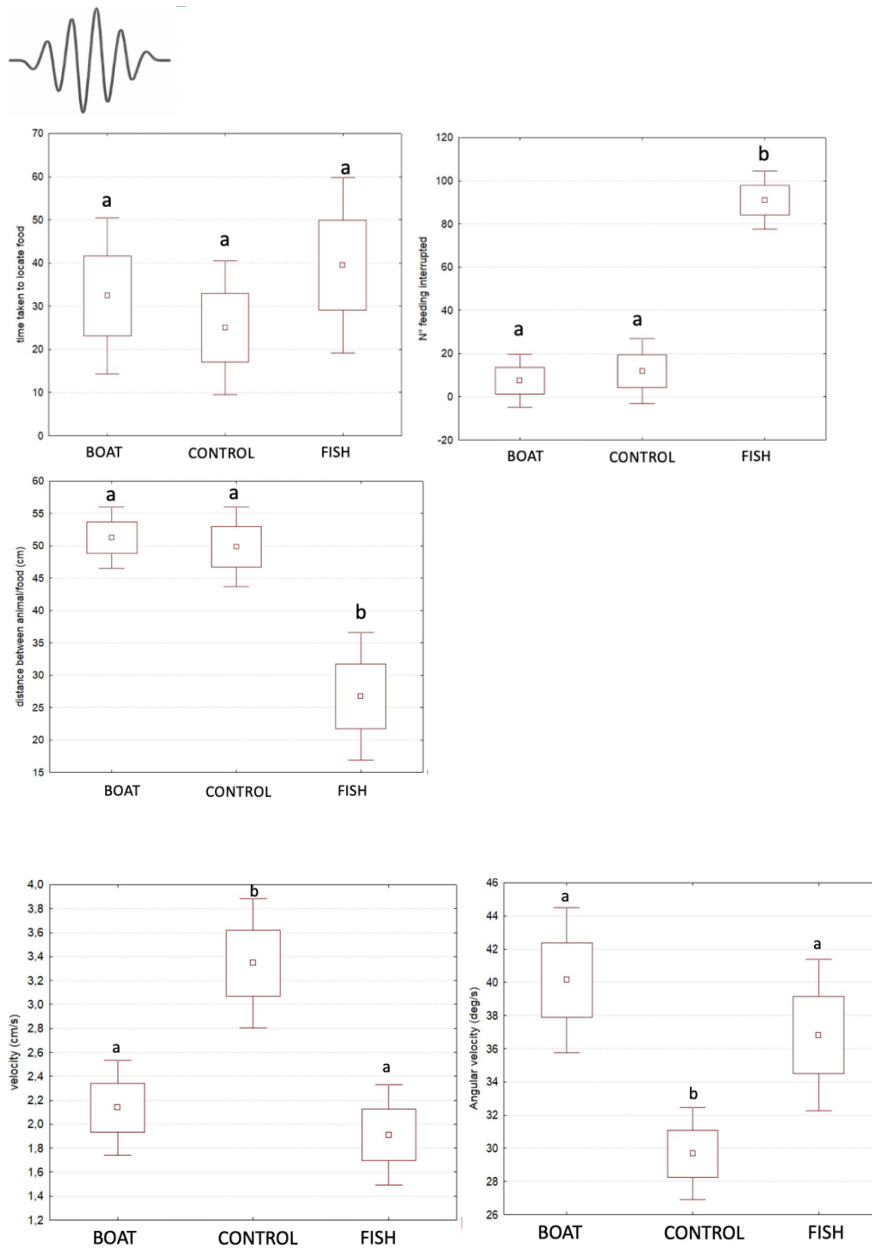
From the analysis of the considered behavioral parameters, the “time spent on local food” did not show significant differences among the treatments (Kruskal-Wallis multiple comparison test:  $H=0.059$   $p=0.97$ ) (Figure 25, Table 12). For the parameter “n° interrupted feeding”, the analysis showed significant differences when the animals were





subjected to the fish treatment, in particular in the animals subjected to the predatory fish stimulus, they were significantly greater than in the control and the boat stimulus (Kruskal - Wallis multiple comparison test  $H=30.72$   $p<0.001$ ) (Figure 25, Table 12). For the animal/food distance parameter, the analysis highlighted significant differences when the animals were subjected to the fish treatment, in particular in the animals subjected to the fish stimulus predators are significantly smaller than the control and boat stimulus (Kruskal-Wallis multiple comparison test  $H=12.44$   $p<0.001$ ) (Figure 25, Table 12).

