Responses of Aquatic Invertebrates to Anthropogenic Sound Across Different Life History Stages

---

Edward L. Bolger

A thesis submitted in partial fulfilment of the requirements of Edinburgh Napier University, for the award of Doctor of Philosophy

July 2022

I declare that the work in this thesis was carried out in accordance with the requirements of Edinburgh Napier University's Research Degrees Framework and that it has not been submitted for any other academic award or professional qualification. Except where indicated by specific reference in the text, the work is my own. Work done in collaboration with, or with the assistance of, others, is indicated as such at the start of each chapter. My own contributions to any collaborative work are here also highlighted. Any views expressed in this thesis are my own, and do not represent the views of Edinburgh Napier University.

SIGNED: .....[redacted]..... DATE: 01/07/2022

I declare that all work within this thesis has been planned, carried out, and reported on, with consideration of, and in accordance with, Edinburgh Napier University's Code of Practice on Research Integrity.

SIGNED: .....[redacted]..... DATE: 01/07/2022

Abstract

Underwater soundscapes have grown considerably louder over the previous century due to increases in anthropogenic noise, with increasing evidence that man-made sound can negatively impact aquatic fauna. Despite their vital ecological roles, and increasing commercial importance, the effects of sound on aquatic invertebrates are understudied in comparison to marine mammals and fish, despite their ability to perceive sound. Even fewer studies have considered the responses of *early life* aquatic invertebrates to underwater sound, despite their often greater vulnerability to environmental stressors compared to adults.

Through a series of controlled laboratory experiments, five model species (Norway lobster *Nephrops norvegicus* (L.), European lobster *Homarus gammarus* (L.), the European flat oyster *Ostrea edulis* (L.), the veined squid *Loligo forbesii* (Steenstrup, 1857) and the water flea *Daphnia magna* (Straus, 1820)) were exposed to playbacks of anthropogenic noise. Two key knowledge gaps were addressed in this thesis: whether, and how 1) continuous noise (the most common anthropogenic noise source in the field) affects the early life stages of aquatic invertebrates and 2) noise impacts on aquatic invertebrates carry over from one life stage to the next e.g. from larva to juvenile or from mother to offspring.

The early life stages of four out of the five model species studied were negatively impacted by noise, demonstrating that noise susceptibility is shared across a functionally and taxonomically diverse range of early life aquatic invertebrates. The experiments also revealed that impacts can carry over from larva to juvenile (*N. norvegicus*) and from mother to offspring (*D. magna*). The scientific evidence generated here will inform future research, legislative decision makers and managers of the importance of considering early life invertebrates as receptors of anthropogenic noise impacts, allowing them to generate more adequate mitigation strategies to uphold their commitments to limiting the impacts of noise on the marine environment.

Acknowledgements

Firstly, I would like to thank my director of studies Professor Karen Diele for her endless enthusiasm, ideas, advice and encouragement. With unwavering support, Karen has always pushed to me to produce my best work and helped me to pursue some unforgettable scientific adventures. Thanks also to my second supervisor Dr Rob Briers for all the advice, encouragement, chats in the hallway and skilful ability to calmy reduce complex problems to simple solutions. I would like to thank Fiona Stainsby for her advice on my project and my wider development as an academic in her capacity as my independent panel chair.

I would like to thank my funder Edinburgh Napier University for the financial support and provision of facilities throughout my PhD. Thanks also go to the Marine Alliance for Science and Technology for Scotland (MASTS) for their financial assistance for enrolment into their graduate school and their Post-Graduate Certificate in Researcher Professional Development. The Nesbitt family and the staff of St Abbs Marine Station are thanked for their generous ability to always find time, space and enthusiastic support for the numerous experiments I have carried out at St Abbs Marine Station. I would also like to thank the staff of the Danish Shellfish Centre for hosting me for the duration of my oyster larvae experiment (chapter 6), and particular thanks to Camille Saurel and Pedro Freitas for their warm welcome to Nykøbing Mors and to Pascal Barreau for keeping my spirits up during periods of troublesome larval rearing.

I would like to acknowledge the contribution of Dr Mark Hartl of Heriot-Watt University who helped with the design and implementation of the biochemical assays used in chapter 3 and kindly hosted me at Heriot Watt University to conduct this work. Thanks also to all the students and volunteers who have helped me in various ways over the years, from cleaning tanks to water-changing larval rearing vessels, there are too

many of you to mention but you were all appreciated, and I hope you got something out of it.

The support of friends and colleagues has been invaluable. I'd like to thank the staff and students of the MASTS graduate school for providing a haven of fellow marine scientists that I could turn to for support and encouragement. Thanks to Dr Matt Wale for all the advice, discussion and pub quizzes. Thanks also to Craig Stenton who was a true partner and steadfast support during the various obstacles we had to scale together. Thanks to Sophie Lane for the good times, support and the beautiful watercolours featured in this thesis. I am lucky to have some lifelong and close friends, both from Giggleswick and Durham, who kept me sane and provided some much-needed reminders that life goes on outside of PhD work, particularly over Covid-lockdown zoom chats. There are too many to mention but they know who they are and know they are appreciated. Thanks to Fergus Voigt for persuading me to pick up my trumpet again; nights spent gigging around the UK with Objectified were a well needed diversion from invertebrate larvae. Finally, I would like to thank my family, specifically my parents Kate and Guy, and my sister Emily, for all the encouragement, laughter, endless support and cups of tea.

Contents

List of Figures and Tables

List of Abbreviations *(and definitions where appropriate)*

ANOVA: Analysis of variance

c: Speed of sound, in water this is approximately 1,480 m s^{-1}

dB: Decibel, a relative unit of measurement widely used in acoustics. The dB is a logarithmic unit, describing the ratio between a measured value and a reference value. In this thesis, the dB is used to describe sound intensity.

DPH: Days post hatching

EC: European Commission

EU: European Union

f: Frequency, the number of times a periodic function repeats in a given time. In acoustics, this is measured as cycles per second (unit: Hertz)

GES: Good environmental status, a concept defined within the MSFD and consisting of 11 qualitative descriptors

Hz: Hertz, the unit of frequency for sound. One hertz in one cycle per second.

kHz: kilohertz (1000 Hz)

MPA: Marine Protected Area

MSFD: Marine Strategy Framework Directive, an EU directive put in place to protect the marine ecosystem and biodiversity

OECD: Organisation for Economic Co-operation and Development

OSPAR: The Convention for the Protection of the Marine Environment of the North-East Atlantic

p: Sound pressure, the difference between the pressure produced by a sound wave and the ambient level of pressure (under silent conditions) at a single point in space. This scalar quantity is measured as force per unit area, with unit Pascals (Pa)

Pa: Pascals, the SI derived unit of pressure

PM: Particle motion, a vector measure that describes the movement of a particle relative to its resting position in order to transfer the energy of an acoustic wave.

PSD: Power spectral density, the power carried by a sound wave per unit frequency

RMS: Root-mean-square, an averaging technique often used to characterise the intensity of continuous sound

ROS: Reactive oxygen species

RTE: Responses till exhaustion

SD: Standard deviation

SI: International System of Units

SE: Standard error

SPL: Sound pressure level, usually described as an RMS average of instantaneous pressure levels over time and given as a logarithmic decibel in relation to a reference pressure, where SPL is $20\log_{10}$ of the ratio between measured RMS pressure and the reference pressure

λ: Wavelength

Chapter 1

General Introduction

E. L. Bolger

Chapter 1

General Introduction

The overall topic of this thesis is the response of aquatic invertebrates to anthropogenic (man-made) sound across different life history stages. A review of the trends (in taxa, sounds, life stages, and response parameters studied) in stressor research (and underwater sound research in particular) is presented which was used to identify knowledge gaps and generate the overarching hypotheses pertaining to the thesis topic (chapter 2). A series of controlled laboratory experiments, designed to address the identified knowledge gaps are then reported on (chapters 3-7), followed by a synthesis of all experimental results and their wider implications (chapter 8). Firstly however, this chapter will introduce the key concepts of aquatic sound, the importance of sound to invertebrates, and the current legislative framework concerning noise pollution. The overall aim, objectives and the structure of the thesis will also be outlined.

1.1 – Sound in the aquatic environment

The realm beneath the surface of the water has long been considered to be a "silent world" (Cousteau and Dumas, 1953). In fact, the aquatic environment, much like the terrestrial environment, is filled with sounds. These underwater sounds can be produced by natural sources or can be man-made (anthropogenic). Natural ocean sound encompasses abiotic sources such as crashing waves, wind, rain, tidal flows, lightning, thermal sound (the high-frequency vibration of energetic water molecules) and the various sonic consequences of tectonic activity ranging from seismic movements to earthquakes and volcanic eruptions. This abiotic environmental sound is supplemented with biotic sounds, from actively-produced signals such as marine mammal calls (Berta et al., 2015), the 'drumming' sounds produced by courting fish (Engen and Folstad, 1999) and snapping shrimp 'pops' (Butler et al., 2017), to the by-products of biological

activity such as the scraping sounds produced by the mouthparts of feeding sea urchins (Coquereau et al., 2016).

In the aquatic environment light travels poorly, whereas sound propagates faster and further than in air, driving many animals to evolve mechanisms to utilise underwater sound as a primary source of long distance information (Popper and Hawkins, 2016). Aquatic mammals, fish and invertebrates use underwater sound for communication (Janik, 2014; Smott et al., 2018; Zenone et al., 2019), predator detection (Randall Hughes et al., 2014), orientation towards desirable habitats (Lillis et al., 2015; Simpson et al., 2004; Vermeij et al., 2010) and prey location and capture (Berta et al., 2015). Over the previous century, human activity in aquatic environments, and the sounds that it produces, has increased considerably (Frisk, 2012), leading to growing concerns over the potential impacts of this additional sound on aquatic organisms (Williams et al., 2015). Economic growth has necessitated (and been driven by) increases in shipping, oil and gas exploration and extraction and more recently, the expansion of renewable energy developments. These activities have caused background sound levels in the oceans to rise globally (Hildebrand, 2009). Low-frequency ocean sound (20 – 200 Hz) has increased by 10 – 100 fold over the previous century, predominantly due to increases in commercial shipping (Tyack, 2008). Whereas sound is a term that can be used to describe any acoustic wave, noise is defined as a sound that is unwanted or harmful and therefore always has a negative connotation. In this thesis, noise is defined as unwanted or harmful sound with respect to the aquatic fauna that are exposed to it.

To understand how these increases in anthropogenic sound relate to natural sound, and how it may affect the animals that encounter them, it is important to first understand the characteristics of sound and how it behaves in aquatic environments.

1.1.1 - The fundamentals of underwater acoustics

Sound is a mechanical wave that propagates through a medium via the vibration of the particles that make up said medium. In elastic substances such as air and water, sound travels away from its source as a longitudinal pressure (*P*) wave composed of alternating compressions and rarefactions of the medium. Sound is therefore composed of two different parameters, the pressure waves that pass through the medium, and the displacement of the individual particles within the medium that is necessary to transfer the waves (Figure 1.1).

The change in measured pressure from the ambient level at a single location caused by the compression or rarefaction of a passing sound wave is known as sound pressure. Sound pressure is most often characterised as the sound pressure level (SPL), which is a relative measure of the amplitude of a pressure wave expressed on a decibel logarithmic scale. The SPL (decibels (dB)) is calculated as:

$$20\log\left(\frac{P_{measured}}{P_{ref\ water}}\right)$$

Where $P_{measured}$ is the pressure amplitude of a sound wave (measured in Pascals (Pa)) and $P_{ref\ water}$ is a defined reference pressure level, which in water is 1 µPa. In contrast, the reference pressure for air $P_{ref\ air}$ is 20 µPa. For this reason, care must be taken when comparing in-air and in-water SPLs.

The second component of sound is particle motion – the oscillation of individual molecules within the medium to facilitate the transference of the pressure waves (Figure 1.1). Unlike SPL which is a scalar measure, particle motion is described as a vector, with both a magnitude *and* a direction. The movement of particles can be described in three ways: 1) displacement (m), 2) velocity (m s^{-1}) or 3) acceleration (m s^{-2}).

The speed of sound in water (c) is related to the frequency (Hertz (measured as the wave cycles per second)) and wavelength (m) of the sound pressure waves in the following way:

$$c = f\lambda$$

Where f = frequency and λ = wavelength. This means that for a given sound speed, low-frequency sound waves have long wavelengths. The speed of sound in water is dependent on temperature, salinity and depth, but can simply be stated to be 1500 m s^{-1}, approximately four times faster than in air (340 m s^{-1}). This means sound travels further in water before it begins to lose energy to the surrounding medium (attenuation). Attenuation is also frequency dependent, with higher frequency sound waves attenuating more quickly. This is of significance to marine environments, as many anthropogenic sound sources occupy lower frequency ranges and can therefore travel over great distances. To illustrate this, the Heard Island feasibility test demonstrated that low frequency (57 Hz) sound signals at a SPL of 206dB (re: 1 µPa) could be transmitted by underwater speakers in the Antarctic waters of Heard Island, and could then be detected 3 hours later 16,800 km away off the coast of Bermuda (Baggeroer and Munk, 1992).

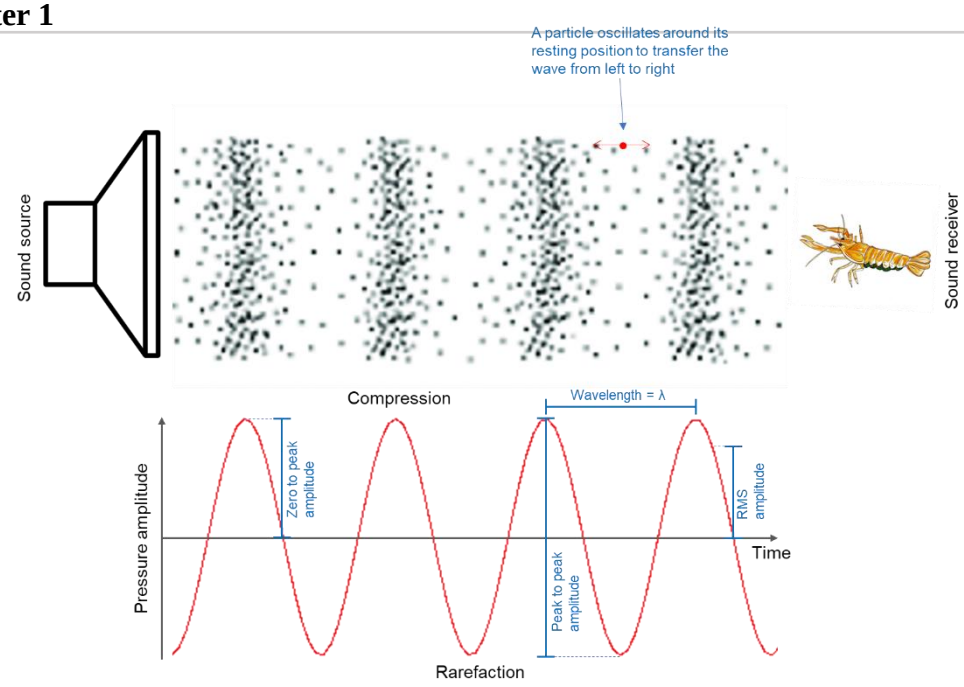


Figure 1.1 The movement of a sound wave through an elastic medium. As the wave moves from left to right, areas of high pressure (compression) are followed by areas of low pressure (rarefaction). For these waves to propagate, it is necessary for individual particles to oscillate back and forth as they receive energy from a particle to the left and transfer this energy to a particle to their right (signified by the red particle). These are the two key components of sound: pressure waves and particle motion.

Whilst underwater sound pressure can be measured with a hydrophone (the aquatic equivalent of a microphone) relatively simply, the associated particle motion cannot always be reliably calculated from the measured pressure. This is because sound pressure and particle motion, whilst related, propagate and behave in different ways. In the far-field sound waves travel as a plane and pressure becomes proportional to particle motion. The far-field is usually calculated as the distance from the sound source that is greater than or equal to:

$$\frac{\pi D^2}{\lambda}$$

Where D = the greatest dimension of the sound source (m) and λ = sound wavelength (m).

In this scenario, the relationship between sound pressure (p) and particle motion (v) is described by a fluid property called the acoustic impedance (Z), where $Z = pv$. In free-field conditions, where sound waves propagate without interference, Z can also be described as the product of the fluid dependent c and ρ, where ρ is density.

However, the assumptions of free-field conditions often do not apply in aquatic environments, due to water surfaces or bottom substrates reflecting sound waves in unpredictable ways causing interference to propagation. In the near-field of the sound source particle motion and pressure decay at different rates, further complicating the relationship between the two parameters. For these reasons, when wanting to characterise a given sound field, in most circumstances particle motion must be measured, rather than back-calculated from measured sound pressure (Nedelec et al., 2016). This is of particular importance for tank based sound exposure studies, where an experimental animal may be exposed to sound waves that are in the near-field of an underwater speaker and tank boundaries will reflect sound waves, affecting the sound field within the tanks. Experimental aquaria can however be built to exacting specifications and both pressure and particle motion can be measured in multiple locations in the tank to adequately characterise the sound field and consider it in any analyses and interpretations. As particle motion is a vector, it is usually measured with a tri-axial accelerometer, so that movements in the x,y and z axes can be taken into account.

Once particle motion and sound pressure have been measured with x-y-z accelerometers and hydrophones respectively, there are several ways of presenting the outputs produced by these devices. The appropriate reporting of sound measurements predominantly depends on whether the sound is continuous or impulsive. As the energy content of continuous sound remains relatively constant over time, the most important component of such sound to animal hearing is thought to be the average amplitude. For

this reason, continuous sound should be reported as an average sound pressure level and an average particle velocity (or acceleration). The averaging method most used is root-mean-square (RMS) (Merchant et al., 2015). Impulsive sound is transient in comparison to continuous sound. In other words, high-amplitude sound pulses are interspersed between periods of low-amplitude sound levels (common source examples include underwater explosions and pile-driving). The most important characteristics of these sounds is the peak amplitude levels of the sound pulses, the duration of the pulses and the number of pulses in a given time period. These are reported as zero-to-peak or peak-to-peak sound pressure levels ($SPL_{z\text{-}p}$, $SPL_{p\text{-}p}$) , zero-to-peak or peak-to-peak sound velocity levels ($SVL_{z\text{-}p}$, $SVL_{p\text{-}p}$), energy per sound pulse (single strike sound exposure level (SEL_{SS})) and the cumulative energy received over time (SEL_{cum}) (Van der Graaf et al., 2012).

1.2 – Underwater sound and invertebrates

Since concerns over the impacts of anthropogenic sound on aquatic animals first arose in the 1970s (e.g. (Myrberg, 1978)), research has historically focused on marine mammals (Wale et al., 2021; Williams et al., 2015). These organisms produce clear and recognisable sounds for communication and hunting, and mass strandings were linked to military sonar usage therefore making them logical candidates for initial research (Myrberg, 1978; Richardson et al., 1995). Exposure of marine mammals to anthropogenic noise is now known to cause shifts in foraging and diving behaviour, physical injury and hearing loss (Gomez et al., 2016; Gordon et al., 2003; Madsen et al., 2006).

The research field then developed to encompass fish, where anthropogenic noise can variously cause stress (for definition see section 2.3.2), increase oxygen consumption, disturb foraging and anti-predator behaviour, cause mechanical injury and

reduce early-life survival. For examples and comprehensive reviews, see Harding et al. (2018); Hawkins and Popper (2017); Popper and Hastings (2009); Radford et al. (2016); Sierra-Flores et al. (2015); Simpson et al. (2016); Slabbekoorn et al. (2010).

In comparison to marine mammals and fish, aquatic invertebrates have received far less research attention (Wale et al., 2021; Williams et al., 2015). As such this thesis aims to increase their representation within the noise impact literature, for the reasons outlined below.

Invertebrates are taxonomically the most diverse form of eukaryotic life (Mora et al., 2011). They play vital ecological roles, e.g. they form key links in food webs (New and Yen, 1995), filter water and control eutrophication (Officer et al., 1982), increase nutrient cycling, bioturbate sediments (Snelgrove et al., 1997) and form habitat for other species through e.g. reef-building (Coen et al., 2007) or burrowing (Needham et al., 2010). Due to their important role in food web trophic dynamics, they are often described as a keystone species (Miner et al., 2012; Rodriguez-Perez et al., 2019; Rosa et al., 2012).

Aquatic invertebrates are also commercially important globally (Bondad-Reantaso et al., 2012; Botta et al., 2020; Pauly et al., 2002), making up 15% of global wild capture tonnage and 34% of global trade value in 2018 (FAO, 2020). They form an increasingly important component of wild capture fisheries and aquaculture production, since as many finfish stocks become overfished and/or stringently managed, fishers are diversifying their effort to more abundant or less restricted invertebrate stocks (Anderson et al., 2011).

Aquatic animals perceive sound in varying ways. Sound pressure waves are detected by the compression and expansion of sensory systems, whereas particle motion is detected as sensors are vibrated or ‘shaken’. To detect sound pressure waves, the

walls of a sensory system must oscillate enough to stimulate sensory hair cells. This is achieved in an aquatic environment with air-filled organs which are found in marine mammals (mammalian ears) and many fish (swim bladders). The lower density of air inside such organs allows them to expand and contract sufficiently for hair cell stimulation with passing pressure waves (Gomez et al., 2016; Hawkins and Popper, 2017). Invertebrates, as far as we know, do not possess organ systems that are likely involved in sound pressure wave detection. They do however possess a range of mechanoreceptors that are likely to be involved in the perception of the particle motion component of sound. Many invertebrates are covered in sensory hairs (setae) across their outer body surfaces which can detect particle motion (Popper et al., 2001). Molluscs and crustaceans also often possess statocyst organs which are thought to detect particle motion (Jézéquel, et al., 2021; Kaifu et al., 2008). Statocysts are fluid filled sacs with a denser mass called a statolith. The statolith lies against sensory hair cells that line the inner walls of the statocyst. The bodies of aquatic animals are of a similar density to water, so the body vibrates with particle motion as a sound wave passes through. The inertia of the statolith causes it to move in relation to the vibrating statocyst that surrounds it, thereby stimulating the sensory hairs in contact with it. Finally, crustaceans possess chordotonal organs which are associated with the joints of flexible body appendages. In water, some of these appendages can vibrate alongside the oscillation of water surrounding them. Highly sensitive chordotonal systems have been described in the joints associated with the antennal flagellum in crayfish and lobsters (Bender et al., 1984; Tazaki, 1977).

Given the diversity of both the sensory systems described above and the morphology, physiology and life-history strategies of invertebrates, predictions of likely responses to underwater sound are difficult to make. For example, low frequency anthropogenic noise has been shown to induce the settlement of ascidian larvae and

delay the settlement of barnacle larvae (Branscomb and Rittschof, 1984; McDonald et al., 2014). Depending on the amplitude, frequency and temporal characteristics of the sound wave, and the sensitivity of the invertebrate to each combination of these sound characteristic variables, a wide range of responses to sound could be observed, from mortality to subtle behavioural changes. The range of possible responses to noise are summarised in Table 1.1.