For the velocity parameter, the analysis highlighted significant differences when the animals are subjected to the fish treatment and the boat treatment, in particular the animals subjected to both stimuli tend to significantly decrease the velocity compared to the control ones (Kruskal-Wallis multiple comparison test  $H =16.57$   $p<0.001$ ) (Figure 25, Table 12). For the angular velocity parameter, the analysis highlighted significant differences when the animals were subjected to the fish treatment and the boat treatment, in particular animals subjected to both stimuli tended to significantly increase the angular velocity compared to the control (Kruskal-Test of Wallis multiple comparison  $H=12.87$   $p<0.001$ ) (Figure 25, Table 12).



**Figure 25.** Behavioral parameters in the different treatments (boat stimulus, control and fish stimulus). Shows mean  $\pm$  25th to 75th percentile; error bars:  $1.96^* \pm SE$ ; The different letters indicate significant differences ( $p < 0.05$ ) between the different treatments

**Table 12.** K-W Multiple Comparisons  $z'$  values per behavioral parameters, in red the values with  $p < 0.05$ .

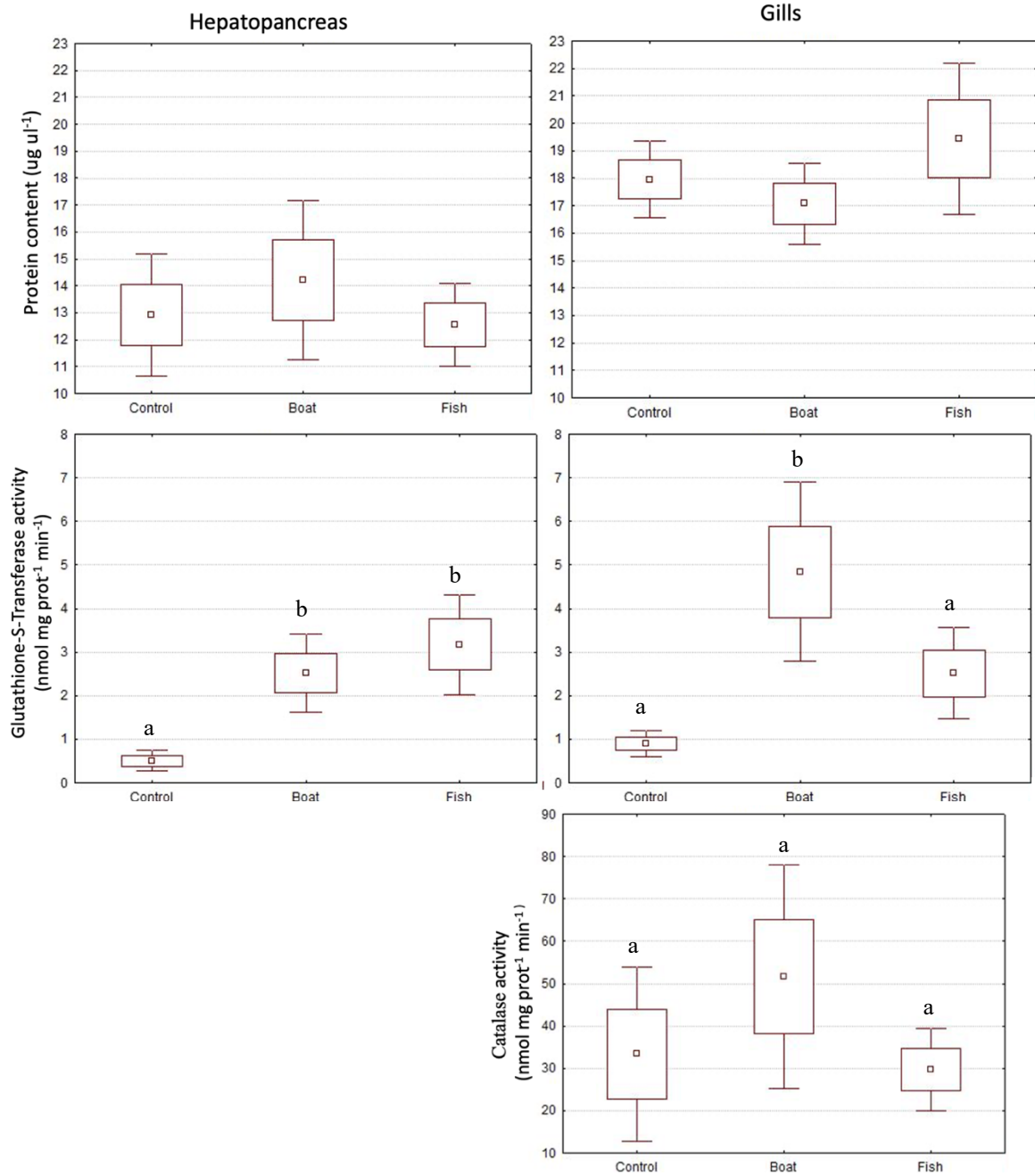
Behavioral parameters		Boat	Control	Fish
Time taken to locate food	Boat		0.21	0.19
	Control	0.21		0.022
	Fish	0.19	0.022	
N° feeding interrupted	Boat		0.25	4.24
	Control	0.25		4.65
	Fish	4.24	4.65	