Table 1.1 The potential negative impacts of anthropogenic sound on aquatic invertebrates with associated example references

Impact	Type of effect	Example references
Mortality	Immediate or delayed death	(McCauley et al., 2017; Nedelec et al., 2015)
Auditory damage	Damage to statocysts or sensory hair cells	(Day et al., 2019; Solé et al., 2013)
Stress	Endocrine, immune and metabolic changes that may lead to internal damage (e.g. oxidative stress) or other impacts listed in this table	(Celi et al., 2015; Wale et al., 2019)
Behavioural disturbance	Acute responses e.g. tail-flicking, change in swimming activity, defensive posturing, antennule beating	(Aimon et al., 2021; Carter et al., 2020; Mooney et al., 2016; Wale et al., 2013)
	Chronic responses e.g. reduced feeding success, reduced anti-predator response, altered relationship to habitat and shelter, long term changes in movement activity	
Masking	Reduced communication with conspecifics	(Jézéquel, Bonnel, et al., 2021)
	Reduced ability to respond to acoustic signals e.g. larvae orienting towards a reef	
Fitness	Reduced reproductive success, reduced fecundity, reduced offspring growth and survival	(de Soto et al., 2013; Nedelec et al., 2015)

Few studies on the impacts of noise on invertebrates have been carried out in comparison to fish and mammals (Popper et al., 2020; Williams et al., 2015). Of the 468 papers on animals presented at the previous four 'Effects of Noise on Aquatic Life' conferences (Popper and Hawkins, 2012; Popper and Hawkins, 2016), 64% involved marine mammals, 26% fish, and 10% invertebrates (Popper et al., 2020). Of the studies that do focus on invertebrates, even fewer have considered early life stage invertebrates or carry over effects from one life stage to another. The most recent published review of invertebrate noise impact studies identified only 3 out of 15 focusing on early life stages (de Soto, 2016), despite the fact that invertebrate larvae are often particularly vulnerable to environmental stress (Dupont et al., 2013; Egilsdottir et al., 2009; Pineda et al., 2012; Wood et al., 2015). The overall low survival of the sensitive larval stages is considered to be a bottleneck for the reproductive success and recruitment of invertebrates with a larval phase (Byrne, 2011; Fitzgibbon et al., 2014). The earliest life stages can also be eggs which are attached to the substrate or passively moving with currents, and therefore unable to move away from unwanted sound exposure (de Soto, 2016; Nedelec et al., 2015). Given the rising noise levels in aquatic environments, the importance of early life stages to invertebrate success, and the lack of information regarding the ways in which these stages may be impacted by underwater sound and noise, there is a clear need to study a wider range of aquatic invertebrates and to work towards an understanding of how they are impacted by noise across life history stages. With most studies focusing on the acute effects of noise, little information exists on whether exposure of mothers to noise affects the offspring, or whether noise impacts in early life carry over to adulthood. These questions must be answered if commitments to international and domestic conservation objectives are to be met.

1.3 – The legislative framework

The increasing levels of anthropogenic noise globally (Frisk, 2012) and concern over their impacts on aquatic fauna have led to its international recognition as a pollutant in conventions such as the OSPAR Convention (Götz et al., 2009) and the Convention on Biological Diversity (CBD, 2012). The European Union has enacted legislation such as the Marine Strategy Framework Directive (MSFD) (Marine Strategy Framework Directive, 2008) which requires member states to act to achieve "Good Environmental Status" (GES) in their territorial waters. Descriptor 11 of GES is: "Introduction of energy, including underwater noise, is at levels that do not adversely affect the marine environment" (Borja et al., 2010). Whilst this is good progress towards mitigating the harms that noise may cause to fauna, there remains a knowledge gap as to how many, and how, invertebrate species are affected by noise (Van der Graaf et al., 2012). This knowledge gap must be addressed if legislation such as the MSFD is to be implemented in an effective and informed way.

1.4 – Thesis aims and outline

In this thesis I will begin to address the current lack of knowledge concerning the effects of anthropogenic noise on aquatic invertebrates, with a particular focus on the understudied early life stages. Firstly, I will summarize the trends and current state of the field in aquatic invertebrate noise impact literature. I will use this literature review to identify specific knowledge gaps regarding aquatic invertebrate responses to noise (chapter 2). I will then address these knowledge gaps by considering behavioural, physiological and biochemical impacts of noise on a taxonomically diverse range of study species as they transition through key early life stages (chapters 3-7). I will also use a model species to test for the potential for chronic noise exposure to have impacts on invertebrates across multiple generations – a concept yet to be addressed in any species (chapter 7).

Five model species will be considered. Two decapod crustacean species of the Nephropidae family, the European lobster *Homarus gammarus* (L.) and the Norway lobster *Nephrops norvegicus* (L.) and two mollusc species, the European flat oyster *Ostrea edulis* (L.) and the veined squid *Loligo forbesii* (Steenstrup, 1857). Finally, a keystone freshwater crustacean, the water flea *Daphnia magna* (L.), was chosen as a model species to test for and explore the multi-generational effects of noise exposure. The work in this thesis utilises playbacks of anthropogenic noise using underwater speakers in controlled laboratory conditions. This allowed for greater control over exposure conditions, eliminating any confounding variables, and enabled care to be taken in the long-term rearing of small and sensitive early life stage invertebrate larvae, with exacting requirements for feeding and water parameters.

By monitoring the developmental rate and success of the early life stages of the chosen study species and combining this data derived from behavioural, physiological and biochemical response parameters, a more holistic understanding of the way anthropogenic noise impacts invertebrates can be generated, provided much needed information on how impacts on crucial early life stages may affect recruitment to the wider population. The methods used here can be replicated for other studies focusing on early-life noise impacts and *D. magna* has emerged as a suitable model organism for further study of the multigenerational impacts of noisy environments on planktonic invertebrates.

1.5 – Objectives

This thesis has the following objectives:

- To identify knowledge gaps concerning the responses of aquatic invertebrates to underwater sound, particularly the early life stages
- To use the identified knowledge gaps to guide the generation of experimental hypotheses
- To develop methodologies for the simultaneous rearing and anthropogenic noise exposure of invertebrate larvae
- To test for and characterise anthropogenic noise-induced impacts on the chosen response parameters of a taxonomically diverse range of 5 species of early life crustaceans and molluscs
- To investigate whether any observed anthropogenic noise-induced changes carry over from one life stage to the next. For example, larvae to juvenile or mother to offspring
- To investigate the suitability of *Daphnia magna* (L.) as a model organism for exploring the effects of multi-generational anthropogenic noise exposure

1.6 – Thesis Structure

The structure of this thesis is described below. A summary of the 5 aquatic invertebrate species chosen for the experimental chapters (chapters 3-7), the response parameters studied, and the sound playbacks used for each species is displayed in figure 1.2.

- **Chapter 1 - General Introduction**

- **Chapter 2 - Stress, Sound, and Invertebrate Larvae: Trends, Methods and Knowledge Gaps**

  The study of anthropogenic noise impacts on the early life stages of invertebrates has increased over previous decades. The trend in publications of this research in comparison to other types of stressors on invertebrate larvae will be outlined and discussed. The existing literature on how early life invertebrates respond to underwater sound will be analysed, highlighting existing knowledge gaps.

- **Chapter 3 - Effects of anthropogenic noise on the early life stages of *Nephrops norvegicus***

  Here I describe the commercial and ecological importance of *N. norvegicus* and highlight the overlap of its spatial distribution with busy shipping lanes and the noise they produce. I investigate noise playback impacts on the survival, developmental duration, oxygen consumption, oxidative stress biomarkers and anti-predator behaviour of *N. norvegicus*, the first study to focus on the larvae of this species in the context of underwater noise. Differences between noise exposure regimes with differing amounts of noise are explored, as are the effects of noise pre-exposure on the acute responses to subsequent repeat exposures.

- **Chapter 4 - Effects of anthropogenic noise on the early life stage development of *Homarus gammarus***

  Continuing the work in chapter 3, the same experimental noise exposure set up is used to test for effects of anthropogenic noise on *H. gammarus* larvae. Survival and developmental duration data of noise-exposed larvae are reported for the first time for this species and the findings observed for *N. norvegicus* and *H. gammarus* are compared.

- **Chapter 5 - Effects of anthropogenic noise on the oxygen consumption rate of *Loligo forbesii* embryos**

  Here, the commercial and ecological importance of this squid species is briefly reviewed, and the current literature on squid hearing presented. The effects of acute ship noise playbacks on the oxygen consumption of squid embryos are then reported and the effect of noise pre-exposure on the acute responses to subsequent exposures explored. The implications of the results to our understanding of squid hearing and the potential ecological implications are discussed.

- **Chapter 6 - Effects of anthropogenic noise on the survival and settlement behaviour of European oyster *Ostrea edulis* larvae**

  The once abundant *O. edulis* is currently the subject of concerted restoration efforts across the North-East Atlantic to restore this reef-forming bivalve to its former extent. Given that larvae of other oyster species have been shown to modify settlement rates in response to sound, the effects of anthropogenic noise on the settlement behaviour of *O. edulis* is tested and

reported on here. The implications for restoration efforts in a noisy North-East Atlantic are discussed.

- **Chapter 7 - Multi-generational effects of anthropogenic noise on the water flea *Daphnia magna***

  Given that stress impacts can carry over from one generation to the next, in the context of this thesis the question arises whether, and how, noise exposure of larval invertebrates impacts their adult life, and whether noise-exposed mothers pass on impacts to their offspring. There is no study to date that has addressed this question. The biology and ecology of *D. magna* and its suitability as a multi-generational model organism is first discussed. Then the impacts of anthropogenic noise on survival, development, and reproduction over four generations are reported and the impacts of these on population growth modelled. The implications of these results for the ecology of freshwater systems and future noise impact research are discussed.

- **Chapter 8 – Synthesis**

  This final chapter is a summation of all results generated and methods used throughout the thesis. I discuss how the results sit within the broader context of existing noise impact literature. I highlight the implications for the research field and stakeholders. Finally, I suggest refinements and adjustments to the research methods conducted here and propose future avenues for further research to effectively expand the field underwater noise research.

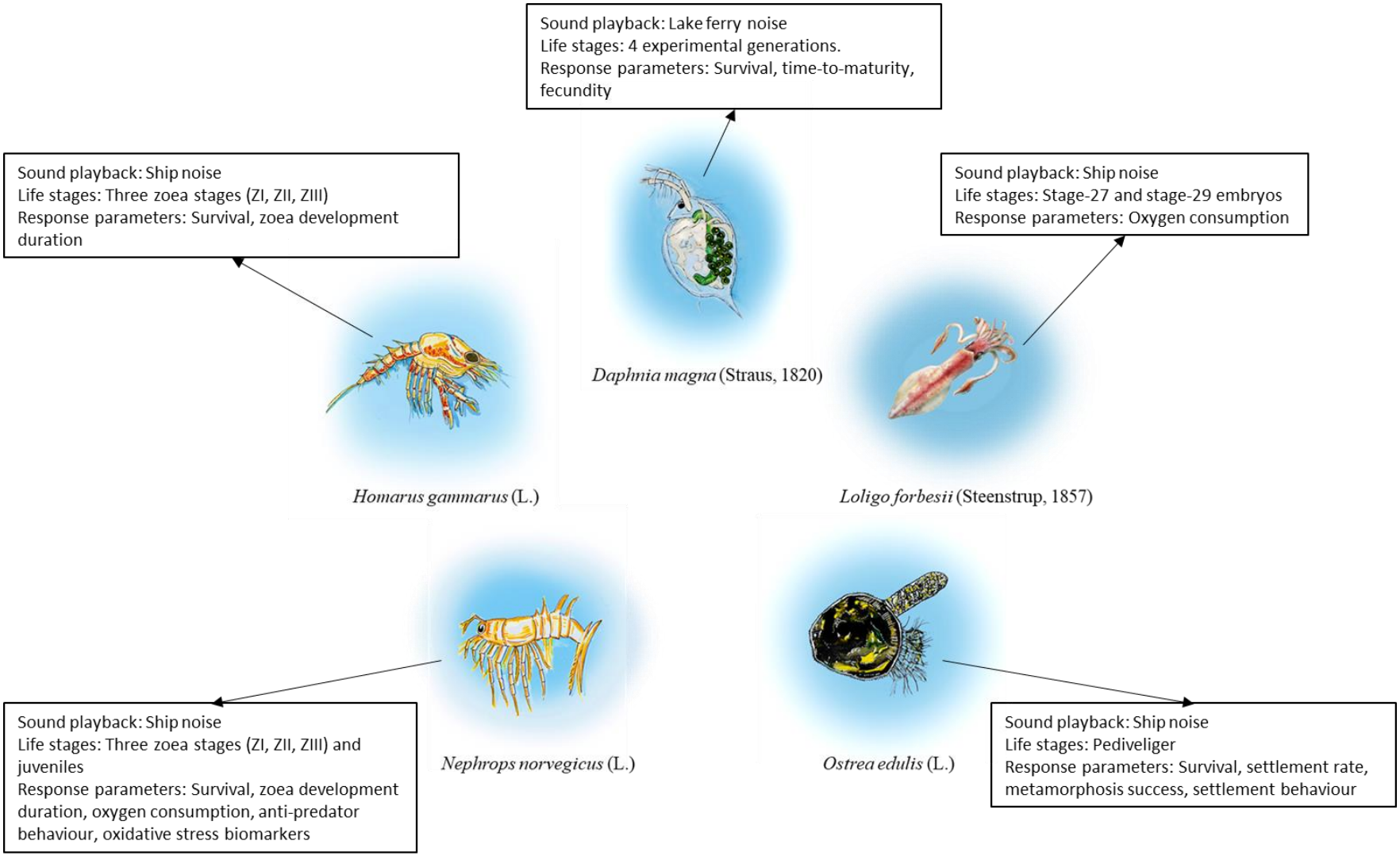


Figure 1.2 The five model aquatic invertebrate species considered in the controlled laboratory sound playback experiments reported in this thesis. The life stages studied, sound playbacks used, and the response parameters measured are also summarised for each species. For picture credits, see appendix C

1.8 – References

Aimon, C., Simpson, S. D., Hazelwood, R. A., Bruintjes, R. and Urbina, M. A. (2021). Anthropogenic underwater vibrations are sensed and stressful for the shore crab *Carcinus maenas*. *Environmental Pollution*, *285*, 117148. https://doi.org/10.1016/j.envpol.2021.117148

Anderson, S. C., Flemming, J. M., Watson, R. and Lotze, H. K. (2011). Rapid global expansion of invertebrate fisheries: Trends, drivers, and ecosystem effects. *PLoS ONE*, *6*(3), 1–9. https://doi.org/10.1371/journal.pone.0014735

Baggeroer, A. and Munk, W. (1992). The Heard Island Feasibility Test. *Physics Today*, *45*(9), 22–30. https://doi.org/10.1063/1.881317

Bender, M., Gnatzy, W. and Tautz, J. (1984). The antennal feathered hairs in the crayfish: a non-innervated stimulus transmitting system. *Journal of Comparative Physiology A*, *154*(1), 45–47. https://doi.org/10.1007/BF00605388

Berta, A., Sumich, J. L. and Kovacs, K. M. (2015). Sound Production for Communication, Echolocation, and Prey Capture. In *Marine Mammals* (pp. 345–395). Elsevier. https://doi.org/10.1016/B978-0-12-397002-2.00011-9

Bondad-Reantaso, M. G., Subasinghe, R. P., Josupeit, H., Cai, J. and Zhou, X. (2012). The role of crustacean fisheries and aquaculture in global food security: Past, present and future. *Journal of Invertebrate Pathology*, *110*(2), 158–165. https://doi.org/10.1016/j.jip.2012.03.010

Borja, Á., Elliott, M., Carstensen, J., Heiskanen, A. S. and van de Bund, W. (2010). Marine management - Towards an integrated implementation of the European marine strategy framework and the water framework directives. *Marine Pollution Bulletin*, *60*(12), 2175–2186. https://doi.org/10.1016/j.marpolbul.2010.09.026

Botta, R., Asche, F., Borsum, J. S. and Camp, E. V. (2020). A review of global oyster aquaculture production and consumption. *Marine Policy*, *117*, 103952. https://doi.org/10.1016/j.marpol.2020.103952

Branscomb, E. S. and Rittschof, D. (1984). An investigation of low frequency sound waves as a means of inhibiting barnacle settlement. *Journal of Experimental Marine Biology and Ecology*, *79*(2), 149–154. https://doi.org/10.1016/0022-0981(84)90215-6

Butler, J., Iv, M. J. B. and Gaff, H. (2017). Snap , crackle , and pop : Acoustic-based model estimation of snapping shrimp populations in healthy and degraded hard-bottom habitats. *Ecological Indicators*, *77*, 377–385. https://doi.org/10.1016/j.ecolind.2017.02.041

Byrne, M. (2011). Impact of ocean warming and ocean acidification on marine invertebrate life history stages: Vulnerabilities and potential for persistence in a changing ocean. *Oceanography and Marine Biology: An Annual Review*, *49*, 1–42. https://doi.org/10.1016/j.marenvres.2011.10.00

Carter, E. E., Tregenza, T. and Stevens, M. (2020). Ship noise inhibits colour change, camouflage, and anti-predator behaviour in shore crabs. *Current Biology*, *30*(5), R211–R212. https://doi.org/10.1016/j.cub.2020.01.014

CBD. (2012). Scientific Synethesis on the Impacts of Underwater Noise on Marine and Coastal Biodiversity and Habitats. *Subsidiary Body on Scientific, Technical and Technological Advice*.

Celi, M., Filiciotto, F., Vazzana, M., Arizza, V., Maccarrone, V., Ceraulo, M., Mazzola, S. and Buscaino, G. (2015). Shipping noise affecting immune responses of European spiny lobster (*Palinurus elephas*). *Canadian Journal of Zoology*, *93*, 113–121. https://doi.org/10.1139/cjz-2014-0219

Coen, L. D., Brumbaugh, R. D., Bushek, D., Grizzle, R., Luckenbach, M. W., Posey, M. H., Powers, S. P. and Tolley, S. G. (2007). Ecosystem services related to oyster restoration. *Marine Ecology Progress Series*, *341*, 303–307. https://doi.org/10.3354/meps341303

Coquereau, L., Grall, J., Chauvaud, L., Gervaise, C., Clavier, J., Jolivet, A. and Di Iorio, L. (2016). Sound production and associated behaviours of benthic invertebrates from a coastal habitat in the north-east Atlantic. *Marine Biology*, *163*(5), 1–13. https://doi.org/10.1007/s00227-016-2902-2

Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 Establishing a Framework for Community Action in the Field of Marine Environmental Policy (Marine Strategy Framework Directive), 19 (2008).

Cousteau, J.-Y. and Dumas, F. (1953). *The Silent World*. Harper & Brothers Publishers

Day, R. D., McCauley, R. D., Fitzgibbon, Q. P., Hartmann, K. and Semmens, J. M. (2019). Seismic air guns damage rock lobster mechanosensory organs and impair righting reflex. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1907). https://doi.org/10.1098/rspb.2019.1424

de Soto, N. A. (2016). Peer-Reviewed Studies on the Effects of Anthropogenic Noise on Marine Invertebrates: From Scallop Larvae to Giant Squid. In A. N. Popper & A. Hawkins (Eds.), *The Effects of Noise on Aquatic Life II* (pp. 17–26). Springer New York. https://doi.org/10.1007/978-1-4939-2981-8_3

de Soto, N. A., Delorme, N., Atkins, J., Howard, S., Williams, J. and Johnson, M. (2013). Anthropogenic noise causes body malformations and delays development in marine larvae. *Scientific Reports*, *3*(1), 2831. https://doi.org/10.1038/srep02831

Dupont, S., Dorey, N., Stumpp, M., Melzner, F. and Thorndyke, M. (2013). Long-term

and trans-life-cycle effects of exposure to ocean acidification in the green sea urchin *Strongylocentrotus droebachiensis*. *Marine Biology*, *160*, 1835–1843. https://doi.org/10.1007/s00227-012-1921-x

Egilsdottir, H., Spicer, J. I. and Rundle, S. D. (2009). The effect of CO2 acidified sea water and reduced salinity on aspects of the embryonic development of the amphipod *Echinogammarus marinus* (Leach). *Marine Pollution Bulletin*, *58*(8), 1187–1191. https://doi.org/10.1016/j.marpolbul.2009.03.017

Engen, F. and Folstad, I. (1999). Cod courtship song: a song at the expense of dance? *Canadian Journal of Zoology*, *77*(4), 542–550. https://doi.org/10.1139/cjz-77-4-542

FAO. (2020). The State of World Fisheries and Aquaculture 2020. Sustainability in action. In *Fao*. https://doi.org/https://doi.org/10.4060/ca9229en

Fitzgibbon, Q. P., Jeffs, A. G. and Battaglene, S. C. (2014). The Achilles heel for spiny lobsters: The energetics of the non-feeding post-larval stage. *Fish and Fisheries*, *15*(2), 312–326. https://doi.org/10.1111/faf.12018

Frisk, G. V. (2012). Noiseonomics: The relationship between ambient noise levels in the sea and global economic trends. *Scientific Reports*, *2*(1), 2–5. https://doi.org/10.1038/srep00437

Gomez, C., Lawson, J. W., Wright, A. J., Buren, A. D., Tollit, D. and Lesage, V. (2016). A systematic review on the behavioural responses of wild marine mammals to noise: The disparity between science and policy. *Canadian Journal of Zoology*, *94*(12), 801–819. https://doi.org/10.1139/cjz-2016-0098

Gordon, J., Gillespie, D., Potter, J., Frantzis, A., Simmonds, M. P., Swift, R. and Thompson, D. (2003). A review of the effects of seismic surveys on marine

mammals. *Marine Technology Society Journal*, *37*(4), 16–34. https://doi.org/10.4031/002533203787536998

Götz, T., Hastie, G., Hatch, L. T., Raustein, O., Southall, B., Tasker, M. and Thomsen, F. (2009). Overview of the impacts of anthropogenic underwater sound in the marine environment Biodiversity Series. *OSPAR Biodiversity Series*.

Harding, H. R., Gordon, T. A. C., Hsuan, R. E., Mackaness, A. C. E., Radford, A. N. and Simpson, S. D. (2018). Fish in habitats with higher motorboat disturbance show reduced sensitivity to motorboat noise. *Biology Letters*, *14*(10). https://doi.org/10.1098/rsbl.2018.0441

Hawkins, A. D. and Popper, A. N. (2017). A sound approach to assessing the impact of underwater noise on marine fishes and invertebrates. *ICES Journal of Marine Science*, *74*(3), 635–651. https://doi.org/10.1093/icesjms/fsw205

Hildebrand, J. A. (2009). Anthropogenic and natural sources of ambient noise in the ocean. *Marine Ecology Progress Series*, *395*, 5–20. https://doi.org/10.3354/meps08353

Janik, V. M. (2014). Cetacean vocal learning and communication. *Current Opinion in Neurobiology*, *28*, 60–65. https://doi.org/10.1016/j.conb.2014.06.010

Jézéquel, Y., Bonnel, J. and Chauvaud, L. (2021). Potential for acoustic masking due to shipping noise in the European lobster (*Homarus gammarus*). *Marine Pollution Bulletin*, *173*, 112934. https://doi.org/10.1016/j.marpolbul.2021.112934

Jézéquel, Y., Jones, I. T., Bonnel, J., Chauvaud, L., Atema, J. and Mooney, T. A. (2021). Sound detection by the American lobster (*Homarus americanus*). *Journal of Experimental Biology*, *224*(6). https://doi.org/10.1242/jeb.240747

Kaifu, K., Akamatsu, T. and Segawa, S. (2008). Underwater sound detection by

cephalopod statocyst. *Fisheries Science*, *74*(4), 781–786. https://doi.org/10.1111/j.1444-2906.2008.01589.x

Lillis, A., Bohnenstiehl, D. R. and Eggleston, D. B. (2015). Soundscape manipulation enhances larval recruitment of a reef-building mollusk. *PeerJ*, *3*, e999. https://doi.org/10.7717/peerj.999

Madsen, P., Wahlberg, M., Tougaard, J., Lucke, K. and Tyack, P. (2006). Wind turbine underwater noise and marine mammals: implications of current knowledge and data needs. *Marine Ecology Progress Series*, *309*, 279–295. https://doi.org/10.3354/meps309279

McCauley, R. D., Day, R. D., Swadling, K. M., Fitzgibbon, Q. P., Watson, R. A. and Semmens, J. M. (2017). Widely used marine seismic survey air gun operations negatively impact zooplankton. *Nature Ecology & Evolution*, *1*(7), 0195. https://doi.org/10.1038/s41559-017-0195

McDonald, J. I., Wilkens, S. L., Stanley, J. A. and Jeffs, A. G. (2014). Vessel generator noise as a settlement cue for marine biofouling species. *Biofouling*, *30*(6), 741–749. https://doi.org/10.1080/08927014.2014.919630 M4 - Citavi

Merchant, N. D., Fristrup, K. M., Johnson, M. P., Tyack, P. L., Witt, M. J., Blondel, P. and Parks, S. E. (2015). Measuring acoustic habitats. *Methods in Ecology and Evolution*, *6*(3), 257–265. https://doi.org/10.1111/2041-210X.12330

Miner, B. E., De Meester, L., Pfrender, M. E., Lampert, W. and Hairston, N. G. (2012). Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1735), 1873–1882. https://doi.org/10.1098/rspb.2011.2404

Mooney, T. A., Samson, J. E., Schlunk, A. D. and Zacarias, S. (2016). Loudness-

dependent behavioral responses and habituation to sound by the longfin squid (*Doryteuthis pealeii*). *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *202*(7), 489–501. https://doi.org/10.1007/s00359-016-1092-1

Mora, C., Tittensor, D. P., Adl, S., Simpson, A. G. B. and Worm, B. (2011). How many species are there on earth and in the ocean? *PLoS Biology*, *9*(8), 1–8. https://doi.org/10.1371/journal.pbio.1001127

Myrberg, A. A. (1978). Ocean noise and the behavior of marine animals: relationships and implications. In J. L. Fletcher & R. B. Busnel (Eds.), *Effects of noise on wildlife*. Academic press.