<b>Distance between animal/food</b>	Boat		0.009	3.06
	Control	0.009		3.05
	Fish	3.06	3.05	
<b>Velocity</b>	Boat		3.02	0.84
	Control	3.02		3.87
	Fish	0.84	3.87	
<b>Angular velocity</b>	Boat		3.49	1.04
	Control	3.49		2.45
	Fish	1.04	2.45	

#### 4.2.2 Biochemical parameters

The protein content did not change in fish and boat treatments in either of the two tissues considered, hepatopancreas (Kruskal - Wallis multiple comparison test  $H=0.26$   $p>0.05$ ) and gills (Kruskal - Wallis multiple comparison test  $H=1.46$   $p>0.05$ ) (Figure 26 and Table 13). In contrast, the activity of Glutathione-S-Transferase (GST) significantly increased in the hepatopancreas after exposure to both stimuli, boat and fish stimuli (Kruskal - Wallis multiple comparison test  $H=12.7$   $p>0.01$ ) (Figure 26 and Table 13). While in the gills, GST activity showed a significant increase only after exposure to boat noise (Kruskal - Wallis multiple comparison test  $H=10.03$   $p<0.01$ ) (Figure 26 and Table 13). Due to technical problems, it was not possible to calculate the catalase values in the hepatopancreas, while in the gills no significant differences were observed in CAT activity following exposure to the treatments (Kruskal - Wallis multiple comparison test  $H=2.47$   $p>0.05$ ) (Figure 26 and Table 13).



**Figure 26.** Biochemical parameters in the different treatments (boat stimulus, control and fish stimulus). Shows mean  $\pm$  25th to 75th percentile; error bars:  $1.96^* \pm \text{SE}$ ; The different letters indicate significant differences ( $p < 0.05$ ) between the different treatments



**Table 13.** K-W Multiple Comparisons  $z'$  values per biochemical parameters, in red the values with  $p < 0.05$ .

Tissues	Biochemical parameters	Control	Boat	Fish
<b>Hepatopancreas</b>	Protein content	Control		0.05
		Boat	0.46	0.40
		Fish	0.05	0.40
<b>Gills</b>	Protein content	Control		0.43
		Boat	0.76	1.19
		Fish	0.43	1.19
<b>Gills</b>	Catalase (CAT)	Control		0.19
		Boat	1.44	1.30
		Fish	0.19	1.30
<b>Hepatopancreas</b>	Glutathione-S-Transferase (GST)	Control		<b>3.34</b>
		Boat	<b>2.93</b>	0.57
		Fish	<b>3.34</b>	0.57
<b>Gills</b>	Glutathione-S-Transferase (GST)	Control		1.79
		Boat	<b>3.16</b>	1.41
		Fish	1.79	1.41

### 4.3 Discussions

In this study, the effect of administering boat sounds and a biological sounds from a predator fish (*Pogonias courbina*) on the biochemical parameters and their potential influence on the foraging behaviour of the crab *Cyrtograpsus angulatus* were evaluated. Parameters such as the time taken to locate food, the number of feeding interruptions, and the distance between the animal and food were considered. It was observed that anthropogenic noise does not appear to directly affect the foraging behaviour; the animals did not show changes in food detection or interruptions during feeding. These findings are consistent with those of Hubert et al. (2018), who, in their study on *Carcinus maenas* and *Crangon crangon*, demonstrated that exposure to a broad-spectrum of anthropogenic sounds did not influence the feeding activity of the animals studied. However, while anthropogenic noise does not directly affect the foraging behaviour of *Cyrtograpsus angulatus*, it does have effects on the locomotion of the animals. As crustaceans are



mobile species, their locomotor movements are highly linked to important ecological and biological aspects.