Nedelec, S. L., Campbell, J., Radford, A. N., Simpson, S. D., Merchant, N. D. and Fisher, D. (2016). Particle motion: the missing link in underwater acoustic ecology. *Methods in Ecology and Evolution*, *7*(7), 836–842. https://doi.org/10.1111/2041-210X.12544

Nedelec, S. L., Radford, A. N., Simpson, S. D., Nedelec, B., Lecchini, D. and Mills, S. C. (2015). Anthropogenic noise playback impairs embryonic development and increases mortality in a marine invertebrate. *Scientific Reports*, *4*, 5891. https://doi.org/10.1038/srep05891

Needham, H. R., Pilditch, C. A., Lohrer, A. M. and Thrush, S. F. (2010). Habitat dependence in the functional traits of *Austrohelice crassa*, a key bioturbating species. *Marine Ecology Progress Series*, *414*, 179–193. https://doi.org/10.3354/meps08726

New, T. R. and Yen, A. L. (1995). Ecological Importance and Invertebrate Conservation. *Oryx*, *29*(3), 187–191. https://doi.org/10.1017/S0030605300021116

Officer, C., Smayda, T. and Mann, R. (1982). Benthic Filter Feeding: A Natural Eutrophication Control. *Marine Ecology Progress Series*, *9*, 203–210. https://doi.org/10.3354/meps009203

Pauly, D., Christensen, V., Guénette, S., Pitcher, T. J., Sumaila, U. R., Walters, C. J., Watson, R. and Zeller, D. (2002). Towards sustainability in world fisheries. *Nature*, *418*(6898), 689–695. https://doi.org/10.1038/nature01017

Pineda, M. C., McQuaid, C. D., Turon, X., López-Legentil, S., Ordóñez, V. and Rius, M. (2012). Tough Adults, Frail Babies: An Analysis of Stress Sensitivity across Early Life-History Stages of Widely Introduced Marine Invertebrates. *PLoS ONE*, *7*(10), e46672. https://doi.org/10.1371/journal.pone.0046672

Popper, A. and Hawkins, A. (2012). *The Effects of Noise on Aquatic Life*. *November*, 695. https://doi.org/10.1007/978-1-4939-2981-8

Popper, A. N. and Hastings, M. C. (2009). The effects of anthropogenic sources of sound on fishes. *Journal of Fish Biology*, *75*(3), 455–489. https://doi.org/10.1111/j.1095-8649.2009.02319.x

Popper, A. N. and Hawkins, A. (2016). *The Effects of Noise on Aquatic Life II* (Vol. 875). https://doi.org/10.1007/978-1-4939-2981-8

Popper, A. N., Hawkins, A. D. and Thomsen, F. (2020). Taking the Animals' Perspective Regarding Anthropogenic Underwater Sound. *Trends in Ecology and Evolution*, *35*(9), 787–794. https://doi.org/10.1016/j.tree.2020.05.002

Popper, A. N., Salmon, M. and Horch, K. W. (2001). Acoustic detection and communication by decapod crustaceans. *Journal of Comparative Physiology - A Sensory, Neural, and Behavioral Physiology*, *187*(2), 83–89. https://doi.org/10.1007/s003590100184

Radford, A. N., Lèbre, L., Lecaillon, G., Nedelec, S. L. and Simpson, S. D. (2016). Repeated exposure reduces the response to impulsive noise in European seabass. *Global Change Biology*, *22*(10), 3349–3360. https://doi.org/10.1111/gcb.13352

Randall Hughes, A., Mann, D. A. and Kimbro, D. L. (2014). Predatory fish sounds can alter crab foraging behaviour and influence bivalve abundance. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1788). https://doi.org/10.1098/rspb.2014.0715

Richardson, W. J., Greene Jr, C. R., Malme, C. I. and Thomson, D. H. (1995). *Marine mammals and noise*. Academic press.

Rodriguez-Perez, A., James, M., Donnan, D. W., Henry, T. B., Møller, L. F. and Sanderson, W. G. (2019). Conservation and restoration of a keystone species: Understanding the settlement preferences of the European oyster (*Ostrea edulis*). *Marine Pollution Bulletin*, *138*, 312–321. https://doi.org/10.1016/j.marpolbul.2018.11.032

Rosa, R., Pimentel, M. S., Boavida-Portugal, J., Teixeira, T., Trübenbach, K. and Diniz, M. (2012). Ocean warming enhances malformations, premature hatching, metabolic suppression and oxidative stress in the early life stages of a keystone squid. *PLoS ONE*, *7*(6). https://doi.org/10.1371/journal.pone.0038282

Sierra-Flores, R., Atack, T., Migaud, H. and Davie, A. (2015). Stress response to anthropogenic noise in Atlantic cod *Gadus morhua* L. *Aquacultural Engineering*, *67*, 67–76. https://doi.org/10.1016/j.aquaeng.2015.06.003

Simpson, S. D., Meekan, M. G., McCauley, R. D. and Jeffs, A. (2004). Attraction of settlement-stage coral reef fishes to reef noise. *Marine Ecology Progress Series*, *276*(1), 263–268. https://doi.org/10.3354/meps276263

Simpson, S. D., Radford, A. N., Nedelec, S. L., Ferrari, M. C. O., Chivers, D. P., McCormick, M. I. and Meekan, M. G. (2016). Anthropogenic noise increases fish mortality by predation. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms10544

Slabbekoorn, H., Bouton, N., van Opzeeland, I., Coers, A., ten Cate, C. and Popper, A. N. (2010). A noisy spring: The impact of globally rising underwater sound levels on fish. *Trends in Ecology and Evolution*, *25*(7), 419–427. https://doi.org/10.1016/j.tree.2010.04.005

Smott, S., Monczak, A., Miller, M. E. and Montie, E. W. (2018). Boat noise in an estuarine soundscape – A potential risk on the acoustic communication and reproduction of soniferous fish in the May River, South Carolina. *Marine Pollution Bulletin*, *133*, 246–260. https://doi.org/10.1016/j.marpolbul.2018.05.016

Snelgrove, P. V. R., Henry Blackburn, T., Hutchings, P. A., Alongi, D. M., Frederick Grassle, J., Hummel, H., King, G., Koike, I., Lambshead, P. J. D., Ramsing, N. B. and Solis-Weiss, V. (1997). The importance of marine sediment biodiversity in ecosystem processes. *Ambio*, *26*(8), 578–583. https://doi.org/10.2307/4314672

Solé, M., Lenoir, M., Durfort, M., López-Bejar, M., Lombarte, A. and André, M. (2013). Ultrastructural Damage of *Loligo vulgaris* and *Illex coindetii* statocysts after Low Frequency Sound Exposure. *PLoS ONE*, *8*(10), e78825. https://doi.org/10.1371/journal.pone.0078825

Tazaki, K. (1977). Nervous responses from mechanosensory hairs on the antennal flagellum in the lobster, *Homarus gammarus* (L.). *Marine Behaviour and Physiology*, *5*, 1–18.

Tyack, P. L. (2008). Implications for Marine Mammals of Large-Scale Changes in the Marine Acoustic Environment. *Journal of Mammalogy*, *89*(3), 549–558.

https://doi.org/10.1644/07-MAMM-S-307R.1

Van der Graaf, A. J., Ainslie, M. A., André, M., Brensing, K., Dalen, J., Dekeling, R. P. A., Robinson, S., Tasker, M. L., Thomsen, F. and Werner, S. (2012). *European Marine Strategy Framework Directive Good Environmental Status (MSFD-GES): Report of the Technical Subgroup on Underwater noise and other forms of energy*. *February*, 75.

Vermeij, M. J. A., Marhaver, K. L., Huijbers, C. M., Nagelkerken, I., Simpson, S. D. and Stephen, D. (2010). Coral Larvae Move toward Reef Sounds. *PLoS ONE*, *5*(5), 3–6. https://doi.org/10.1371/journal.pone.0010660

Wale, M. A., Briers, R. A. and Diele, K. (2021). Marine invertebrate anthropogenic noise research – Trends in methods and future directions. *Marine Pollution Bulletin*. https://doi.org/10.1016/j.marpolbul.2021.112958

Wale, M. A., Briers, R. A., Hartl, M. G. J., Bryson, D. and Diele, K. (2019). From DNA to ecological performance: Effects of anthropogenic noise on a reef-building mussel. *Science of The Total Environment*, *689*, 126–132. https://doi.org/10.1016/j.scitotenv.2019.06.380

Wale, M. A., Simpson, S. D. and Radford, A. N. (2013). Noise negatively affects foraging and antipredator behaviour in shore crabs. *Animal Behaviour*, *86*(1), 111–118. https://doi.org/10.1016/j.anbehav.2013.05.001

Williams, R., Wright, A. J., Ashe, E., Blight, L. K., Bruintjes, R., Canessa, R., Clark, C. W., Cullis-Suzuki, S., Dakin, D. T., Erbe, C., Hammond, P. S., Merchant, N. D., O'Hara, P. D., Purser, J., Radford, A. N., Simpson, S. D., Thomas, L. and Wale, M. A. (2015). Impacts of anthropogenic noise on marine life: Publication patterns, new discoveries, and future directions in research and management. *Ocean and Coastal Management*, *115*, 17–24.

https://doi.org/10.1016/j.ocecoaman.2015.05.021

Wood, H. L., Eriksson, S. P., Nordborg, M. and Styf, H. K. (2015). The effect of environmental stressors on the early development of the Norway lobster *Nephrops norvegicus* (L.). *Journal of Experimental Marine Biology and Ecology*, *473*, 35–42. https://doi.org/10.1016/j.jembe.2015.08.009

Zenone, A., Ceraulo, M., Ciancio, J. E., Buscaino, G., D'Anna, G., Grammauta, R., Mazzola, S. and Giacalone, V. M. (2019). The use of 3-axial accelerometers to evaluate sound production in European spiny lobster, *Palinurus elephas*. *Ecological Indicators*, *102*, 519–527. https://doi.org/10.1016/j.ecolind.2019.02.064

Chapter 2

Stress, Sound and Invertebrate Larvae: Trends, Methods and Knowledge Gaps

Chapter 2

Stress, Sound and Invertebrate Larvae: Trends, Methods and Knowledge Gaps

Abstract

As evidenced by a systematic review of all peer-reviewed publications published in the field since 1990, the study of anthropogenic sound impacts on the early life stages of invertebrates has increased over previous decades but is sparse (3% of studies since 2010) compared to studies involving other stressors and many unknowns remain. This chapter utilises a bibliometric analysis of early life aquatic invertebrate stressor research and aquatic invertebrate sound response research to identify trends and current knowledge gaps. Past studies mostly investigated whether natural habitat-associated sounds change the developmental rate, orientation and settlement of invertebrate larvae. In contrast, whether and how ubiquitous anthropogenic noise sources such as shipping impacts early life invertebrates is understudied. The literature review also revealed a complete lack of information on whether any noise induced effects may carry over from early life into adulthood or from mothers to offspring. The two gaps of knowledge identified above must be addressed if we are to understand the realistic long-term impacts of noisy environments on aquatic invertebrates over multiple iterations of their life cycles. Using the identified knowledge gaps, experimental hypotheses for this thesis are then generated and stated herein.

2.1 - Trends in invertebrate larvae stressor research - a bibliometric analysis

By assessing how the field of early life invertebrate stressor impact research has changed over time, it is possible to determine: 1) how well-represented underwater sound impacts are in the literature compared to other stressor impacts and 2) how much attention has been paid to different invertebrate taxonomic groups. This will in turn provide information as to whether there is a scarcity of knowledge concerning the responses of early life invertebrates to noise and which taxonomic groups, if any, require further study.

2.1.1 - Methods

To assess how the field of stressor impact research has changed over time, in terms of stressors and taxa studied, literature searches utilising the ISI Web of Science database were carried out on 27th August 2021. This database was chosen as it is a common standard for bibliometric analyses, has a well-defined coverage and offers convenient search result refining and bibliography export functions. Systematic review literature search techniques were applied, modifying methods used by Williams *et al.* (2015), to provide opportunity for future repetition of the same review process. The search terms contained Boolean operators and were designed to return the maximum number of relevant papers as possible, whilst maintaining specificity to the search topic. These terms can be found in Table 2.1. Search terms were entered into the 'Topic' field and the Web of Science Core Collection was queried for papers published from 1900-2021. The results were further refined to contain only 'Article' document types in the English language. Therefore, this review is composed of peer-reviewed articles only. Where necessary, search terms were enclosed in quotation marks to remove large numbers of unrelated papers. Some search terms were truncated with a * symbol to capture papers containing variations of word beginnings or endings (Table 2.1). Bibliographic data was downloaded and stored as an excel spreadsheet for later analysis

by choosing the 'Full Record with Cited References' download option. Whilst attempts were made to formulate search terms with high specificity, many irrelevant papers were returned and these were removed after exporting bibliographic data. Irrelevance occurred mainly due to homonyms of sound and noise e.g. the geographic term 'sound' meaning an ocean inlet or channel. After editing, the first results of the search terms were found to begin in 1991.

All downloaded search results were merged into a single spreadsheet. Each entry in the spreadsheet was given a general taxonomic category and imported into R (R Core Team, 2017). In the R environment, taxa and stressor topic data was summarised by decade and by five-year period. Stressor topic by decade and taxa studied by five-year period were presented graphically to visualise trends over time.

2.1.2 - Results

There has been a clear trend since the 1990s (first paper published in 1991) of a broadening of stressor research with respect to early life invertebrates. Initially, most studies were carried out within a toxicology context, predominantly using established model bivalve larvae (23% of all studies in 1990-1995) and polychaetes (30% of studies 1995-2000) (Figure 2.1) to assess for lethal doses of a variety of chemicals and pharmaceuticals (Figure 2.2). From 2000 onwards, increasing concerns over the potential impacts of stressors on marine ecosystems have led to a proliferation of studies focusing on an ever-wider range of potential stress sources. Whilst the impacts of hypoxia, pH and UV radiation began to be studied in the 2000's, noise impacts on early life invertebrates were not studied until the following decade, this is despite experiments conducted in the 1980s and 1990s having demonstrated the sensitivity of adult invertebrates towards noise (see (Budelmann, 1992a, 1992b; Regnault and Lagardere, 1983)). There are no clear trends in the number of different taxa studied over time, other

than a broadening the types of crustacea and echinoderms studied since the year 2000 (figure 2.1)

Given the justified concerns over global warming and associated ocean acidification, since the 2010's research has focused on temperature (28% of studies in 2010s and 24% of studies in 2020s) and pH (28% of studies 2010s and 26% of studies in 2020s) as stressors, along with chemical pollutants (28% of studies in 2010s and 30% of studies in 2020s) (Figure 2.2). In this research landscape there is a need for attention to be paid to other stressors such as noise, which formed 3% of studies in the 2010s and 1% of studies since 2020. Given the fact that unlike many other stressors mentioned here, we are often in direct control of when, where and how we make noise in aquatic systems, robust evidence generation for impacts can be comparably swiftly and meaningfully acted upon.

Table 2.1 The list of search terms used in the ISI Web of Science bibliometric analysis. Boolean operators were used to maximise the returned results. Results were edited to remove literature in irrelevant fields (e.g the search term 'sound' returned results concerning geographical 'sounds' (sea inlet/channels) and 'noise' returned results concerning statistical noise).

Topic	Search Term	Results (Pre/Post Editing)
Chemical stress	marine AND invertebrate AND (larva* OR embry* OR development*) AND (chemical OR contamina* OR pollut* OR pharmaceutical OR metal*) AND (stress* OR impact OR response OR mortality OR behaviour* OR toxic*)	547/258
Hypoxia	marine AND invertebrate AND (larva* OR embry* OR development*) AND (hypox* OR O2 OR oxygen) AND (stress* OR impact OR response OR mortality OR behaviour* OR toxic*)	143/27
pH	marine AND invertebrate AND (larva* OR embry* OR development*) AND (pH OR acid* OR alkali*) AND (stress* OR impact OR response OR mortality OR behaviour* OR toxic*))	371/145
Salinity	marine AND invertebrate AND (larva* OR embry* OR development*) AND (*salin* NOT salina) AND (stress* OR impact OR response OR mortality OR behaviour* OR toxic*)	139/55
Sound	marine AND invertebrate AND (larva* OR embry* OR development*) AND (noise OR sound OR vibrat* OR acoustic) AND (stress* OR impact OR response OR mortality OR behaviour* OR toxic*)	72/12
Temperature	marine AND invertebrate AND (larva* OR embry* OR development*) AND (temperature OR heat OR therm*) AND (stress* OR impact OR response OR mortality OR behaviour* OR toxic*)	451/166
UV Radiation	marine AND invertebrate AND (larva* OR embry* OR development*) AND (ultraviolet OR UV OR UVA OR UVB OR UV-A OR UV-B OR radiation) AND (stress* OR impact OR response OR mortality OR behaviour* OR toxic*)	56/15

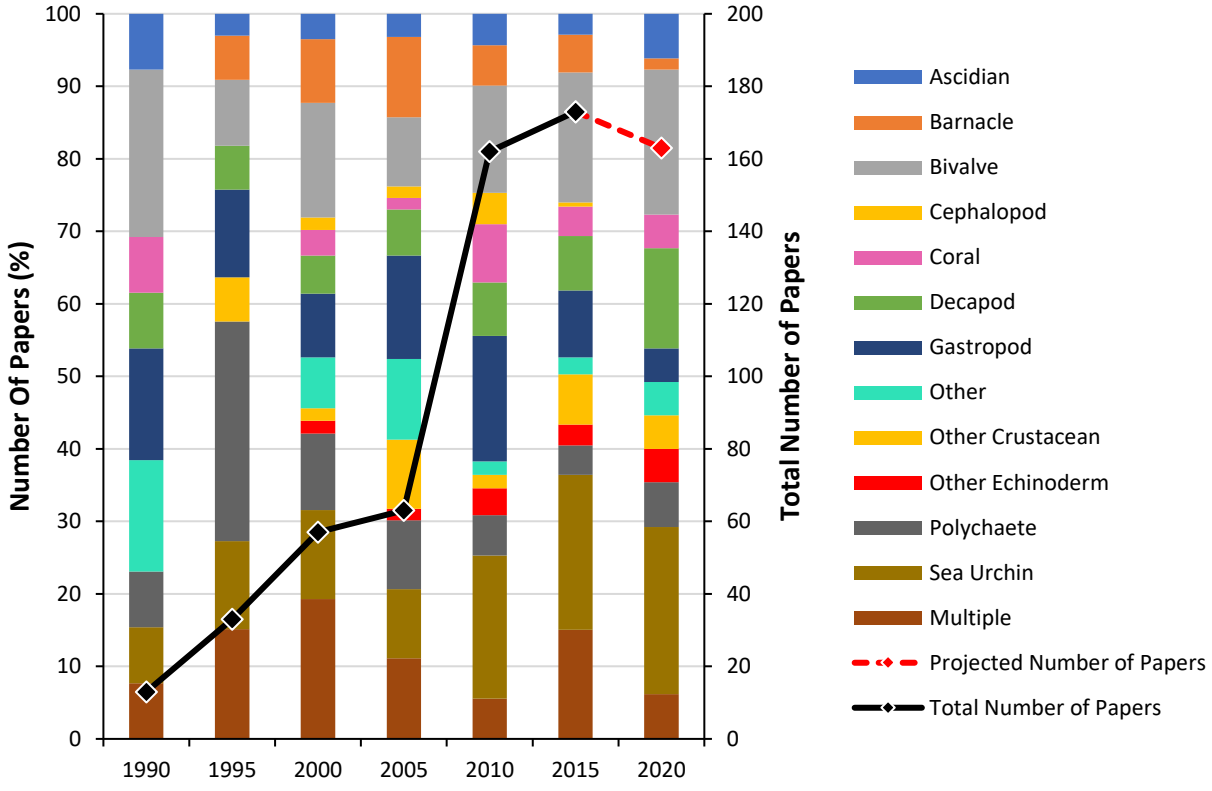


Figure 2.1 Changing patterns in the taxa included in early life stage marine invertebrate stressor research by five-year period. The number of papers that studied each taxa is summarised as a percentage (bar graphs, left y-axis) for each 5-year bin ($1990 \leq t < 1995$, $1995 \leq t < 1999$, etc). The total number of papers published for each 5-year period is also presented (black line graph, right y-axis). As the final five year period (2020-2024) has not been completed, the total number of papers published has been projected assuming the current annual publication rate (32.5 publications $year^{-1}$) remains constant for the remaining years in this period (red dashed line, right y-axis).

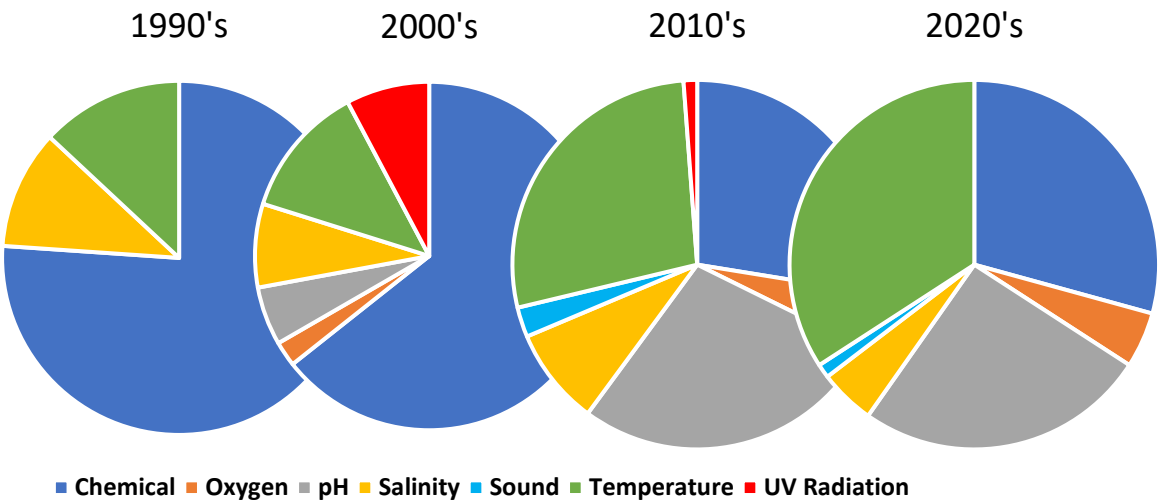


Figure 2.2 Changing patterns in the sources of stress researched in early life stage marine invertebrates by decade. The number of papers that studied each stressor is summarised as a proportion for each 10-year bin ($1990 \leq t < 2000$, $2000 \leq t < 2010$, etc). The number of publications in each group are: 1990s, $n = 46$; 2000s, $n = 129$; 2010s, $n = 421$; 2020s, $n = 82$

2.2 - Underwater sound and early life invertebrates: the state of the art

As seen in section 2.1, the effects of underwater sound on invertebrates are understudied in comparison to other stressors such as temperature and changes in pH, despite the fact that recent work has shown that underwater noise can impact invertebrates in a number of ways, from changes in behaviour (Aimon et al., 2021; Fewtrell and Mccauley, 2012; Mooney et al., 2016; Wale et al., 2013b), to physiology (Solan et al., 2016; Wale et al., 2013a), to biochemistry (Filiciotto et al., 2014; Vazzana et al., 2016). The full extent of the effects of noise on aquatic invertebrates are still largely unknown, given that most species have not yet been studied in this context. This

lack of information is even more stark when one considers the early life stages of invertebrates as will be demonstrated below.