Crabs subjected to boat noise exhibit reduced movement velocity, with many studies showing a decrease in response activity to various behaviors after exposure to anthropogenic boat activities. A decrease in locomotion has been described in *N. granulata* after exposure to various anthropogenic sound sources (Filiciotto et al. 2019, Snitman 2022). In the *Palaemon serratus*, a decrease in encounters between conspecifics and increased resting time was observed (Filiciotto et al., 2014). Additionally, in the lobster *Nephrops norvegicus*, locomotion activity is reduced, and exposure to noise also suppressed burying behaviour (Solan et al. 2016). Boat noise also decreased agonistic behaviour, typical of the *Procambarus clarkii* (Celi et al., 2013). Regarding angular velocity, it tends to increase, with animals tending to rotate more in the presence of noise. This response could be correlated with a distraction effect, as other studies have shown that anthropogenic noise increases the level of distraction in animals (Chan et al. 2010), including crustaceans during foraging behaviour (Wale et al. 2013). In hermit crabs, it has been previously proposed that the distracting effect of ship noise causes interruptions in information gathering by the animals (Chan et al. 2010).

Administering the predator sound (fish) does not alter the food detection time but increases the number of interruptions and decreases the distance between the animal and the food. This, combined with reduced movement velocity and increased body rotation, might suggest an alert behavior by the frightened animal trying to understand where the predator is coming from. Crabs *Panopeus spp.* and *N. granulata* have shown reduced locomotion and decreased feeding time when exposed to acoustic stimuli corresponding to predators (Hughes et al., 2014; Snitman et al., 2022). Buscaino et al. (2011) demonstrated that *Palinurus elephas* exhibit an alert behaviour in the presence of a



predator. Foraging behaviour is influenced differently depending on the source of the administered sound, highlighting that the diversification of responses depends on the sound administered.

There are no studies explaining how *C. angulatus* detects sound, but a recent study conducted by Ceraulo (2022) demonstrated that males emit acoustic signals during agonistic behaviours and the species has behavioral responses during the administration of sounds (Sal Moyano et al., 2021). Behavioral studies are important for evaluating the auditory threshold of species (Popper and Hawkins, 2021), and considering that the species emits signals and responds to them, we may not know the auditory threshold, but we can confirm that it is capable of perceiving them.

Numerous studies in literature confirm the susceptibility of crustaceans to acoustic pollution, as evidenced not only by behavioural changes but also by variations in biochemical parameters (Celi et al. 2012, 2015, Filiciotto et al. 2014, 2016).

In this study, three biochemical parameters were considered: total protein and the enzymatic activity of GST and CAT in two tissues of the animal, namely gills and hepatopancreas. Both gills and hepatopancreas are highly susceptible to environmental variations. Gills, which are in direct contact with the environment for the exchange of water and gases (Henry et al. 2012), and the hepatopancreas, representing the central oxidative reactions organ of crustaceans (Vogt G 2020).

The administration of boat sounds or fish sounds did not result in significant changes in total proteins in the tissues we considered. This finding aligns with the results of Celi et al. (2012) in their study on the effects of sweep on *Procambarus clarikii*, where total proteins present in the hemolymph remained unchanged after exposure to acoustic stress. However, other studies on lobsters have shown increases in total proteins. For instance, in *Palinurus elephas*, animals subjected to acoustic stress exhibited significant increases



in total proteins present in the hemolymph (Filiciotto et al., 2014). Similarly, in *Palaemon serratus*, total proteins increased both in the hemolymph and in the brain in animals stressed by noise (Filiciotto et al., 2016). Additionally, Snitman et al. (2022) studied total protein in gills of *Neohelice granulata*, observing an increase in total proteins in animals subjected to boat noise as well as in those subjected to predator sounds. Changes in total proteins are used as an indicator of stress in crustaceans, not only for acoustic stress but also for other stressors (Chen et al. 1994, Chen & Cheng 1995), although it appears to depend on various variables including sex, size, and environmental factors (Chen & Cheng 1993).

In the hepatopancreas, the activities of GST tend to significantly increase in animals subjected to both predator and boat stimuli. GST possesses diverse properties implicated in detoxification during oxidative stress (Sherratt & Hayes 2001). Several studies have showed that anthropogenic noise causes stress to organisms, and the risk of predation can also increase oxidative stress (Regnault & Lagardere 1983, Hughes et al. 2014). Antioxidant enzymes, such as GST or CAT, represent efficient defense systems and protective mechanisms against oxidative stress and for the maintenance of cellular homeostasis (Jemec et al. 2010). From the results, we observed how the antioxidant activity of GST increases in the hepatopancreas in animals subjected to anthropogenic noise as well as predator sounds. This increase in activity can be explained by the fact that the hepatopancreas is an organ where numerous oxidative reactions occur and is also the site of free radical generation (Röszer 2014, Vogt G 2020). In the gills, a different response occurs in animals subjected to the two considered stimuli. Boat noise increases enzyme activity in the gills, unlike predator sounds, which do not vary enzyme activity. This demonstrates how GST activity depends not only on the type of stimulus administered but also on the tissue considered. A similar response was seen in *Dreissena*





*polymorpha*, where the biomarker response depended on the tissue and environmental contaminant levels to which it was subjected (Osman et al. 2007), and also in *Neohelice granulata*, where GST activity was influenced by the type of tissue and sound stress considered (Snitman et al. 2022).

Regarding CAT, results were obtained only in the gills, showing no change in enzyme activities in animals subjected to the sounds considered. CAT is a significant bioindicator in invertebrates, although it exhibits wide intraspecific variability (Livingstone 2003). CAT appears to increase in the gills of other crustaceans subjected to pollutants (Borković et al. 2008), as well as in those exposed to acoustic stress (Snitman et al. 2022). Noise from boats caused an increase in GST activity and no change in CAT activity in the gills of *Cyrtograpsus angulatus*. Some authors suggest that the absence of changes in antioxidant enzyme activity in situations where animals are subjected to stress is due to the activation of other antioxidant enzymes, thus inhibiting the activation of other defense mechanisms (Power & Sheehan 1996, Almeida et al. 2005).



## 5. Conclusions

In this PhD project, we used *Cherax spp* and *Cyrtograpsus angulatus* species to explore the effect of anthropogenic noise on crustacean using both the behavioural, the biochemical and genes expression changes. In the behavioural study, we found that these species emit acoustic signals. To verify that these sounds are used in communication, other studies would be necessary, but considering that animals modify their behaviour in the presence of a conspecific or in response to their signal, which can be chemical, postural or acoustic, we could hypothesise that these animals use these signals to communicate with conspecifics, to court each other, to try to keep the group cohesive, and avoid clashes. Furthermore, the species is able to perceive sounds, in fact exposure to noise has altered the behavioural patterns and biochemical responses of *Cherax destructor* and *Cherax quadricarinatus*. Increased motility or fighting events cause animals to use their energy reserves for behaviours that are not essential for their survival. A decrease in enzymatic activity highlighted that acoustic signals can negatively influence the health status of these animals. On the other hand, since it is known that biochemical responses depend both on the species and on the acoustic stimulus used, the absence of variations in other biochemical parameters in our study does not exclude the lack of effects but suggests to further evaluate the effects of acoustic signals different. From a molecular point of view, we are also witnessing an increase in the expression of genes attributed to stress and lysozyme, an antimicrobial enzyme that plays an essential immune role in crustaceans. Lysozyme certainly plays an important role in achieving homeostasis under conditions of acoustic stress, although further investigations are needed to confirm this, furthermore the increased expression level of lysozyme in response to acoustic stress provides further evidence in support of the close link between the environment and immune capacity in shrimp. Regarding the effects of noise and



predator sounds on the foraging behavior of the crab *Cyrtograpsus angulatus*, it was found that anthropogenic noise does not have a direct effect on foraging behavior but leads to increased GST activity in the hepatopancreas and in the gills, confirming that the acoustic stimulus causes stress in animals, unlike biological sound which directly modifies foraging behaviour, increasing the number of interruptions in feeding. Both stimuli lead to a decrease in locomotor activities, confirming the effects of the stimuli on the species. Furthermore, the comparison between biological and anthropogenic sounds provides valuable information on animals' reactions to different types of sounds, indicating the complexity of organisms' responses to different sounds. Finally, this project highlights not only that anthropogenic noise has effects on aquatic organisms, but the change in essential behaviours for species or a variation in parameters essential for immune defense show the need to adopt management measures to mitigate these impacts. Understanding the mechanisms through which noise influences behavior and physiological functions is fundamental to developing effective strategies. Furthermore, the research demonstrates the importance of an integrated vision that starts from the visible effects of noise, at a behavioural level, up to the molecular one. This approach can provide a comprehensive view of the effects of this pollutant in a context increasingly influenced by human activities.

Furthermore, with the increase in aquaculture facilities, the number of aquatic species farmed is always increasing, these can be equally sensitive to noise and prolonged exposure to noise of anthropogenic origin could negatively influence the growth, well-being and reproduction of the species. Therefore, noise management should be considered an integral part of sustainable agricultural practices, with the implementation of measures to reduce noise exposure and mitigate its negative effects on farmed species. This



approach would not only help improve the welfare conditions of farmed animals, but could also have positive impacts on the productivity and quality of aquatic products.

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