There are already a number of recent and comprehensive reviews of the known impacts of noise on aquatic invertebrates so far (de Soto, 2016; Di Franco et al., 2020; Hawkins and Popper, 2016; Wale et al., 2021), so this literature review will not replicate the work already completed by others. Instead, here I will identify: 1) trends in the way early life stage aquatic invertebrates have been studied (in terms of response parameters and sound exposures) in comparison to adults and 2) existing knowledge gaps regarding the impact of noise on aquatic invertebrates.

2.2.1 - Methods

To illustrate the trends (in life stages studied, sound exposures used, and response parameters measured) in the study of underwater sound and how sound affects early aquatic invertebrate life stages and how these studies fit into the current body of literature, an ISI Web of Science database query was carried out on 27th August 2021, using the same methods as in section 2.1.1. However, this time a single search term was used:

(marine OR ocean OR aquatic OR freshwater) AND (sound OR noise OR acoustic) AND (invertebrate)

Bibliographic data was downloaded and categorised into studies that focused on adults or early life stages. Early life stages were defined as any stage from the point of egg fertilisation to the end of the juvenile phase.

The studies that were found to consider early life invertebrates and underwater sound will be critically analysed.

The search term generated 476 results, after removal of irrelevant papers, 37 papers remained.

2.2.2 – Results

When viewed as a comparison between adult and early life, underwater sound effect studies on aquatic invertebrates have given greater consideration to adults than early life stages. A total of 14 studies (38% of total studies) have been conducted on early life invertebrates, compared to 23 (62% of total studies) on adults (Figure 2.3). No studies have focused on both adults and early life invertebrates.

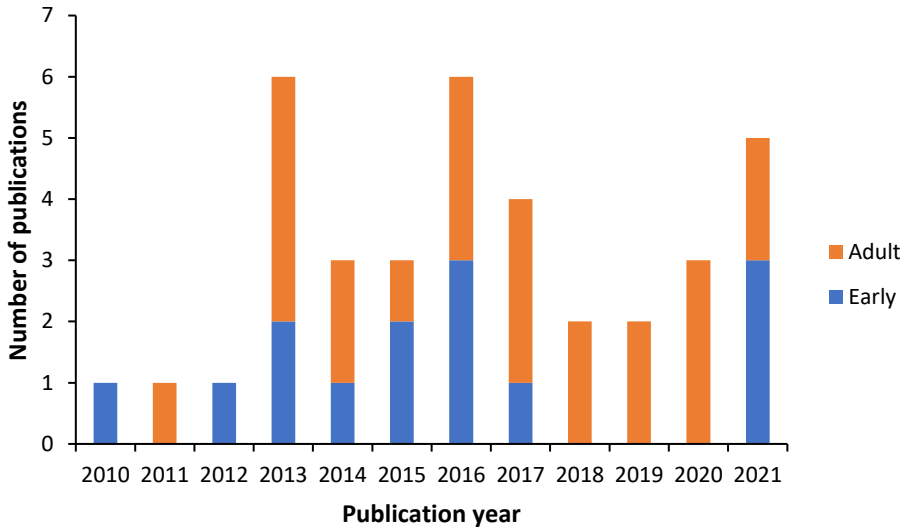


Figure 2.3 Trends in adult versus early-life aquatic invertebrate underwater sound effect research since 2010.

When the noise sources utilised, and response parameters tested, are analysed in more detail, it furthermore becomes clear that early life stages have been researched with different goals in mind when compared to adults.

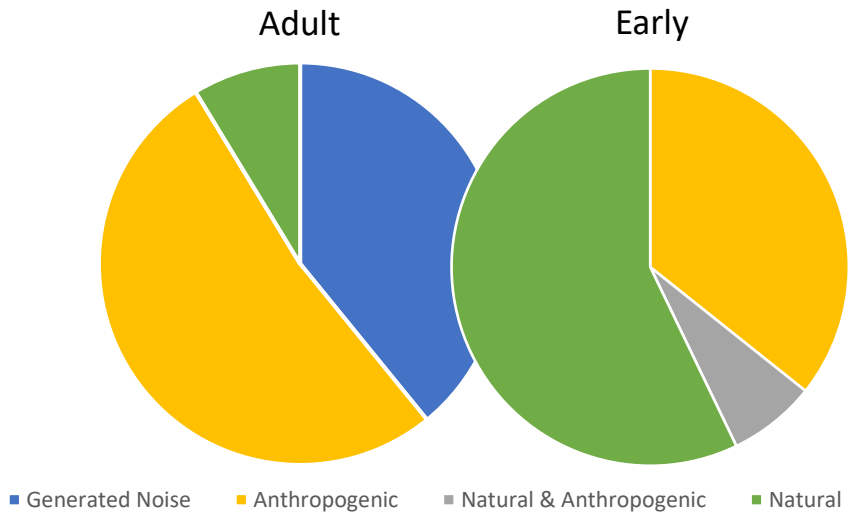


Figure 2.4 The type of sound exposure used in adult versus early-life aquatic invertebrate underwater sound effect research since 2010. Generated noise means a synthetic sound e.g. a pure-tone sine wave or white noise. Adult n = 23, early life n = 14.

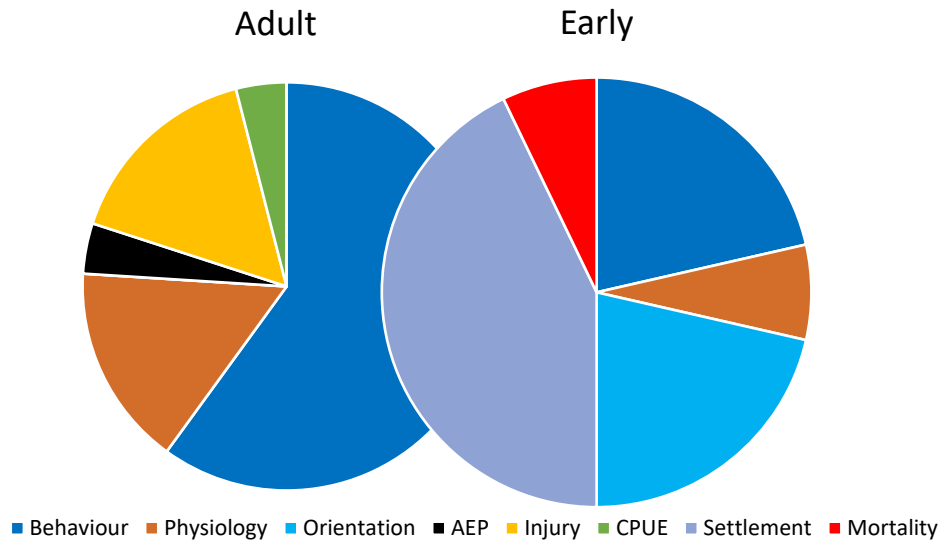


Figure 2.5 The type of response parameter tested in adult versus early-life aquatic invertebrate underwater sound effect research since 2010. AEP = Auditory evoked potential (an electrical brain signal produced in response to acoustic stimulation). CPUE = Catch per unit effort. Adult n = 23, early life n = 14.

In general adult invertebrates have been exposed to anthropogenic and synthetically generated noise (Figure 2.4), to test for: 1) auditory responses, which could be stereotypical behaviours (Mooney et al., 2016) or neurological (Jézéquel et al., 2021); 2) noise disrupted behaviour such a reduced foraging (Jones et al., 2021; Purser and Radford, 2011); 3) noise induced impacts on physiology such as increased oxygen consumption (Aimon et al., 2021; Wale et al., 2013a) and 4) the potential for high amplitude impulsive sound to cause direct mechanical injuries (Day et al., 2019, 2020; Solé et al., 2013a,; 2013b) (Figure 2.5).

In contrast, early life invertebrates have been subjected by researchers to different sound sources, with different response parameters measured. In general, most early life invertebrates have been tested for responses to natural habitat-associated sounds (64% of studies). Researchers have been interested in testing for the way sounds affect:

1) The settlement rate of planktonic larvae (43% of studies), where free-swimming larvae move from the water column to the substrate to begin benthic or sessile juvenile or adult stages (Lillis et al., 2013, 2015).

2) The orientation of free swimming larvae towards or away from the sound source (21% of studies) (Vermeij et al., 2010).

3) Foraging and swimming behaviour (21% of studies) (Aimon et al., 2021).

4) Developmental success (14% of studies) (de Soto et al., 2013).

5) Physiology, such as oxygen consumption (1 study equating to 7%) (Aimon et al., 2021).

Only one study (Day et al., 2016) considered the potential for noise to manifest impacts on marine invertebrates across life history stages. This study exposed egg-bearing *Jasus edwardsii* (Hutton, 1875) females to seismic air gun pulses and found no subsequent effects on the number, morphology, energy content and competency of hatched larvae (Day et al., 2016).

Of all studies returned by the literature search, only one focused on an early life stage freshwater invertebrate (Villalobos et al., 2017), with the remaining articles reporting on marine species.

2.3 - Discussion

Clear trends in the invertebrate noise effect research field have been identified. Not only are early life invertebrates less researched with regard to underwater sound in comparison to their adult counterparts (comprising 38% of search results), but they have also been researched in different ways. The justified excitement over the discovery that natural habitat-associated sound can both orient invertebrate larvae (Sal Moyano et al., 2021; Vermeij et al., 2010) and promote settlement (Jolivet et al., 2016; Lillis et al., 2013; Pine et al., 2016; Stanley et al., 2012, 2015) has caused the research field to tip in favour of pursuing this type of research. This has left two major knowledge gaps to a great extent unaddressed for early life invertebrates in comparison to adults, presented in the following section

2.3.1 – Knowledge gaps

2.3.1.1 – Knowledge gap 1

How do invertebrate larvae respond to ship or boat sound, akin to some of the most prevalent noise pollution sources in the medium to long term? Our contemporary North-East Atlantic, to give an example, with increasingly busy shipping lanes (Farcas et al., 2020; Merchant et al., 2016) and growing areas planned to lease for offshore renewable developments (Myhr et al., 2014; Nedwell and Howell, 2004; Watson et al., 2019) is inhabited by a wide range of commercially and ecologically important temperate invertebrate species that have not yet been tested for early life sensitivity to noise exposure. Many of these species may be subject to chronic or repeated exposures to anthropogenic noise during early life phases as they drift as plankton through shipping lanes, or are deposited as sessile eggs adjacent to a noise-producing offshore installation in its construction or operational phase.

2.3.1.2 – Knowledge gap 2

Only one study (Day et al., 2016), has so far considered the potential for noise to manifest impacts on aquatic invertebrates across life history stages, and this study exposed a single life stage (eggs) to noise, rather than repeating the noise exposure at later larval stages. Hence, the following knowledge gaps remain**: Does exposure of larvae to noise affect the responses of juveniles and adults to subsequent exposures? Does a noise impacted mother transfer effects to her offspring?** Answering this type of question is now being considered of vital importance to gaining a holistic understanding of how a stressor impacts on organisms in the long term that acute impact studies cannot provide (see (Bell and Hellmann, 2019; Dupont et al., 2013; Ellis et al., 2020)).

2.3.2 – Overarching hypotheses

The work reported in this thesis will address the identified knowledge gaps (section 2.3.2) by testing the following experimental hypotheses (see below) on a taxonomically diverse group of 5 aquatic invertebrate species under controlled laboratory conditions. The hypotheses are generated based on the starting position that exposure to anthropogenic noise playbacks will elicit a 'stress' type response in the subject animal. Stress is often defined and measured differently according to the level of biological organisation (e.g. physiological vs psychological). However, in general terms, stress occurs when a biological control system detects a failure to control a fitness-critical variable (Del Giudice et al., 2018). The response of the biological control systems to stress can include both feedback (reactive) and feedforward (anticipatory) components, and the interplay of these can result in wide-ranging responses. Remaining cognisant of the fact that stress responses can manifest a wide range of resultant biological changes, depending on the make-up of the neuro-endocrine systems involved and the specific ways in which they are activated, an example of how the starting position of noise-induced stress leads to predictions of downstream response parameters is as follows: Noise → Stress → Increased Respiration → Decreased energetic investment into growth → Delayed development. Or to give another example: Noise → Stress → Behavioural change → Reduced larval settlement.

The experimental hypotheses to be tested in this thesis are as follows:

H_1: Underwater noise will increase the oxygen consumption of the exposed early life stages of the study species

(chapters 3 and 5)

H_2: Underwater noise will delay the development of the exposed early life stages of the study species

(chapters 3, 4 and 7)

H_3: Underwater noise will reduce the survival of the exposed early life stages of the study species

(chapters 3 – 7)

H_4: Underwater noise will increase oxidative stress biomarker levels of the exposed early life stages of the study species

(chapter 3)

H_5: Underwater noise will change the behaviour of the exposed early life stages of the study species

(chapters 3 and 6)

H_6: Underwater noise will reduce the larval settlement rate of the exposed early life stages of the study species

(chapter 6)

H_7: Underwater noise will reduce reproductive output/fecundity of the exposed early life stages of the study species

(chapter 7)

2.4 – References

Aimon, C., Simpson, S. D., Hazelwood, R. A., Bruintjes, R. and Urbina, M. A. (2021). Anthropogenic underwater vibrations are sensed and stressful for the shore crab *Carcinus maenas*. *Environmental Pollution*, *285*, 117148. https://doi.org/10.1016/j.envpol.2021.117148

Bell, A. M. and Hellmann, J. K. (2019). An Integrative Framework for Understanding the Mechanisms and Multigenerational Consequences of Transgenerational Plasticity. *Annual Review of Ecology, Evolution, and Systematics*, *50*(1), 97–118. https://doi.org/10.1146/annurev-ecolsys-110218-024613

Budelmann, B. U. (1992a). Hearing in Crustacea. In *The Evolutionary Biology of Hearing* (pp. 131–139). Springer-Verlag.

Budelmann, B. U. (1992b). Hearing in Nonarthropod Invertebrates. In *The Evolutionary Biology of Hearing* (pp. 141–155). Springer-Verlag.

Day, R. D., Fitzgibbon, Q. P., McCauley, R. D., Hartmann, K. and Semmens, J. M. (2020). Lobsters with pre-existing damage to their mechanosensory statocyst organs do not incur further damage from exposure to seismic air gun signals. *Environmental Pollution*, *267*, 115478. https://doi.org/10.1016/j.envpol.2020.115478

Day, R. D., McCauley, R. D., Fitzgibbon, Q. P., Hartmann, K. and Semmens, J. M. (2019). Seismic air guns damage rock lobster mechanosensory organs and impair righting reflex. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1907). https://doi.org/10.1098/rspb.2019.1424

Day, R. D., McCauley, R. D., Fitzgibbon, Q. P. and Semmens, J. M. (2016). Seismic air gun exposure during early-stage embryonic development does not negatively affect

spiny lobster *Jasus edwardsii* larvae (Decapoda:Palinuridae). *Scientific Reports*, *6*(1), 22723. https://doi.org/10.1038/srep22723

de Soto, N. A. (2016). Peer-Reviewed Studies on the Effects of Anthropogenic Noise on Marine Invertebrates: From Scallop Larvae to Giant Squid. In A. N. Popper & A. Hawkins (Eds.), *The Effects of Noise on Aquatic Life II* (pp. 17–26). Springer New York. https://doi.org/10.1007/978-1-4939-2981-8_3

de Soto, N. A., Delorme, N., Atkins, J., Howard, S., Williams, J. and Johnson, M. (2013). Anthropogenic noise causes body malformations and delays development in marine larvae. *Scientific Reports*, *3*(1), 2831. https://doi.org/10.1038/srep02831

Del Giudice, M., Buck, C. L., Chaby, L. E., Gormally, B. M., Taff, C. C., Thawley, C. J., Vitousek, M. N. and Wada, H. (2018). What Is Stress? A Systems Perspective. *Integrative and Comparative Biology*, *58*(6), 1019–1032. https://doi.org/10.1093/icb/icy114

Di Franco, E., Pierson, P., Di Iorio, L., Calò, A., Cottalorda, J. M., Derijard, B., Di Franco, A., Galvé, A., Guibbolini, M., Lebrun, J., Micheli, F., Priouzeau, F., Risso-de Faverney, C., Rossi, F., Sabourault, C., Spennato, G., Verrando, P. and Guidetti, P. (2020). Effects of marine noise pollution on Mediterranean fishes and invertebrates: A review. *Marine Pollution Bulletin*, *159*(March), 111450. https://doi.org/10.1016/j.marpolbul.2020.111450

Dupont, S., Dorey, N., Stumpp, M., Melzner, F. and Thorndyke, M. (2013). Long-term and trans-life-cycle effects of exposure to ocean acidification in the green sea urchin *Strongylocentrotus droebachiensis*. *Marine Biology*, *160*, 1835–1843. https://doi.org/10.1007/s00227-012-1921-x

Ellis, L. A., Kissane, S., Hoffman, E., Brown, J. B., Valsami-Jones, E., Colbourne, J. and Lynch, I. (2020). Multigenerational Exposures of Daphnia Magna to Pristine

and Aged Silver Nanoparticles: Epigenetic Changes and Phenotypical Ageing Related Effects. *Small*, *16*(21), 2000301. https://doi.org/10.1002/smll.202000301

Farcas, A., Powell, C. F., Brookes, K. L. and Merchant, N. D. (2020). Validated shipping noise maps of the Northeast Atlantic. *Science of the Total Environment*, *735*, 139509. https://doi.org/10.1016/j.scitotenv.2020.139509

Fewtrell, J. L. and Mccauley, R. D. (2012). Impact of air gun noise on the behaviour of marine fish and squid. *Marine Pollution Bulletin*, *64*(5), 984–993. https://doi.org/10.1016/j.marpolbul.2012.02.009

Filiciotto, F., Vazzana, M., Celi, M., Maccarrone, V., Ceraulo, M., Buffa, G., Stefano, V. Di, Mazzola, S. and Buscaino, G. (2014). Behavioural and biochemical stress responses of *Palinurus elephas* after exposure to boat noise pollution in tank. *Marine Pollution Bulletin*, *84*(1–2), 104–114. https://doi.org/10.1016/j.marpolbul.2014.05.029

Hawkins, A. D. and Popper, A. N. (2017). A sound approach to assessing the impact of underwater noise on marine fishes and invertebrates. *ICES Journal of Marine Science*, *74*(3), 635–651. https://doi.org/10.1093/icesjms/fsw205

Jézéquel, Y., Jones, I. T., Bonnel, J., Chauvaud, L., Atema, J. and Mooney, T. A. (2021). Sound detection by the American lobster (*Homarus americanus*). *Journal of Experimental Biology*, *224*(6). https://doi.org/10.1242/jeb.240747

Jolivet, A., Tremblay, R., Olivier, F., Gervaise, C., Sonier, R., Genard, B. and Chauvaud, L. (2016). Validation of trophic and anthropic underwater noise as settlement trigger in blue mussels. *Scientific Reports*, *6*(1), 33829. https://doi.org/10.1038/srep33829

Jones, I. T., Peyla, J. F., Clark, H., Song, Z., Stanley, J. A. and Mooney, T. A. (2021).

Changes in feeding behavior of longfin squid (*Doryteuthis pealeii*) during laboratory exposure to pile driving noise. *Marine Environmental Research, 165*, 105250. https://doi.org/10.1016/j.marenvres.2020.105250

Lillis, A., Bohnenstiehl, D. R. and Eggleston, D. B. (2015). Soundscape manipulation enhances larval recruitment of a reef-building mollusk. *PeerJ, 3*, e999. https://doi.org/10.7717/peerj.999

Lillis, A., Eggleston, D. B. and Bohnenstiehl, D. R. (2013). Oyster larvae settle in response to habitat-associated underwater sounds. *PLoS ONE*, *8*(10), 21–23. https://doi.org/10.1371/journal.pone.0079337

Merchant, N. D., Brookes, K. L., Faulkner, R. C., Bicknell, A. W. J., Godley, B. J. and Witt, M. J. (2016). Underwater noise levels in UK waters. *Nature Publishing Group, November*, 1–10. https://doi.org/10.1038/srep36942

Mooney, T. A., Samson, J. E., Schlunk, A. D. and Zacarias, S. (2016). Loudness-dependent behavioral responses and habituation to sound by the longfin squid (*Doryteuthis pealeii*). *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *202*(7), 489–501. https://doi.org/10.1007/s00359-016-1092-1

Myhr, A., Bjerkseter, C., Ågotnes, A. and Nygaard, T. A. (2014). Levelised cost of energy for offshore floating wind turbines in a life cycle perspective. *Renewable Energy*, *66*, 714–728. https://doi.org/10.1016/j.renene.2014.01.017

Nedwell, J. and Howell, D. (2004). A review of offshore windfarm related underwater noise sources. *Cowrie Rep*, 1–63. https://doi.org/10.1093/cid/cir102

Pine, M. K., Jeffs, A. G. and Radford, C. A. (2016). Effects of Underwater Turbine Noise on Crab Larval Metamorphosis. *Advances in Experimental Medecine and*

Biology, *875*, 847–851. https://doi.org/10.1007/978-1-4939-2981-8

Purser, J. and Radford, A. N. (2011). Acoustic noise induces attention shifts and reduces foraging performance in three-spined sticklebacks (*Gasterosteus aculeatus*). *PLoS ONE*, *6*(2). https://doi.org/10.1371/journal.pone.0017478

R Core Team. (2017). R: A language and environment for statistical computing URL https://www.R-project.org/. *R Foundation for Statistical Computing, Vienna, Austria*.

Regnault, N. and Lagardere, J.-P. (1983). Effects of ambient noise on the metabolic level of *Crangon crangon* (Decapoda, Natantia). *Marine Ecology Progress Series*, *11*, 71–78. https://doi.org/10.3354/meps011071

Sal Moyano, M., Ceraulo, M., Hidalgo, F., Luppi, T., Nuñez, J., Radford, C., Mazzola, S., Gavio, M. and Buscaino, G. (2021). Effect of biological and anthropogenic sound on the orientation behavior of four species of brachyuran crabs. *Marine Ecology Progress Series*, *669*, 107–120. https://doi.org/10.3354/meps13739

Solan, M., Hauton, C., Godbold, J. A., Wood, C. L., Leighton, T. G. and White, P. (2016). Anthropogenic sources of underwater sound can modify how sediment-dwelling invertebrates mediate ecosystem properties. *Scientific Reports*, *6*(1), 20540. https://doi.org/10.1038/srep20540

Solé, M., Lenoir, M., Durfort, M., López-Bejar, M., Lombarte, A. and André, M. (2013a). Ultrastructural Damage of *Loligo vulgaris* and *Illex coindetii* statocysts after Low Frequency Sound Exposure. *PLoS ONE*, *8*(10), e78825. https://doi.org/10.1371/journal.pone.0078825

Solé, M., Lenoir, M., Durfort, M., López-Bejar, M., Lombarte, A., Van Der Schaar, M. and André, M. (2013b). Does exposure to noise from human activities compromise

sensory information from cephalopod statocysts? *Deep-Sea Research Part II: Topical Studies in Oceanography*, *95*, 160–181. https://doi.org/10.1016/j.dsr2.2012.10.006

Stanley, J. A., Hesse, J., Hinojosa, I. A. and Jeffs, A. G. (2015). Inducers of settlement and moulting in post-larval spiny lobster. *Oecologia*, *178*(3), 685–697. https://doi.org/10.1007/s00442-015-3251-4

Stanley, J. A., Radford, C. A. and Jeffs, A. G. (2012). Location, location, location: Finding a suitable home among the noise. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1742), 3622–3631. https://doi.org/10.1098/rspb.2012.0697

Vazzana, M., Celi, M., Maricchiolo, G., Genovese, L., Corrias, V., Quinci, E. M., de Vincenzi, G., Maccarrone, V., Cammilleri, G., Mazzola, S., Buscaino, G. and Filiciotto, F. (2016). Are mussels able to distinguish underwater sounds? Assessment of the reactions of *Mytilus galloprovincialis* after exposure to lab-generated acoustic signals. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *201*, 61–70. https://doi.org/10.1016/j.cbpa.2016.06.029

Vermeij, M. J. A., Marhaver, K. L., Huijbers, C. M., Nagelkerken, I. and Simpson, S. D. (2010). Coral larvae move toward reef sounds. *PLoS ONE*, *5*(5), 3–6. https://doi.org/10.1371/journal.pone.0010660

Villalobos, G., Alison, J. and Christopher, M. D. (2017). Environmental noise reduces predation rate in an aquatic invertebrate. *Journal of Insect Conservation*, *21*(5), 839–847. https://doi.org/10.1007/s10841-017-0023-y

Wale, M. A., Briers, R. A. and Diele, K. (2021). Marine invertebrate anthropogenic noise research – Trends in methods and future directions. *Marine Pollution*

Bulletin. https://doi.org/10.1016/j.marpolbul.2021.112958

Wale, M. A., Simpson, S. D. and Radford, A. N. (2013a). Size-dependent physiological responses of shore crabs to single and repeated playback of ship noise. *Biology Letters*, *9*(2), 20121194. https://doi.org/10.1098/rsbl.2012.1194

Wale, M. A., Simpson, S. D. and Radford, A. N. (2013b). Noise negatively affects foraging and antipredator behaviour in shore crabs. *Animal Behaviour*, *86*(1), 111–118. https://doi.org/10.1016/j.anbehav.2013.05.001

Watson, S., Moro, A., Reis, V., Baniotopoulos, C., Barth, S., Bartoli, G., Bauer, F., Boelman, E., Bosse, D., Cherubini, A., Croce, A., Fagiano, L., Fontana, M., Gambier, A., Gkoumas, K., Golightly, C., Latour, M. I., Jamieson, P., Kaldellis, J., … Wiser, R. (2019). Future emerging technologies in the wind power sector: A European perspective. *Renewable and Sustainable Energy Reviews*, *113*, 109270. https://doi.org/10.1016/j.rser.2019.109270

Williams, R., Wright, A. J., Ashe, E., Blight, L. K., Bruintjes, R., Canessa, R., Clark, C. W., Cullis-Suzuki, S., Dakin, D. T., Erbe, C., Hammond, P. S., Merchant, N. D., O'Hara, P. D., Purser, J., Radford, A. N., Simpson, S. D., Thomas, L. and Wale, M. A. (2015). Impacts of anthropogenic noise on marine life: Publication patterns, new discoveries, and future directions in research and management. *Ocean and Coastal Management*, *115*, 17–24. https://doi.org/10.1016/j.ocecoaman.2015.05.021

Chapter 3

Effects of anthropogenic noise on the early life stages of *Nephrops norvegicus*

Chapter 3

Effects of anthropogenic noise on the early life stages of *Nephrops norvegicus*

Abstract:

Anthropogenic noise can negatively affect marine invertebrate behaviour and physiology. Most research has focused on adults, with no studies previously exploring the effects of the ship motor no ise on planktonic crustacean larvae. This study investigated the responses of larval Norway lobster *Nephrops norvegicus* (L.) to playbacks of ship noise in the laboratory. *N. norvegicus* larvae (zoea stages I, II and III) were reared individually in acoustically transparent cups and exposed to three different sound treatments using underwater speakers (n = 83 larvae for each group): 'Busy' treatment, consisting of frequent ship noise playback (totalling 12 hours of noise exposure per 24 hours); 'Occasional' treatment, consisting of less frequent ship noise playback (totalling 3 hours of noise exposure per 24 hours) and 'Ambient' treatment, consisting of constant ambient sound exclusive of any ship noise (Control). Both 'Busy' and 'Occasional' groups showed significantly delayed development (3.36% and 4.39% delays respectively) compared to ambient sound controls. Juveniles reared under ship noise had reduced escape response stamina ('Busy' and 'Occasional' stamina significantly reduced by 33.3% and 45.9% respectively), demonstrating that noise impacts carry over to later life stages. Acute ship noise exposure caused increased (25.9%) oxygen consumption in naïve larvae, whereas noise pre-exposed larvae *decreased* (-13.1%) oxygen consumption in response to noise playbacks. Taken together, these findings suggest that anthropogenic noise elicits physiological responses in *N. norvegicus* that lead to developmental impacts over multiple exposures, carrying-over to juvenile life stages.

3.1 - Introduction

Since the industrial revolution, human activities in the marine environment have led to an increase in underwater noise. Low-frequency sound levels (10 – 500 Hz), have increased by at least 100-fold globally over the last century (Hildebrand, 2009). This is predominantly caused by growing numbers of large commercial vessels and seismic surveys (Ross, 2005). This increase in ambient sound levels was first suggested to be potentially harmful to marine fauna in 1971, with concern over its impacts on long-range baleen whale communication (Payne and Webb, 1971). Since then, the effects of underwater noise on marine fauna have been increasingly studied, with the majority of effort focused upon marine mammal and fish species (Williams et al., 2015). Invertebrates, including crustaceans, have received far less attention in comparison; the effects of anthropogenic noise on this large and diverse taxonomic group remains largely unclear (Morley et al., 2014; Popper and Hawkins, 2016; Williams et al., 2015). Recently, marine noise has become recognised internationally as a pollutant of importance in legislation such as the European Commission Marine Strategy Framework Directive (Borja et al., 2010; Tasker et al., 2010; Van der Graaf et al., 2012). There is therefore an imperative to increase the ability of this type of legislation to effectively manage noise levels in relation to invertebrates, by improving knowledge of what noise sources are of relevance, their ranges of effect and the species of concern.

Studies focusing on direct mechanical damage of high energy impulsive sounds on adult invertebrates have reported mixed results, even within the same species. Acute exposure of adult snow crab *Chionocetes opilio* (O. Fabricius, 1788) to seismic survey pulses (received sound pressure levels: 197-220 dB re: 1 µPa (zero-peak)) in the field caused no significant mechanical damage to hepatopancreas, heart, ovary or statocyst tissues (Christian et al., 2003). In contrast, the seismic pulses caused bruised hepatopancreas and ovary tissue to adult *C. opilo* in a different location (received

pressure levels not reported) (DFO, 2004). Larvae of the dungeness crab (*Cancer magister* (Dana, 1852)) exposed to a single airgun pulse (230.9 (±1.0) dB peak-peak re: 1 µPa) from a seven gun array showed no changes in developmental time or mortality compared to a control group (156 to 168 dB peak-peak re 1 µPa), suggesting a resistance of crustacean larvae to high amplitude impulsive sound (Pearson et al., 1994). It should be noted however, that received levels in the control group were particularly high in the Pearson et al. study (1994), and caution should be applied in attempts to extrapolate this apparent resilience to other crustacean species.

Whilst the amplitude is lower than impulsive noise pollution sources such as pile-driving or seismic surveys, shipping noise is the most ubiquitous anthropogenic noise source and is the dominant contributor to ambient sound levels in shallow-water areas near shipping lanes surrounding the UK (Harland et al., 2005). Whilst no direct lethal effects of continuous noise such as shipping have been reported for crustaceans, sub-lethal behavioural and physiological effects on Decapoda have been reported in a range of species; adult *Nephrops norvegicus* (L.) is reported to exhibit repressed burying and bioirrigation behaviour and reduced locomotion activity in response to ship sound playbacks (received sound pressure levels: 135–140 dB re: 1 µ Pa (rms)) (Solan et al., 2016). Increased metabolic rates of adult brown shrimp, *Crangon crangon* (L.), in response to increased ambient sound levels (+25 dB μbar^{-1}) and of adult shore crabs *Carcinus maenas* (L.) in response to ship noise playbacks (148–155 dB rms re: 1 µPa) have also been reported (Regnault and Lagardere, 1983; Wale et al., 2013a).

Most decapod crustaceans pass through multiple planktonic life stages before reaching adulthood and exhibit r-strategist reproductive traits, with an initial larval abundance that vastly outnumbers the adult population but low larval survival (MacArthur and Wilson, 1967; Parry, 1981). For this reason it has been suggested that early life stages act as a population bottleneck for species with a larval stage, and any

stressor that affects larval survival has the potential to cause significant consequences at the population level (Byrne, 2011). Despite this, no studies to date have assessed the potential effects of elevated levels of continuous shipping noise - the most widespread anthropogenic noise - on the development of crustacean larvae.

Shellfish are historically the UK's most valuable (£/tonne) fishing resource (Dixon et al., 2017), with *N. norvegicus* commanding the second highest price per tonne (£3270) of any fished species in 2016. In 2016, 30,900 tonnes of *N. norvegicus* were landed in the UK, with a value of £100.8 million at first sale, accounting for 31% of the value of total UK shellfish landings.

In Scottish waters, female *N. norvegicus* matures at a carapace length of 21-34 mm, whilst males mature at 29-46 mm (Tuck et al., 2000). Mating usually occurs over the summer months, after which fertilised eggs are extruded onto the female's pleopods, where they are incubated over the winter until larval hatching occurs March-June (Powell and Eriksson, 2013). Hatching occurs at night over a period of successive days (5 – 20), with dozens to several hundred larvae being released each evening. Females exit their burrows and adopt a hatching posture similar to *H. gammarus*, whereby they raise their tails and beat their pleopods to expel newly hatched larva into the water column (Smith, 1987).

N. norvegicus passes through 3 planktonic larval stages (ZI, ZII and ZIII) before settling to the benthos and metamorphosing into the first post-larval stage (PL1) (Johnson and Johnson, 2013; Powell and Eriksson, 2013; Smith, 1987) (Figure 3.1). Whilst PL1 is benthic, and exhibits adult-like morphological characteristics, it is not considered to be a true juvenile until its subsequent moult to stage V (Powell and Eriksson, 2013). Any deleterious impacts during the early life history of this species are likely to have critical importance for the reproductive success and recruitment of the population as a whole (Ligas et al., 2011; Sinclair and IIles, 1989). Adult *N. norvegicus*

exhibit burrowing behaviour and spend the majority of time in tunnel systems (Solan et al., 2016); their distribution is therefore restricted to areas with muddy substrate suitable for burrows (Lordan et al., 2013). Based on *N. norvegicus* presence data, Lordan, Power and Johnson (2013) were able to produce a habitat suitability model using mean annual bottom temperature, salinity, oxygen, mean depth and mean annual surface chlorophyll (Figure 3.2). The core habitat range of this model closely aligns with the most popular shipping routes, with most core habitat coinciding with routes that received over 625,000 unique vessel passages in 2017 (based on MarineTraffic data available at: www.marinetraffic.com) (Figure 3.3). The pelagic stage of *N. norvegicus* can last approximately 7 weeks, depending on water temperatures (Hill, 1990; Powell and Eriksson, 2013), meaning that developing larvae are likely to spend much of their time in close proximity to passing marine vessels and the low frequency noise they produce.

Here, the effect of repeated exposure to ship noise playbacks on i) the survival and developmental duration of each *N. norvegicus* zoea stage (ZI, ZII and ZIII), ii), the tail-flip escape response stamina of PL1 stage iii) oxidative stress biomarkers in PL1 stage and iv) the oxygen consumption of naïve (never having received experimental noise playbacks) versus pre-exposed ZI, ZII and ZIII zoea is investigated. The control playback treatment is compared to two different ship playback regimes: i) 'Busy', with a higher frequency of ship sound occurrences than would likely be experienced by larvae in the wild and ii)'Occasional', with a lower frequency of ship noise occurrences that is more representative of feasible real-world ship noise exposure scenario.

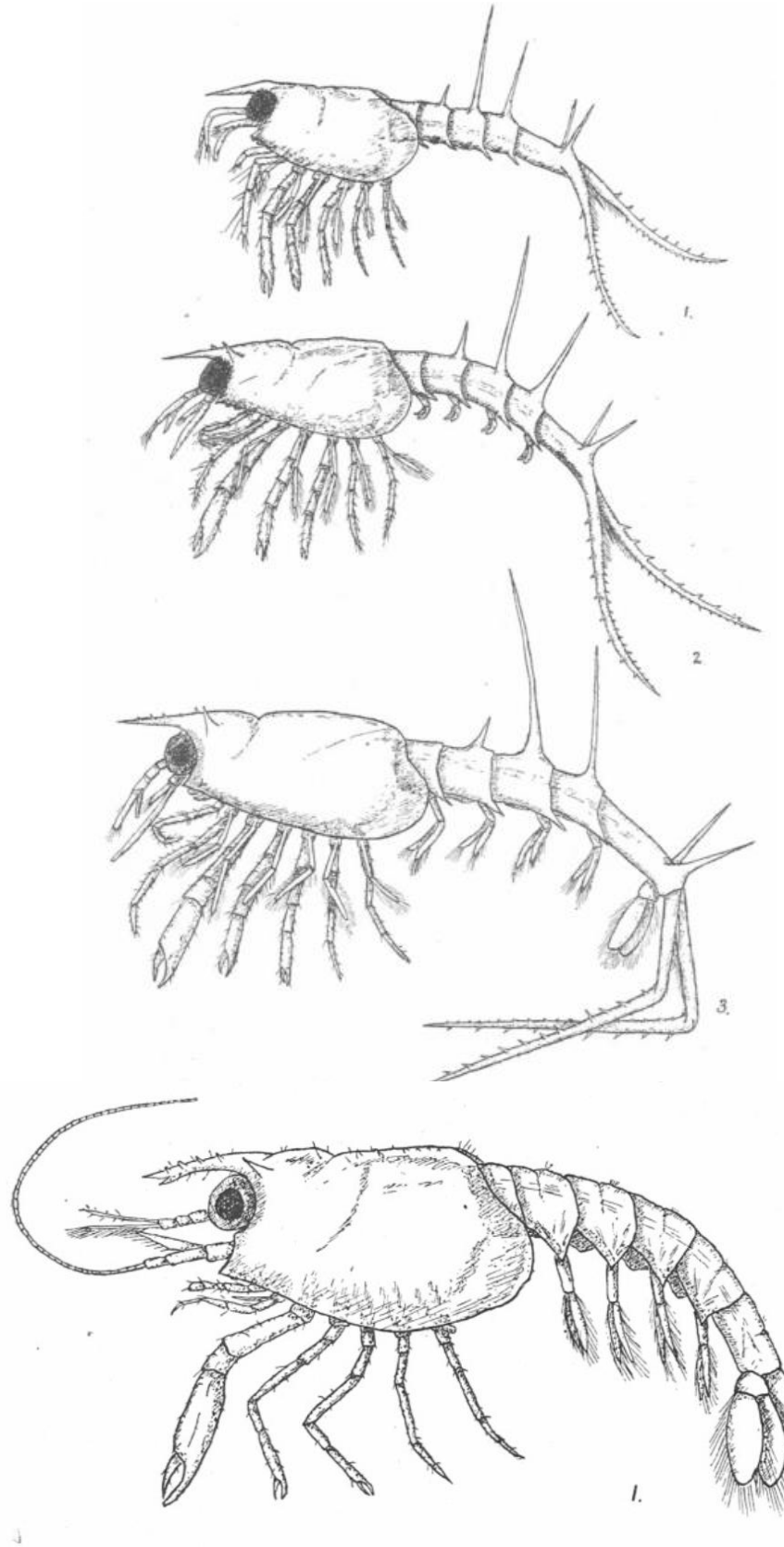


Figure 3.1. Morphology of the zoeal stages I-III (ZI,ZII,ZII) and the first post-larval stage (PL1) of developing *Nephrops norvegicus* (L.), adapted from drawings by Jorgenson (1925). Measurements are average distances from the anterior rostrum tip to the posterior caudal tip, not including the forked caudal appendages found in ZI-ZIII.

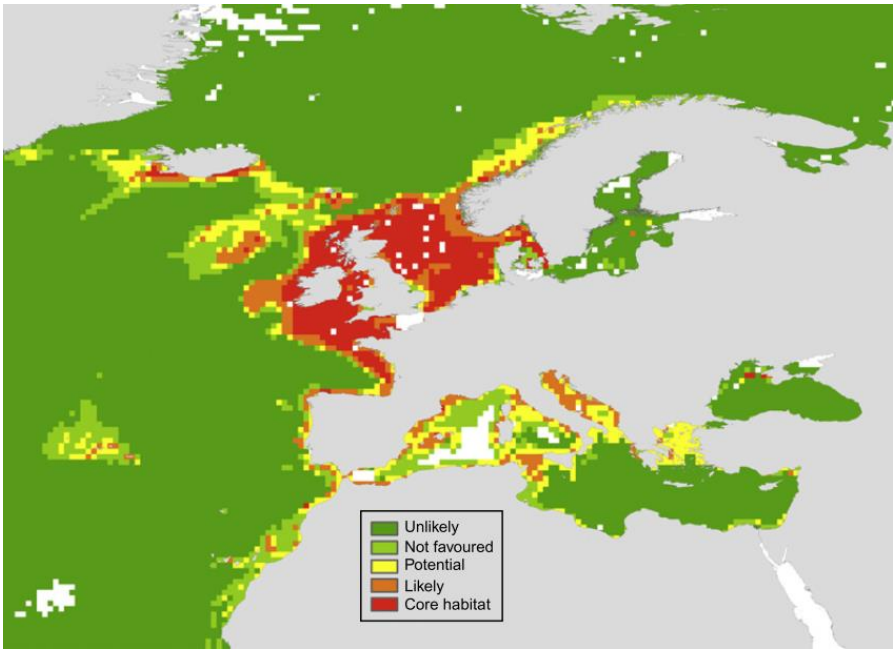


Figure 3.2 MAXENT habitat suitability model for *Nephrops norvegicus* (L.) based on the following predictor variables: mean annual bottom temperature, salinity, oxygen, mean depth and mean annual surface chlorophyll. AUC=0.953. Figure sourced from (Lordan et al., 2013). MAXENT = maximum entropy presence-only species distribution model. AUC = area under the curve.

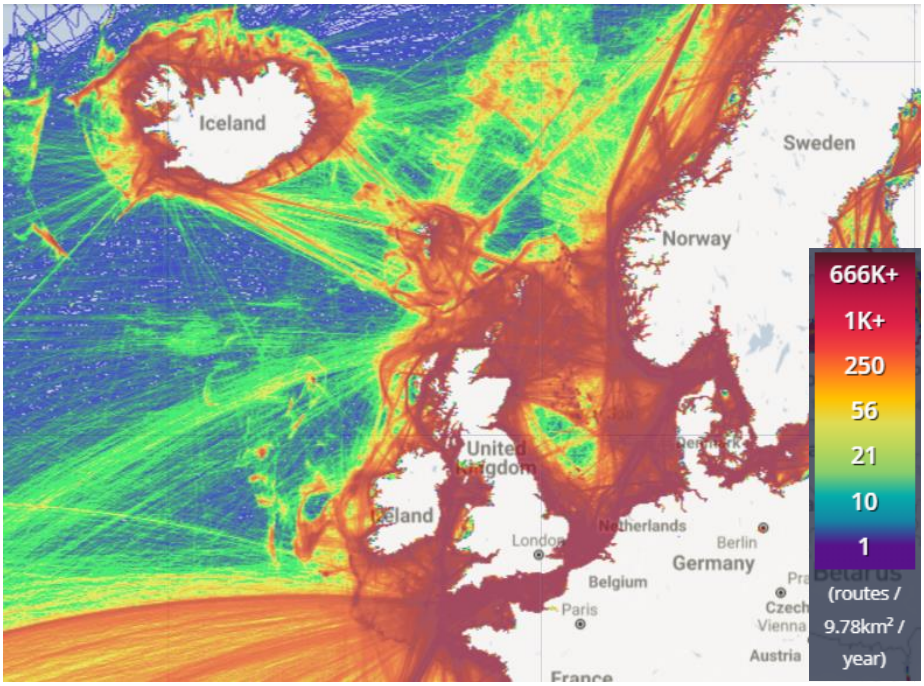


Figure 3.3 Density map of marine traffic routes in the North-East Atlantic during 2017. Based on AIS data from all vessel types. Legend refers to the number of distinct vessels on a daily basis and count positions per km^2 calculated each day over the 2017 period. Data sourced from: https://www.marinetraffic.com/. AIS = Automatic Identification System (as adopted by the International Maritime Organization).

3.2 - Materials and methods

Analysis of developmental duration, survival, tail-flick escape response and oxidative stress biomarkers were conducted on a larval cohort from spring/summer 2017. Oxygen consumption microrespirometry experiments were carried out using a new cohort of larvae in summer 2018. Unless otherwise stated, the following broodstock, hatching and larval maintenance procedures were followed for 2017 and 2018 experiments.

3.2.1 - Broodstock

Berried female *N. norvegicus* were landed at Eyemouth, Scotland (N 55.872008, E -2.087435) in March and were immediately transported to St Abbs Marine Station (N 55.898774, E -2.130516) where they were maintained in rectangular tanks (dimensions: 1600 mm x 1030 mm x 400 mm, 432 L volume) on a continuous flow-through system at ambient seawater temperature (7.41 ± 0.5 ^{O}C) and salinity 33.98 ± 0.3 ppt. Stocking density was 10 per tank. The tanks contained a 10 cm layer of sandy substrate to allow natural burrowing behaviour and were dimmed constantly with 75% shade netting. The broodstock was fed frozen cooked mussel (*Mytilus edulis* (L.)) *ad libitum* twice weekly, removing any uneaten food after each feeding session. This diet has previously been used to successfully rear berried *N. norvegicus* to larvae hatching (Rotllant et al., 2001). In preparation for larval hatching, the berried females were separated into individual cylinder-conic upweller tanks (20 L water volume, dimensions: top diameter 300 mm, depth of cylindrical section 150 mm, depth of conical section 150 mm), with a PVC tube to emulate a burrow shelter, shade netting over the tank surface (75% shade rating) and a mesh fitted to prevent escape of released larvae through the outflow, as in Rotllant et al. (2001). The upweller tanks were also connected to the flow-through system. To keep disturbance at a minimum, females were fed a frozen mussel weekly, with any

uneaten food removed at each feeding session. The conical tanks were checked at 10:00 each morning for hatched stage I zoeae (ZI).

3.2.2 - Hatching

3.2.2.1 - 2017

On 22 May, two females began the hatching process. Each day, during the period 11:00-12:00, all hatched larvae were transferred to individual rearing chambers in the sound exposure set-up using a wide-mouthed pipette. Hatched ZI larvae on a given day were distributed equally between 3 sound treatment tanks (Busy, Occasional and Ambient). Sound treatment tank dimensions were 1600 x 1030 x 690 mm, with a volume of 837 L. On the 10th day of hatching, all 83 rearing cups in each sound treatment tank were occupied with a single ZI larva. Each of the two females contributed larvae to 50% of the rearing cups.

3.2.2.2 - 2018

On 20th July, another two females began the hatching process. Again, each day, during the period 11:00-12:00, all hatched larvae were transferred to individual rearing chambers in the sound exposure set-up using a wide-mouthed pipette. Hatched ZI larvae on a given day were distributed equally between 2 sound treatment tanks (Occasional and Ambient). Again, sound treatment tank dimensions were 1600 x 1030 x 690 mm, with a volume of 837 L. On 6th day of hatching, all 83 rearing cups in each sound treatment tank were occupied with a single ZI larva. Each of the two females contributed larvae to 50% of rearing cups.

3.2.3 - Larval maintenance

A 100% water change was carried out every three days by gently pipetting each larva into a new rearing vessel with fresh UV sterilised seawater of the same

temperature. Larvae were fed *Artemia salina* (L.) nauplii enriched with highly unsaturated fatty-acid (HUFA) enrichment solution (ZM Fish Food (2017)) *ad libitum* at a density of 60 nauplii per individual larva per day; this food-type has been shown to promote the greatest survival in laboratory rearing conditions (Rotllant et al., 2001). 2 ml of evenly mixed enriched *A. salina* suspension was pipetted into each rearing cup, ensuring no variation in feed levels occurred between cups. The order of feeding for each sound treatment group was randomised daily and all larvae were fed within a 15-minute period.

3.2.4 - Experimental set-up and design

Each larva was reared individually in an acoustically transparent polypropylene food-grade disposable drinking cup, in 180 ml of UV sterilised seawater (Cup dimensions: top diameter 75 mm, height 100 mm and bottom diameter 45 mm). All rearing cups were suspended in an 837 L water-bath with dimensions 160 x 103 x 69 cm. The tank was connected to a continuous flow-through system, thereby maintaining ambient seawater temperatures in rearing chambers. Temperature differences between water bath tanks were never more than 0.1 ^{o}C. Inflow pipes were positioned sub-surface to avoid additional ambient sound from falling water. Surface seawater in the cups was exposed to the air, allowing gas exchange whilst preventing water mixing between cups. The cup bases rested on a rigid plastic mesh tray with 10 mm^2 openings allowing greater flow for temperature regulation and minimising interference with sound wave propagation. 84 cups were contained within the tray, 83 were occupied by a single ZI larva (giving an initial n = 83 for each sound treatment group), whilst the 84th cup contained a 64k temperature logger (www.onsetcomp.com/products/data-loggers/ua-002-64) (Accuracy ± 0.53°C from 0° to 50°C), set to log every 5 minutes for the duration of the experiment. 440 mm below the centre of the tray and 100 mm above the tank base, a free-hanging Diluvio^{TM} underwater speaker was suspended, facing

upwards. A total of 3 adjacent tanks were set up in this way, each receiving a different sound treatment (Busy, Occasional or Ambient) (Figure 3.4). For 2018 respirometry experiments, due to time constraints on the maximum number of respirometry trials able to be run within a given day, the sound treatment tanks were reduced to 2: Occasional and Ambient. The Busy treatment was removed as it had the least similarity to a 'real-world' sound exposure scenario and the 'Occasional' treatment has already been demonstrated to be sufficient to affect the measured response parameters.

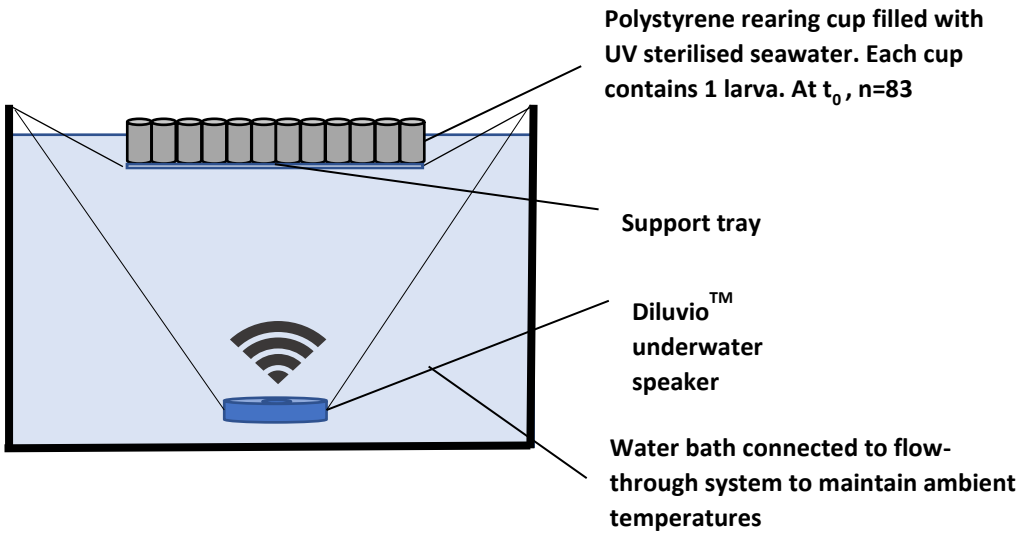


Figure 3.4. Experimental set-up for the rearing and sound exposure of larval *Nephrops norvegicus* (L.). The water bath tank had dimensions 1600 x 1030 x 690 mm. Rearing vessel support tray dimensions were 1200 x 600mm. The Diluvio underwater speaker was free hanging, 100mm above the tank base and 490mm below the support tray. Both the support tray and the underwater speaker were centrally located on the x-y plane of the tank base. Rearing vessels on the shorter edges of the support tray were 200mm from the nearest tank side, whilst rearing vessels on the long edge of the support tray were 215mm from the nearest tank side. A total of 84 polystyrene cup rested on the support tray. At t = 0, 83 cups contained a single ZI larva in 180ml of UV sterilised seawater, with a single cup containing an Onset HOBO temperature/light logger.

3.2.5 - Sound recordings and playback design

Using the Ableton Live Suite software and an M-Audio M-Track Quad, 3 separate audio channels were played from a single Lenovo Thinkpad laptop (Playback sample rate: 96 kHz). Each audio track output from the M-Track Quad was routed through a Samson Servo 300 2 x 150 W amplifier to a Diluvio™ underwater speaker (Frequency Response 20 Hz – 17 kHZ). Three experimental groups were compared using a combination of ambient and ship noise recordings from three major UK ports ((A) Gravesend, (B) Plymouth and (C) Portsmouth), originally recorded by Sophie Nedelec (University of Exeter) and Irene Voellmy (University of Bristol) (Wale et al., 2013a, 2013b) . At each location, during a single day, recordings were made of both ambient sound and the sound generated by a single passing ship at approximately 200 m distance. The ships recorded at each location were: (A) Gravesend: Rio de la Plata, a 286 m long, 64730 tonne container ship, (B) Plymouth: Bro Distributor, a 146 m long, 14500 tonne LPG tanker and (C) Portsmouth: Commodore Goodwill, a 126 m long, 5215 tonne ferry. Ship travel speed was relatively low and remained approximately constant for the duration of recordings (<10 knots). For hydrophone and recorder specifications and calibration information, see: (Wale et al., 2013a, 2013b).

The Ambient group received playbacks of ambient sound recordings; this group served as the control in this experiment. Ambient sound, rather than silence was chosen in order to ensure that any differences observed in ship noise playback groups compared to the ambient group were solely due to the ship sound component of the playback. As there were 3 recording locations, 3 ambient tracks (2 hrs each) were used in the treatment and were cycled between randomly for the duration of the experiment. The Occasional group received playbacks of 15 minutes of ship noise (including a 1-minute fade-in and fade-out), interspersed within 1h45 of corresponding ambient sound recording from the same location, repeated for the duration of the experiment. The

positioning of the 15-minute ship noise block within this 2-hour period could occur at 0 mins, 30 mins, 60 mins, 105 mins. These 4 ship/ambient combinations were created for each recording location (A, B and C) giving a total of 12 possible 2-hour sound playback files for the Occasional group; these 12 playback files were cycled between randomly (Figure. 3.5). The Busy group received 15 minutes of ship noise followed by 15 minutes of corresponding ambient sound recorded from the same location, repeated for the duration of the experiment. As there were 3 recording locations, there were 3 30-minute Busy sound tracks that were cycled between randomly. All experimental tracks were compiled in Audacity 2.1.3 (http://audacity.sourceforge.net/) and exported as 24-bit WAV files for playback.

The structuring of ship sound in the Occasional and Busy groups allowed the comparison of a lower-frequency (in terms of rate of exposure events), randomised exposure regime (Occasional), with a higher-frequency, regular regime (Busy).

The power spectral density (PSD) pressure levels received by the larvae were measured in the rearing cups directly above the Diluvio^{TM} speaker and in two cups that were furthest away from the speaker with a HTI-94-SSQ hydrophone (sensitivity of -165 dB re: 1 V/µPa) with a frequency response of 2 Hz to 30 kHz, recording to a calibrated Roland R-26 digital recorder at a sample rate of 96 kHZ. Sound recordings were stored as 24-bit WAV files. The recorder was calibrated using a signal generator and oscilloscope. This recording system was also used in the locations described above outside of the cups, to assess the acoustic transparency of the HDPE cup material. Recordings were also made to assess background noise levels in each tank. Each recording was 30 s in length.

Particle acceleration (also measured in rearing cups) was measured using a custom-built, calibrated sensor (Sensitivity: 6.6 mV/(m/s^2) developed by the Aquatic Noise Research Group at Edinburgh Napier University (Wale, 2017). This

accelerometer was chosen due to it's small size, allowing recordings to be taken in small tanks and rearing vessels. The sensor was calibrated by exposing it to vibrations of known frequency and amplitude (50, 60, 70, 80, 90, 100 Hz at 1 and 2 Vpp, and 50 Hz at 3, 4, 5, 6, 7, 8, 9, 10 Vpp) on a Controlled Vibration EDP-2424 24 Inch Platform shaker (Crowson Technology, Santa Barbara, California, USA). The response of the sensor was then compared to a reference accelerometer with a known calibration curve (Brüel & Kjær 4508B, Sensitivity 10 mV ms-2, Spectral Noise <343 (µm s-2)√Hz-1 @100Hz, Brüel & Kjær, Nærum, Denmark). The accelerometer output was recorded with a Roland R-26 24-bit recorder. Particle acceleration was recorded separately for all three axes and combined for a single analysis of total acceleration.

Power spectral density graphs were produced in Matlab R2017a using the PAMGuide software package for pressure (Merchant et al., 2015) and paPAM software package for particle acceleration. PSD analyses were conducted on 30 second recordings. The Hann window type was used, with window length of 1 second, 50% window overlap and Discrete Fourier Transformation length of 48 kHz. Root mean square (rms) sound levels were used for comparisons. Ambient group sound recordings were taken whilst ship noise playback was occurring in Busy and Occasional tanks confirming there was no sound transference between tanks.

During ship noise playbacks, there was a peak of 140dB re: 1 μPa^2 Hz^{-1} at 747 Hz and 147dB re: 1 μPa^2 Hz^{-1} at 1590 Hz in the rearing cups of the Busy group. There were peaks of 144dB re: 1 μPa^2 Hz^{-1} at 395 Hz; 140dB re: 1 μPa^2 Hz^{-1} at 747 Hz; 143dB re: 1 μPa^2 Hz^{-1} at 915 Hz and 142dB re: 1 μPa^2 Hz^{-1} at 1566 Hz in the rearing cups of the Occasional group. There were peaks of 116dB re: 1 μPa^2 Hz^{-1} at 120 Hz and 97dB re: 1 μPa^2 Hz^{-1} at 1562 Hz in the rearing cups of the Ambient group (Figure 3.6).

During ship noise playback, there was a particle acceleration peak of 102.4dB re: (1 µm s^{-2})^2 Hz^{-1} at 64 Hz, 98.6dB re: (1 µm s^{-2})^2 Hz^{-1} at 703 Hz and 99dB re: (1 µm s^{-2})^2

Hz^{-1} at 747 Hz in the rearing cups of the Busy group. There were peaks of 101.5dB re: (1 µm s^{-2})^2 Hz^{-1} at 63 Hz, 95.8dB re: (1 µm s^{-2})^2 Hz^{-1} at 571 Hz and 100.1dB re: (1 µm s^{-2})^2 Hz^{-1} at 748 Hz in the rearing cups of the Occasional group. There were peaks of 89.7dB re: (1 µm s^{-2})^2 Hz^{-1} at 250 Hz and 91.63dB re: (1 µm s^{-2})^2 Hz^{-1} at 600 Hz in the rearing cups of the Ambient group (Figure 3.7).

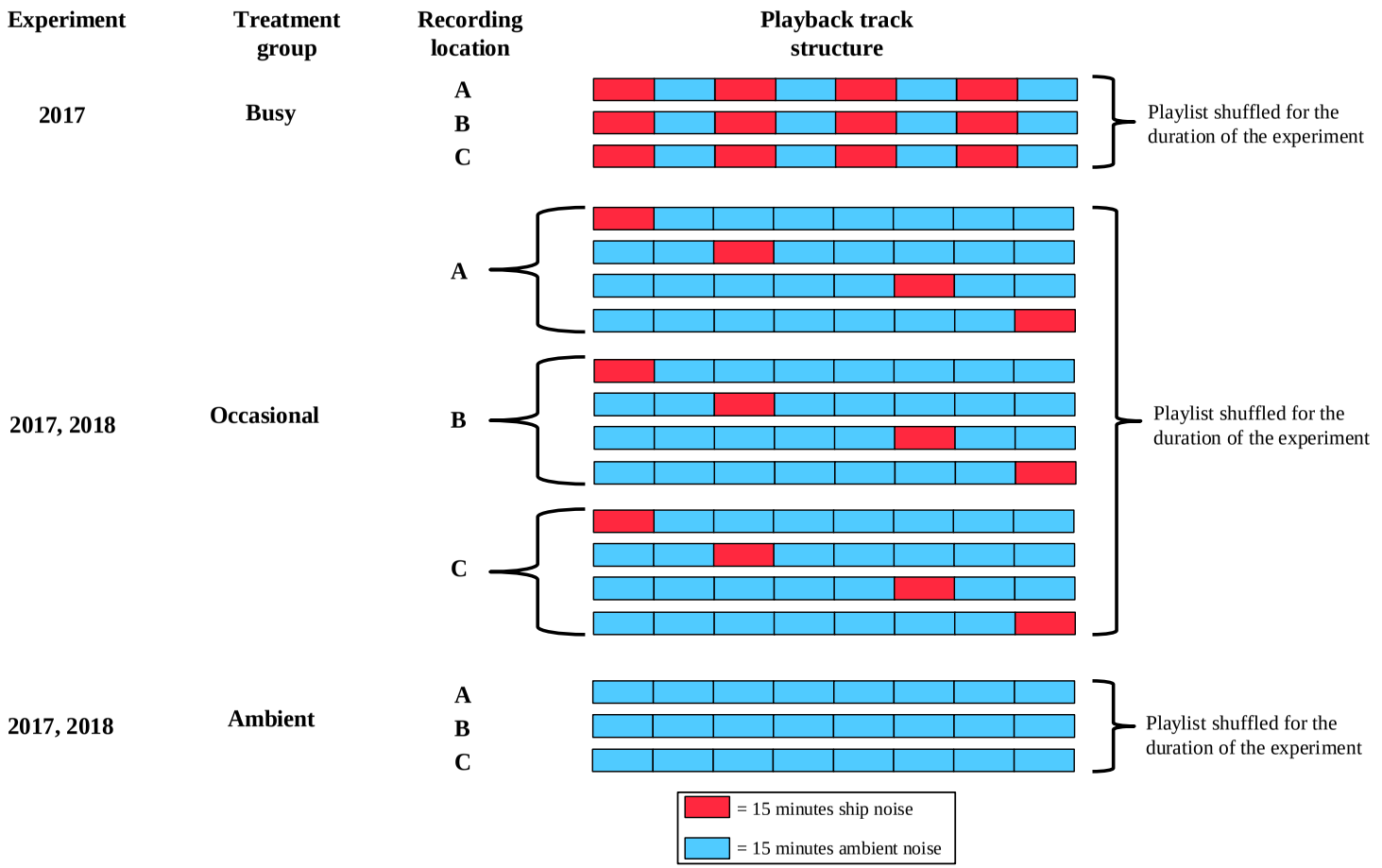


Figure 3.5 The sound playback exposure regime received by developing *Nephrops norvegicus* (L.) larvae in Ambient, Occasional and Busy treatment groups. Sound options were cycled between randomly for the duration of the experiment. **A,B,C** denotes Gravesend, Plymouth and Portsmouth recording locations respectively. The respirometry trials consisted of only Occasional and Ambient treatments.

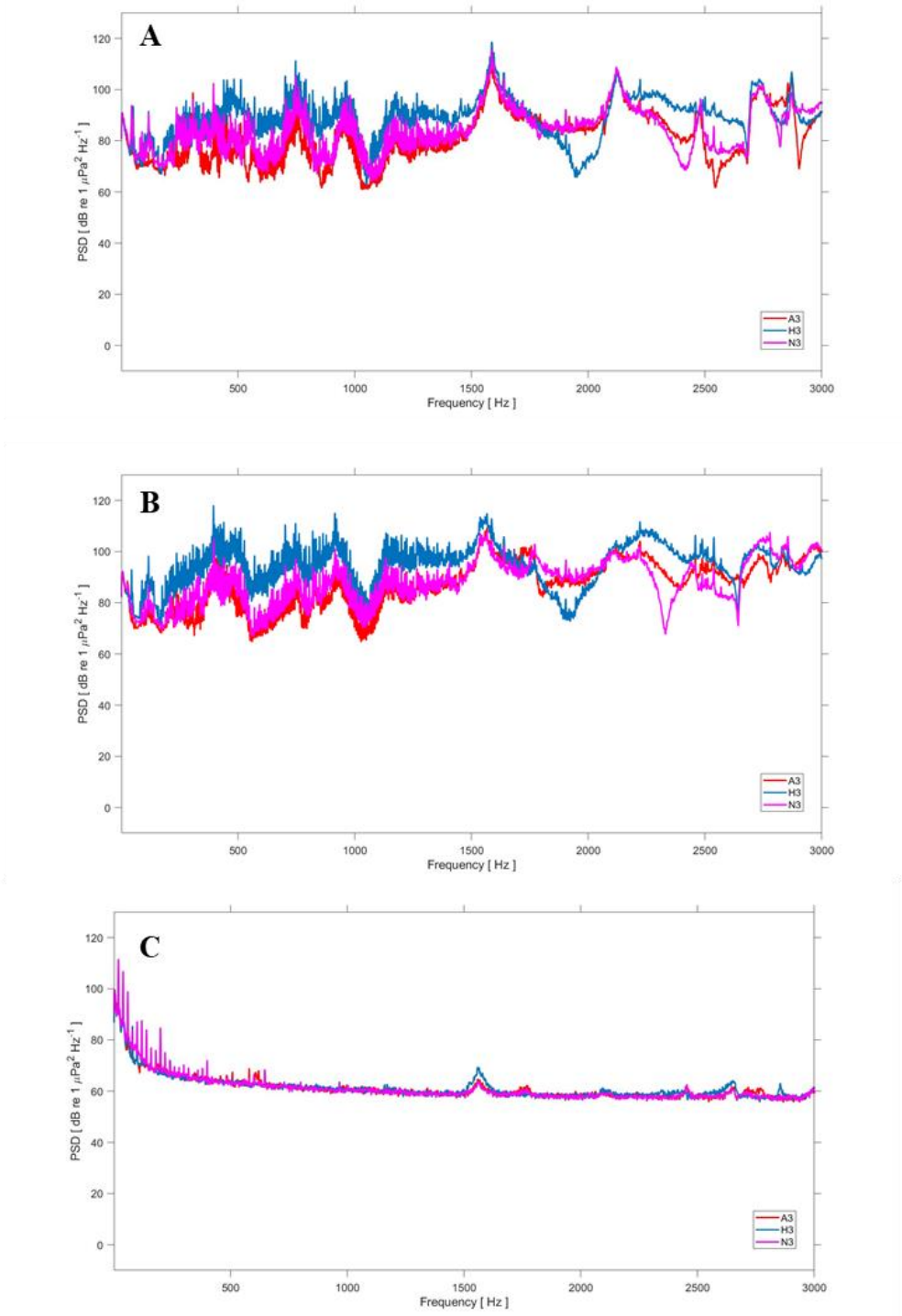


Figure 3.6 Power spectral density plots showing RMS levels (dB re: 1µPa^2 Hz^{-1}) received by developing *Nephrops norvegicus* (L.). Recordings were taken inside rearing cups in **A**) Busy **B**) Occasional and **C**) Ambient treatment groups. In each treatment, recordings were taken directly above the speaker (blue line) and at the furthest cups from the centre moving directly right (pink line) and left (red line). DFT length: 48kHZ, recording lengths 30s, Hann window length: 1s.

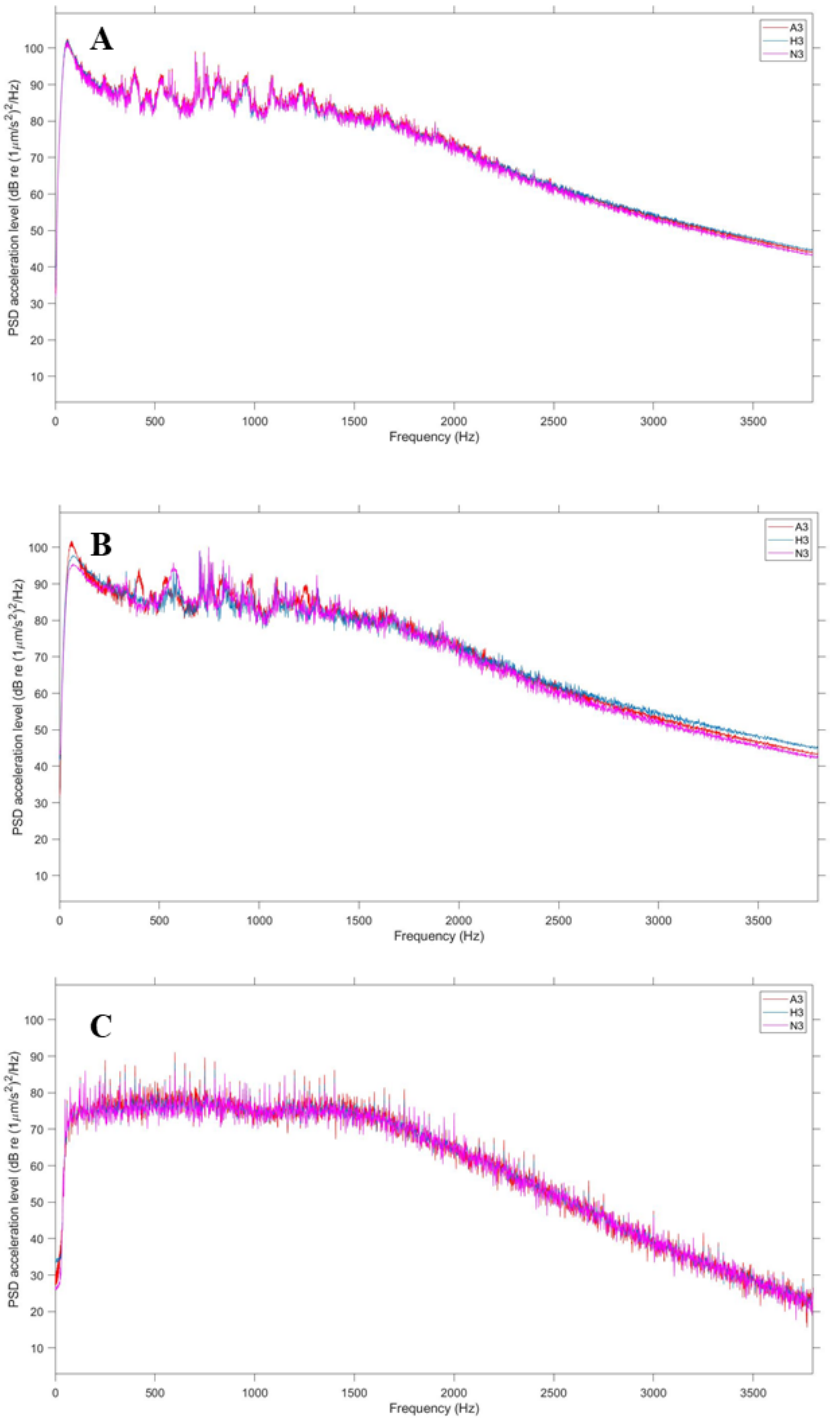


Figure 3.7 Power spectral density plots showing RMS particle acceleration levels (dB re: $(1\mu m\ s^{-2})^2\ Hz^{-1}$) received by developing *Nephrops norvegicus* (L.). Recordings were taken inside rearing cups in **A**) Busy **B**) Occasional and **C**) Ambient treatment groups. In each treatment, recordings were taken directly above the speaker (blue line) and at the furthest cups from the centre moving directly right (pink line) and left (red line). DFT length: 48kHZ, recording lengths 30s, Hann window length: 1s.

3.2.6 - Data collection

3.2.6.1 - Survival and Developmental Stage Duration

Each rearing cup was visually assessed daily for mortality or moulting of the larvae. Transitions between larval stages were clearly observable with the naked eye as there were distinct changes size and morphology and exuviae were clearly visible. Mortality was defined as no observable movement of the larvae for 10 seconds followed by a further 10 seconds of no movement following gentle agitation of the rearing cup water.

3.2.6.2 - Tail-flip escape response

PL1 (Figure 3.1) stage *N. norvegicus* of the same age post moulting (14 days post-moult) were removed from cups and placed in a circular experimental arena filled with UV seawater at the same temperature as the rearing cups. The arena had matte sides and was placed away from noise disturbances and movement. After 5 minutes acclimatisation, a plastic rod was placed ~1 cm anterior of the rostrum to elicit a 'tail-flip' escape response (Newland and Neil, 1990), and then immediately removed. The process was repeated following the completion of each 'tail-flip' response (5 seconds with no tail-flip) until exhaustion (no tail-flip response to 5 consecutive stimuli). All experimental runs were recorded with a DSLR camera located directly above the circular arena and the number of tail flip responses till exhaustion was compared between treatment groups. The same number of individuals from each treatment were analysed in a single day. The experiment took place from 25th May - 13th July 2017. The trial was blind, in that the person conducting the experiment did not know the treatment group of the individual.

3.2.6.3 - Respirometry

Respirometry trials were conducted at the ZI, ZII and ZIII larval stages. In each trial, all larvae were the same age in terms of days post-moult (dpm) or in the case of ZI: days post hatching (dph). ZI larvae were tested at 5 dph, ZII at 5 dpm and ZIII at 5 dpm. Respirometry measurements were carried out with the Unisense Microrespirometry System (Unisense S/A, Aarhus, Denmark). Briefly, for each experimental run, 4 larvae cups were removed from a single rearing tank (ship pre-exposure or control) and placed in a temperature control room (set to ambient seawater temperature). The cups were left to stand for 10 minutes to allow any slight temperature differences between cup water and control room to equilibrate. Each larva was then transferred to a glass microrespirometry chamber (2 ml at ZI, 4 ml at ZII and ZIII) filled with UV sterilised seawater, sealed with a ground glass stopper and placed in a submerged rack in a water-bath tank. A control chamber, with UV sterilised seawater only, was added to the rack to measure O_2 consumption associated with the seawater alone. Lights in the temperature control room were dimmed and all sides of the water bath tank were covered in black felt to ensure larvae were not startled by movement or shadows.

Each chamber was equipped with a capillary pore (400 µm diameter), allowing an oxygen microsensor to be lowered directly into the chamber itself. The pores had a sufficiently small diameter to ensure gas and liquid exchange was negligible (Unisense S/A, Aarhus, Denmark). The oxygen microsensor was calibrated at the experimental temperature, using a two-point calibration curve with endpoints of 0% and 100% O_2 saturation; 0% oxygen saturation was achieved using a saturated solution of sodium dithionate ($Na_2S_2O_6$) and 100% saturation was achieved by bubbling air through UV sterilised seawater for 15 mins. Each chamber was equipped with a glass coated mini-stirrer rotating at 800rpm to prevent the formation of oxygen gradients. Larvae were

prevented from making contact with the stirrer with a stainless steel mesh divider atop a glass cylinder. Individual stirrer heads were located within the chamber rack and produced no heat.

Larvae were acclimated in the water bath tank for 5 minutes before beginning an acute sound playback exposure (ship or ambient). Once sound exposure began, the oxygen microsensor was lowered into the first chamber and allowed to equilibrate before sampling for 10 seconds at a rate of 1 sample s^{-1}. The oxygen microsensor was then immediately lowered into the adjacent chamber where the 10 s sampling run was repeated. Each chamber was sampled for oxygen in this way 5 times, with an interval of 5 minutes between each 10 second sampling session. This gave an experimental time of 20 minutes 50 seconds for each chamber.

3.2.6.4 - Oxidative stress

At 10 days post-moult, stage IV *N. norvegicus* (1^{st} juvenile stage) were removed from the sound exposure system, immediately immersed in liquid nitrogen and stored at -80 ^{o}C until further analysis. Each larva was homogenized with 1:5 vol. of buffer (Tris–HCl 50 mM, 0.15 M KCl, pH 7.4), and centrifuged at 10,000 g for 20 min. The supernatant was extracted and used in all subsequent assays. All assay absorbance readings were taken in an Spectramax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA. Total glutathione (GSH) and thiobarbituric acid reacting substances (TBARS) assays were performed following the methodology of (Smith et al., 2007), which is now a standard methodology for ecotoxicology studies on aquatic invertebrates, as these molecules are highly conserved across taxa in terms of structure and function (Al-Shaeri et al., 2013; Gajda-Meissner et al., 2020). All other Catalase (CAT) and Glutathione peroxidase (GPx) assays were completed using the Cayman Chem Catalase Assay Kit (Item no. 707002) and Gluathione Peroxidase Kit (Item no. 703102). For superoxide dismutase (SOD), the Sigma-Aldrich SOD Determination Kit

(Item no. 19160) was used. For detailed oxidative stress standard operating procedures, see appendix A.

3.2.7 - Statistical analyses

3.2.7.1 - Developmental Stage Duration

Mean values (± Standard Error (SE)) for the duration (days) of stages ZI, ZII,ZIII and overall zoeal development (ZI + ZII + ZIII) were calculated for each sound playback group (Busy, Occasional, Ambient) and displayed graphically. As stage duration data did not meet parametric assumptions (assessed using Shapiro-Wilk and Bartlett tests), differences between median duration values were assessed using Kruskal-Wallis rank sum tests followed by post-hoc Wilcoxon rank sum tests with continuity correction.

3.2.7.2 - Survival

Chi-square comparisons were carried out with 2x3 contingency tables with survival (survived and died) as rows and sound playback group (busy, occasional and ambient) as columns to determine whether there is an overall association of sound playback group and survival. Pairwise chi-square tests were also carried out with 2x2 contingency tables for each sound playback group combination to test for survival differences between specific treatment groups.

The time-dependent mortality rates of different groups were determined using a Kaplan-Meier survival analysis in Graphpad Prism 6.01. A post-hoc log-rank (Mantel-Cox) test was used to compare mortality rates between groups.

3.2.7.3 - Tail-flip escape response

Mean values (± SE) for the number of tail-flip responses until exhaustion (RTE) were calculated for each sound playback group (Busy, Occasional, Ambient) and

displayed graphically. As RTE data met parametric assumptions (Shapiro-Wilk test, W = 0.93, p = 0.054; Bartlett test, $K^2_{(2)} = 2.93$, p = 0.23), differences in mean RTE values between sound treatment groups were assessed using a one-way ANOVA test.

3.2.7.4 - Oxidative stress

For all assays where data met parametric assumptions, a one-way ANOVA test was used to compare mean assay values between each sound playback group (Busy, Occasional, Ambient), otherwise Kruskal-Wallis testing was used to compare median assay values.

3.2.7.5 - Respirometry

A linear regression of oxygen concentration (µMol L^{-1}) decrease for each larval chamber minus the linear regression for the blank chamber (UV filtered seawater only) gave the oxygen consumption rate (OCR) (µMol L^{-1} h^{-1}) for each larva during the acute sound exposure. R^2 values for oxygen consumption regressions were always greater than 0.95.

The independent factors of sound pre-exposure, acute sound exposure and zoeal stage on mean OCR were assessed using a three-way ANOVA. Where significant main effects or effect interactions were found, post-hoc Tukey HSD pairwise testing was used to determine differences between each factor combination group. Data was tested for normality using a Shapiro-Wilk normality test. All statistical analyses were carried out in R version 3.2.4 (R Core Team, 2017).

3.3 - Results

3.3.1 - Developmental Stage Duration

Developmental stage duration was assessed for overall zoeal development (ZI + ZII + ZIII) and for each individual zoeal stage (ZI, ZII and ZIII) in 2017 and 2018.

3.3.1.1 - 2017

There were significant differences in total zoeal duration between sound treatment groups in 2017 (Kruskal-Wallis rank sum test: $X^2_{(2)} = 13.57$, $p = 0.001$). Both the Busy and Occasional groups had a significantly longer total zoeal duration than the Ambient group (Wilcoxon rank sum test, $W = 1996$, $p = 0.03$ and $W = 2100$, $p < 0.01$ respectively). Total zoeal duration in the Occasional group was delayed by 1.36 (4.39% prolonged development) days compared to the Ambient group. The Busy sound group larval duration was delayed by 1.04 days compared to the Ambient sound group (3.36% prolonged development) (Figure 3.7). There was no significant difference between the Busy and Occasional groups. This overall delay in Busy and Occasional groups compared to the Ambient group was not observed consistently throughout development: there were no significant differences in ZI stage duration between sound groups (Kruskal-Wallis rank sum test: 2017, $X^2_{(2)} = 2.62$, $p = 0.27$; 2018, $X^2_{(1)} = 0.80$, $p = 0.37$) (Figure 3.7). Differences in duration were found at ZII stage in 2017 (Kruskal-Wallis rank sum test: $X^2_{(2)} = 7.46$, $p = 0.02$); The busy group had a significantly longer ZII stage than the Occasional and Ambient groups (Wilcoxon rank sum test, $W = 3101$, $p = 0.01$ and $W = 2327.5$, $p = 0.02$ respectively) (Figure 3.7) and was 0.44 days longer than the Ambient group (a delay of 5.03%) (Table 3.1). Differences in duration were also found at ZIII stage (Kruskal-Wallis rank sum test: $X^2_{(2)} = 15.07$, $p < 0.01$). However, it was the Occasional group that had a significantly longer ZIII stage than the

Busy and Ambient groups (Wilcoxon rank sum test, $W = 1465$, $p < 0.01$ and $W = 2118.5$, $p < 0.01$ respectively) (Figure 3.7) and was 0.90 days longer than the Ambient group (a delay of 7.22%) (Table 3.1). The number of larvae transitioning between stages, and the timing of the transitions, from hatching (ZI) to the first postlarval stage (PL1) for all sound treatments over the duration of the experiment is displayed in Figure 3.13.

3.3.1.2 - 2018

The Occasional ship sound treatment once again caused increased total zoeal development duration (Kruskal-Wallis rank sum test: $X^2_{(1)} = 11.01$, $p < 0.001$), with a 1.11 day delay (3.74% prolonged development) compared to the Ambient group. In accord with the observed delays in 2017, the Occasional group's delay did not occur until stage ZIII, where there was a 0.86 day delay (6.95%) (Kruskal-Wallis rank sum test: $X^2_{(1)} = 26.92$, $p < 0.001$) (Figure 3.8).

Table 3.1 The mean (± SE) duration (days) of the A) ZI, B) ZII, C) ZIII and D) Total zoeal (ZI _ZII + ZIII) stages of *Nephrops norvegicus* (L.) in response to different ship sound playback treatments. Equal letters indicate statistically homogeneous groups ($p > 0.05$) calculated using a Kruskall-Wallis test followed by post-hoc Wilcoxon rank sum tests with continuity correction.

A) ZI

Sound group	Mean (± SE) duration (days)	Difference to Ambient mean (days)	% Difference to Ambient mean	Min-Max (days)
Busy	10.08 ± 0.15[a]	+ 0.05	+ 0.50	9-17
Occasional	10.30 ± 0.15[a]	+ 0.27	+2.69	9-15
Ambient	10.03 ± 0.16[a]	N/A	N/A	7-15

B) ZII

Sound group	Mean (± SE) duration (days)	Difference to Ambient mean (days)	% Difference to Ambient mean	Min-Max (days)
Busy	9.19 ± 0.13[a]	+ 0.44	+ 5.03	7-12
Occasional	8.72 ± 0.12[b]	- 0.03	- 0.34	7-12
Ambient	8.75 ± 0.14[b]	N/A	N/A	7-12

C) ZIII

Sound group	Mean (± SE) duration (days)	Difference to Ambient mean (days)	% Difference to Ambient mean	Min-Max (days)
Busy	12.85 ± 0.23[a]	+ 0.38	+ 3.05	11-22
Occasional	13.37 ± 0.17[b]	+ 0.90	+ 7.22	11-17
Ambient	12.47 ± 0.12[a]	N/A	N/A	11-15

D) Total (ZI + ZII + ZIII)

Sound group	Mean (± SE) duration (days)	Difference to Ambient mean (days)	% Difference to Ambient mean	Min-Max (days)
Busy	32.02 ± 0.33[a]	+ 1.04	+ 3.36	29-46
Occasional	32.34 ± 0.25[a]	+ 1.36	+ 4.39	29-38
Ambient	30.98 ± 0.21[b]	N/A	N/A	28-34

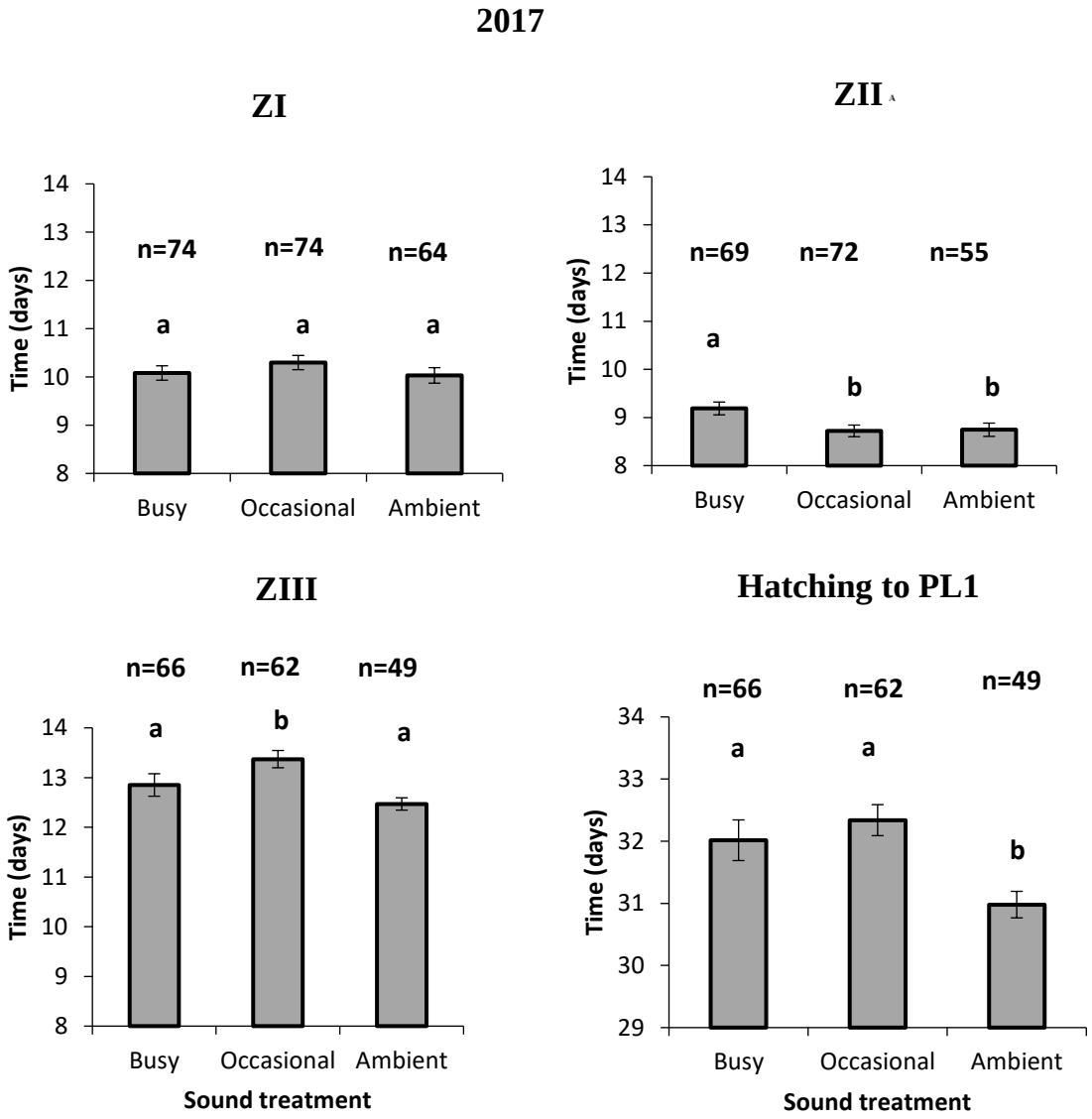


Figure 3.8 Mean (± SE) duration (days) of each planktonic zoeal stage (ZI-ZIII) and hatching to the first post-larval stage (PL1) of *Nephrops norvegicus* (L.) in response to different ship sound playback treatments. Equal letters indicate statistically homogeneous groups ($p > 0.05$) calculated using a Kruskall-Wallis test followed by post-hoc Wilcoxon rank sum tests with continuity correction.

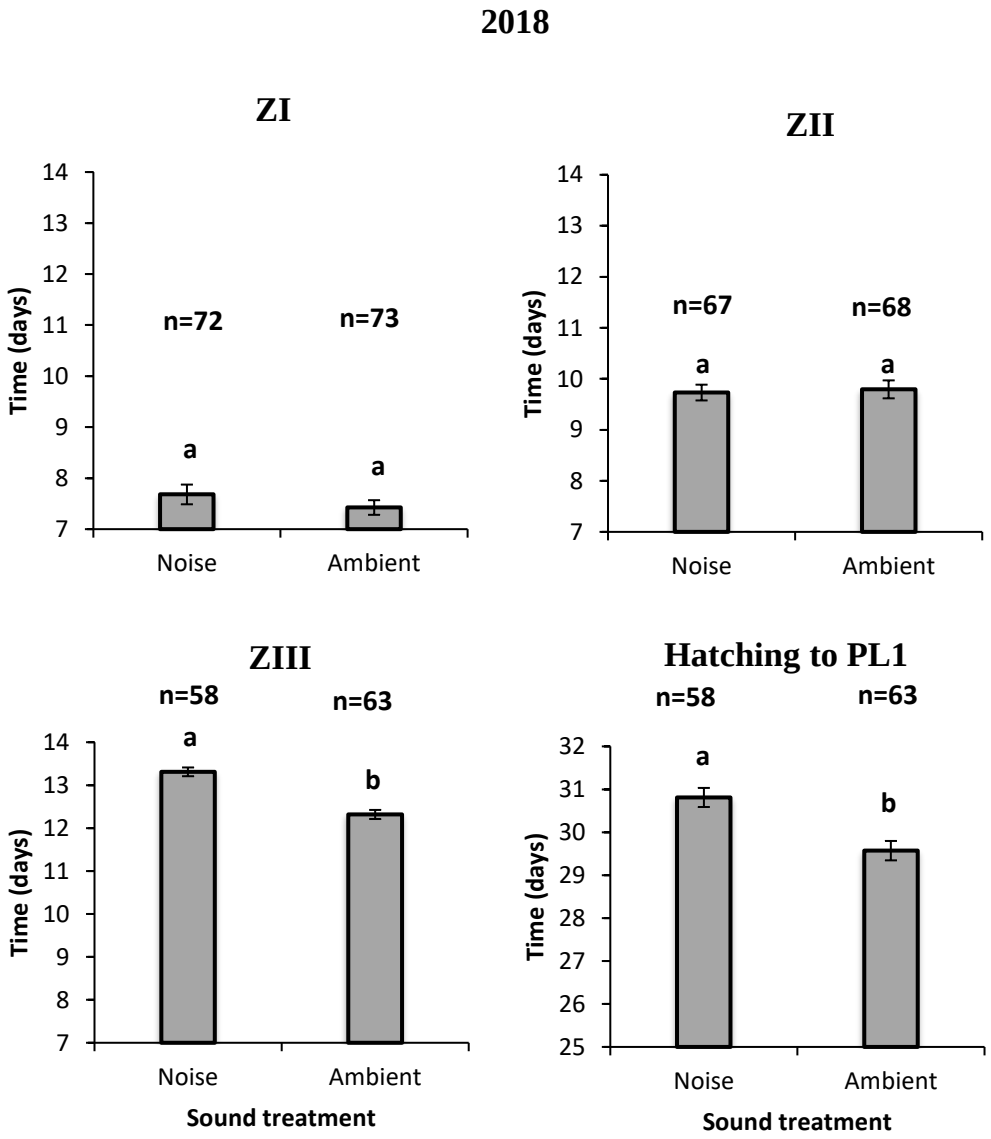


Figure 3.9 Mean (± SE) duration (days) of each planktonic zoeal stage (ZI-ZIII) and hatching to the first post-larval stage (PL1) of *Nephrops norvegicus* (L.) in response to different sound playback treatments (ship noise and ambient). Equal letters indicate statistically homogeneous groups ($p > 0.05$) calculated using a Kruskall-Wallis test followed by post-hoc Wilcoxon rank sum tests with continuity correction.

3.3.2 - Tail-flip escape response

Sound treatment group had a significant effect on the mean number of tail-flick responses until exhaustion (RTE) of 14 days post-moult PL1 stage *N. norvegicus* (One-way ANOVA, $F_{(2,27)} = 6.463$, $p = 0.005$).

Mean RTE in the Busy and Occasional group was significantly reduced by 33.3% and 45.9% respectively when compared to the Ambient group (Tukey HSD tests, $p < 0.05$) (Figure 3.9, Table 3.2), but Busy and Occasional were not significantly different (Tukey HSD test, $p = 0.61$). It should be noted that while the RTE value of 43 in the Ambient group appears to be an extreme value, this value is not an error, as the total number of responses on the video recording is clear to observe, but if removed, the difference between Busy and Ambient becomes marginally insignificant ($p = 0.1$), whilst the difference between Occasional and Ambient remains clear ($p = 0.008$). The 2nd highest RTE value in the Ambient group was 31.

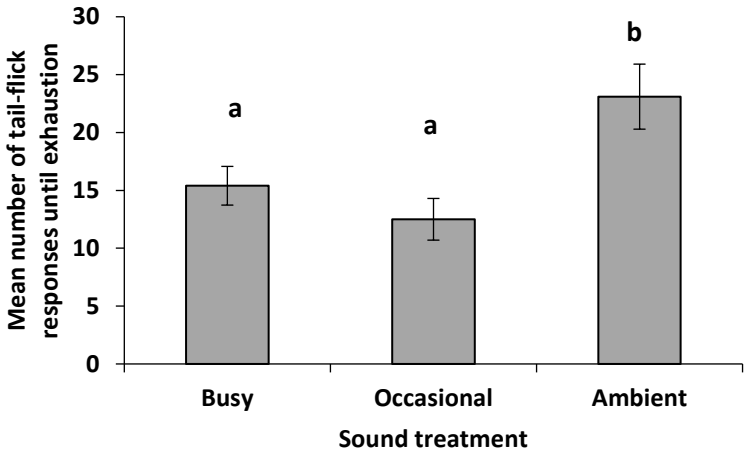


Figure 3.10 Mean (± SE) number of tail-flick responses of the first post-larval stage (stage PL1) *Nephrops norvegicus* (L.) before exhaustion in response to different ship sound playback treatments. Equal letters indicate statistically homogeneous groups ($p > 0.05$) calculated using a one-way ANOVA. N = 10 larvae per sound treatment group.

Table 3.2 Mean (± SE) number of tail-flip responses of first post-larval stage (PL1) *Nephrops norvegicus* (L.) before exhaustion in response to different ship sound playback treatments. Equal letters indicate statistically homogeneous groups ($p > 0.05$) calculated using a one-way ANOVA followed by Tukey's HSD post-hoc pairwise tests.

Sound group	Mean (± SE) tail-flips before exhaustion	Difference to Ambient mean	% Difference to Ambient mean	Min-Max tail-flips before exhaustion
Busy	15.4 ± 1.7^{a}	- 7.7	-33.3	9-25
Occasional	12.5 ± 1.8^{a}	- 10.6	-45.9	3-20
Ambient	23.1 ± 2.8^{b}	N/A	N/A	11-43

3.3.3 - Survival

In 2017, developing *N. norvegicus* had increased mortality overall (hatching to PL1 stage) in the Ambient sound playback group compared to the Occasional and Busy groups ($X^2_{(1)}$ = 3.92, p = 0.048 and $X^2_{(1)}$ = 9.38, p = 0.002 respectively), with 59%, 75% and 82% of the initial 83 individuals reaching PL1 stage in Ambient, Occasional and Busy groups, respectively (Table 3.3) compared to 70% and 76% in Occasional and Ambient groups in 2018 respectively (Figure 3.10B). These overall differences were predominantly caused by a higher mortality rate during the ZI stage in in the Ambient sound playback group compared to Occasional and Busy groups (Figure 3.10). This Ambient mortality rate was significantly higher than the Busy group ($X^2_{(1)}$ = 4.42, p = 0.035) but marginally insignificant compared to the Occasional group ($X^2_{(1)}$ = 3.48, p = 0.062). Significant differences in mortality were no longer observed throughout ZII and ZIII development ($X^2_{(2)}$ = 5.65, p = 0.059 and $X^2_{(2)}$ = 4.21, p = 0.12 respectively) (Figure 3.11). The increase in mortality rate in the Ambient group during ZI predominantly occurred between 4 and 8 days post-hatching (Figure 3.12).

Table 3.3 Survival (%) of *Nephrops norvegicus* (L.) from hatching to the first post-larval stage (PL1) and at each planktonic zoeal stage (ZI to ZIII) in response to different ship sound playback treatments in 2017.

Sound Group	Initial N	Success to PL1 (%)	ZI Success (%)	ZII Success (%)	ZIII Success (%)
Busy	83	82	90	95	96
Occasional	83	75	89	97	86
Ambient	83	59	77	88	88

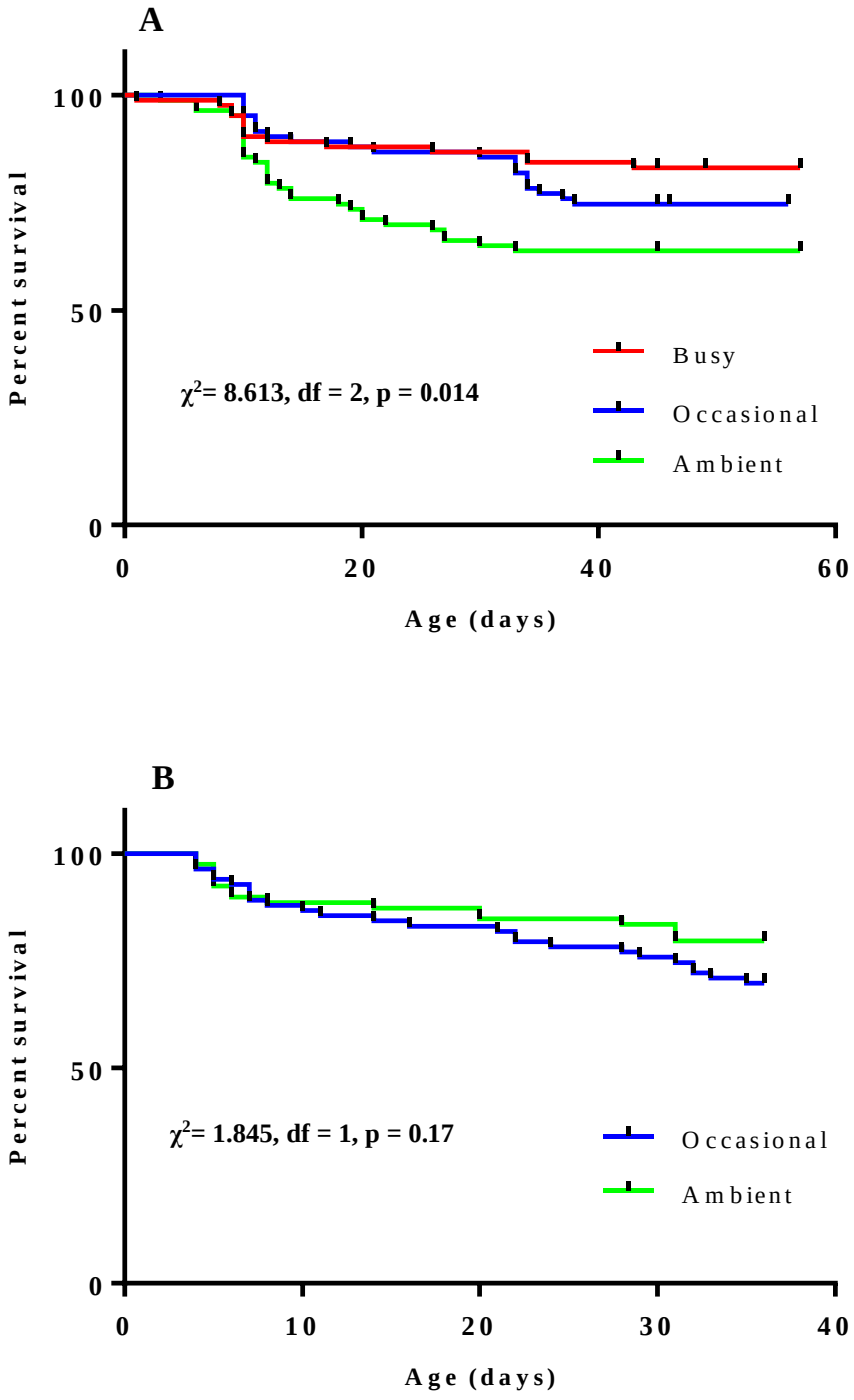


Figure 3.11. Kaplan-Meier survival curves of *Nephrops norvegicus* (L.) in A) 2017 and B) 2018from hatching to the first post -larval stage (PL1) in response to different ship sound playback treatments. % values refer to initial n = 83 larvae in each sound group. Results of a log-rank test for differences between curves is displayed.

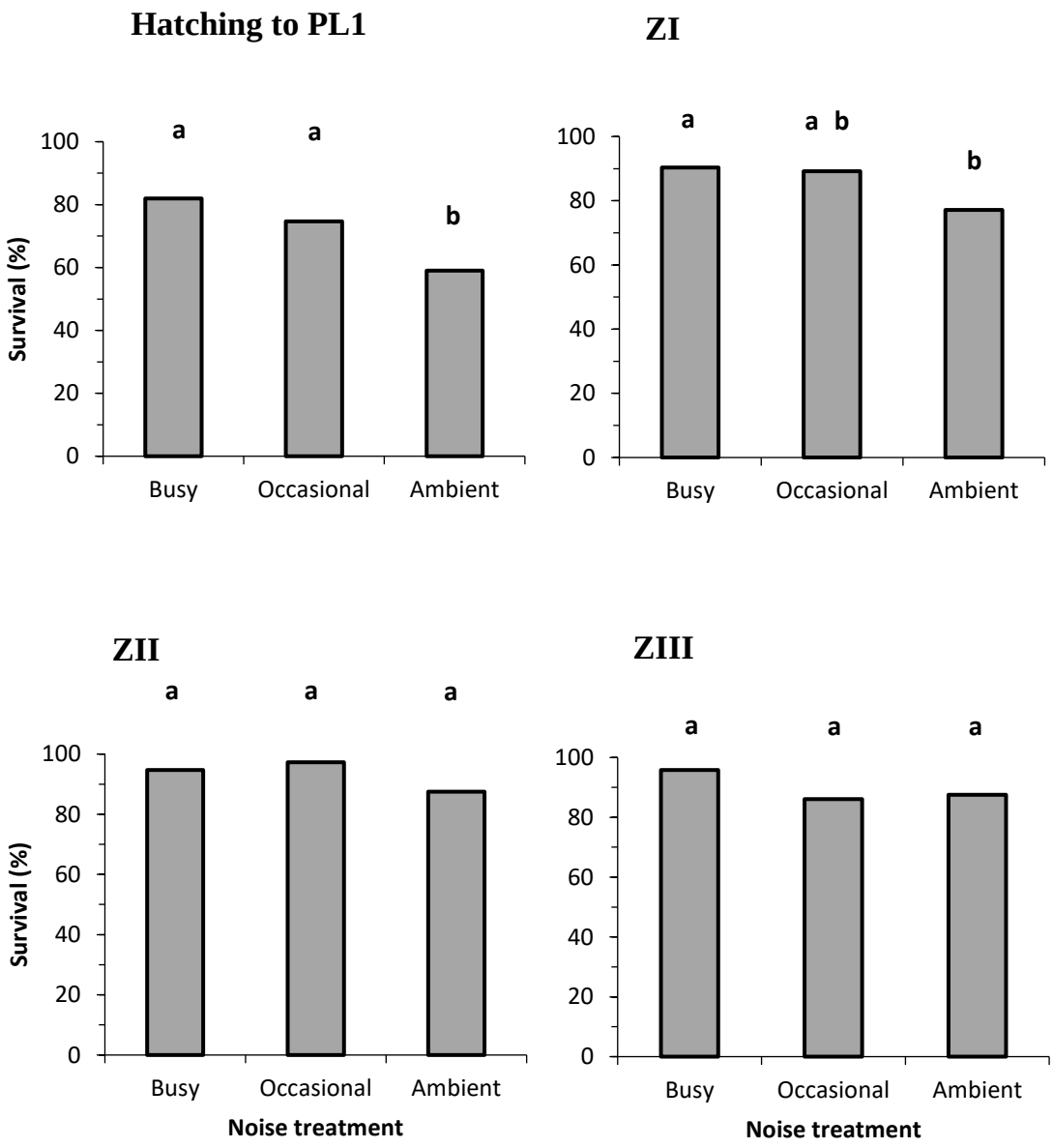


Figure 3.12. Survival (%) of *Nephrops norvegicus* (L.) from hatching to the first post -larval stage (PL1) and at each zoeal stage (ZI to ZIII) in response to different ship sound playback treatments. % values of the ZI and hatching to PL1 stages refer to initial n=83 larvae in each sound group, while for ZII and ZIII each stage value is related to the initial *n* of surviving zoea from the previous stage. Equal letters indicate statistically homogeneous groups ($p > 0.05$) calculated using Chi-square tests

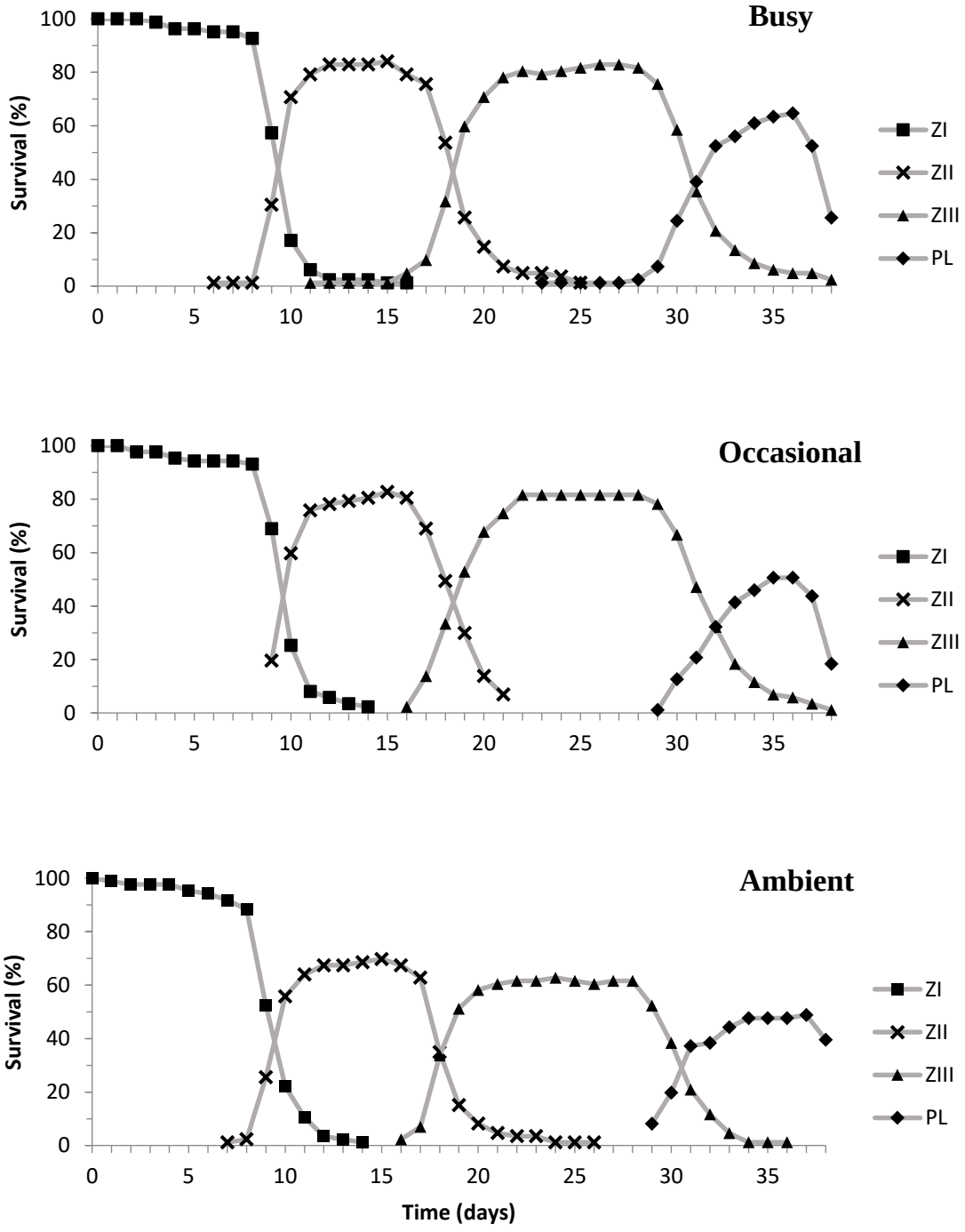


Figure 3.13. Survival (%) and development of *Nephrops norvegicus* (L.) larvae through successive zoeal stages from hatching (ZI) to the first post-larval stage (PL1) in different ship sound playback treatments. (*n* initial = 83 larvae per treatment, % values post-ZI relate to the initial *n* = 83). Data displayed until 38 days post hatching when no ZIII larvae remained.

3.3.4 - Oxidative stress

To assess whether noise exposure throughout larval development causes a build-up of reactive oxygen species (ROS) in the first juvenile stage, a suite of oxidative stress endpoint biomarkers was assayed: SOD, CAT, GPx, GSH and TBARs. The SOD, CAT, GPx, GSH and TBAR assays did not reveal significant differences in oxidative stress as a result of noise playback exposure (Kruskal-Wallis $\chi^2_{(2)} = 2.25$, $p = 0.33$ (SOD); One-way ANOVA $F_{(2,25)} = 1.03$, $p = 0.37$ (CAT); One-way ANOVA $F_{(2,24)} = 0.53$, $p = 0.60$ (GPX); One-way ANOVA $F_{(2,26)} = 0.75$, $p = 0.48$ (GSH); One-way ANOVA $F_{(2,26)} = 0.61$, $p = 0.55$ (TBAR)).

3.3.5 - Respirometry

There was a significant main effect of zoeal stage on the OCR (3-way ANOVA, $F_{(2,108)} = 26.17$, $p < 0.001$), with ZII larvae consuming more oxygen than ZI ($p < 0.001$) and ZIII ($p < 0.001$) larvae (Figure 3.14). The only interaction observed was a highly significant crossover interaction between the factors 'pre-exposure' and 'acute exposure' (3-way ANOVA, $F_{(1,108)} = 26.64$, $p < 0.001$). For this reason, zoeal stage was removed as a factor and Tukey HSD pairwise comparisons for each 'pre-exposure' and 'acute exposure' combination were conducted, averaged across all zoeal stages. Naïve larvae (having never received experimental ship noise playbacks) consumed 25.9% more oxygen when subjected to ship noise ($p < 0.001$). In contrast, pre-exposed larvae consumed 13.1% less oxygen when subjected to a further acute ship noise exposure ($p = 0.038$) (Table 3.4, Figure 3.14). Pre-exposed larvae consumed 17.3% more oxygen than naïve larvae under control conditions ($p = 0.014$).

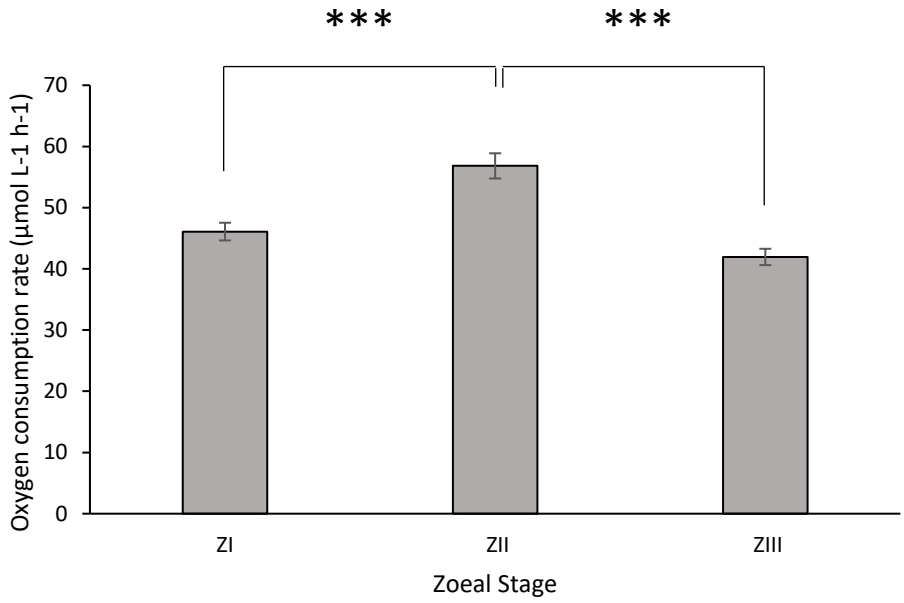


Figure 3.14. Mean (± SE) oxygen consumption rate (µmol L^{-1} h^{-1}) of zoea stages I,II and III *N. norvegicus* averaged across all sound treatments (acute ship noise and control groups of both naïve and pre-exposed larvae). * indicates significantly different groups ($p < 0.05$) calculated using a three-way ANOVA followed by Tukey HSD pairwise testing. N = 40 larvae for each zoeal stage group.

Table 3.4 Mean (± SE) oxygen consumption rate (µmol L^{-1} h^{-1}) of zoea stages I,II and III *N. norvegicus*. * indicates significantly different groups ($p < 0.05$) calculated using a one-way ANOVA followed by Tukey HSD pairwise testing.

Pre-exposure group	Respirometry sound exposure	Mean (± SE) O_2 consumption rate (µmol L^{-1} h^{-1})		
		ZI	ZII	ZIII
Pre-exposed	Ship	45.81± 3.29	52.19 ± 4.08	39.15 ± 3.43
	Ambient	50.13 ± 2.19	58.55 ± 3.52	44.01 ± 1.38
Naive	Ship	49.35 ± 3.89	66.43 ± 2.94	47.17 ± 2.92
	Ambient	42.62 ± 2.31	50.14 ± 4.24	37.36 ± 1.40

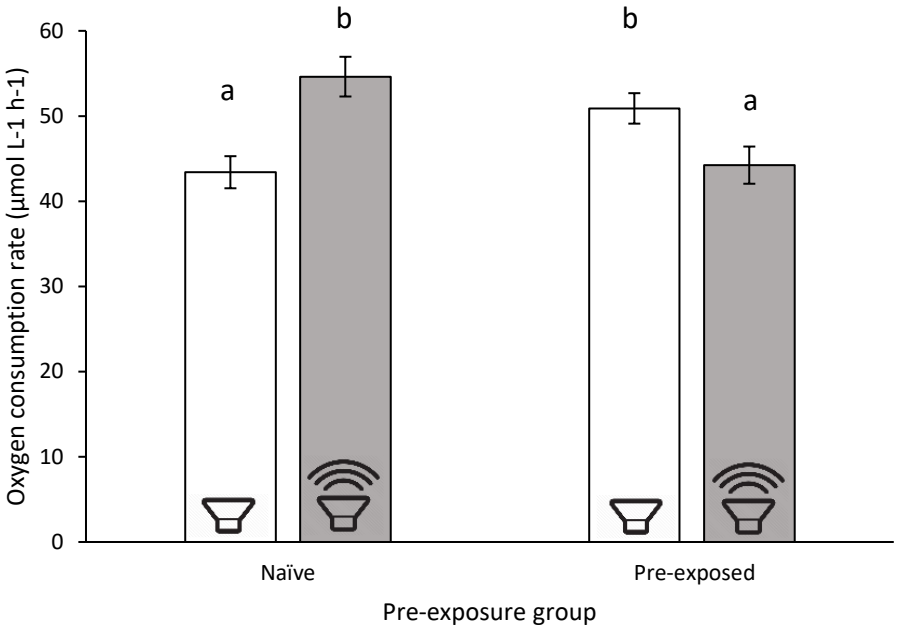


Figure 3.15. Mean (± SE) oxygen consumption rate (µmol L^{-1} h^{-1}) of naïve and pre-exposed *N. norvegicus* averaged across all zoea stages (ZI, ZII and ZIII). Grey and white bars indicate an acute ship noise playback exposure and a control ambient sound playback exposure respectively. Equal letters indicate statistically homogeneous groups ($p > 0.05$) calculated using a three-way ANOVA followed by Tukey HSD pairwise testing. N = 30 larvae for each sound treatment group.

3.4 - Discussion

Playbacks of ship sound significantly increased total *N. norvegicus* zoeal development time in both Occasional and Busy sound treatment groups, whilst also decreasing escape response stamina in the first post-larval stage. Our controlled laboratory experiment therefore suggests that anthropogenic noise has the potential to negatively impact decapod crustaceans. Evidence for reduced developmental success has been reported for gastropod species in response to playbacks of outboard boat motor sound in the field (Nedelec, Radford, et al., 2015), whilst developmental delays and body malformations have been reported for Bivalvia sp. in response to playbacks of seismic survey pulses (de Soto et al., 2013). This is the first study, to our knowledge, to find direct evidence of negative effects of continuous (as opposed to impulsive) anthropogenic sound, characteristic of real-world ship sound, on the larvae of a decapod crustacean. This is further evidence that marine invertebrates, a taxonomic group with significant economic and ecological value (forming an integral component of marine food webs) (Anderson et al., 2011), are worthy of further study in terms of fitness consequences of anthropogenic noise exposure during early life.

The stage duration and tail-flick escape results both suggest a cumulative deleterious effect of ship sound playbacks on early life stage *N. nephrops*. The delays in development changed depending on ontogenetic stage: in 2017 a 5.03% delay (compared to Ambient) in the Busy group at ZII was followed by a 7.22% delay (compared to Ambient) in the Occasional group at ZIII. This Occasional group delay at ZIII was replicated in the 2018 experiment (3.74% delay compared to Ambient). This suggests that whilst the more frequent exposure (15 mins noise, 15 mins ambient) to ship noise playbacks was able to reduce developmental speed of larvae as early as ZII in the Busy group, by the time ZIII was reached the Busy group had developed tolerance to the regular timing of exposure, whilst the Occasional group delay was due to the

cumulative effect of exposure to a less frequent (15 mins within a 2h period) but randomised ship noise playback exposure regime. Put simply, it is not only the frequency of occurrence, but also the randomness of timing of noise exposure that can affect the developmental rate of *N .norvegicus* larvae. When exposure to a stressor (physical, chemical or perceived) is repeated, animals could either increase their tolerance (where repeated stimulations cause increased tolerance levels and reduced responses over time) or conversely, sensitize (where repeated stimulations cause decreased tolerance levels and increased responses over time) (Bejder et al., 2009). If viewed in isolation, the developmental delay results would suggest that the Busy group had increased tolerance at ZIII compared to the Occasional group. However, both noise groups showed reduced escape response stamina in comparison to controls at stage IV (1st juvenile stage), demonstrating that some negative effects are retained beyond the duration and survival of larval stages. Future noise playback experiments with marine invertebrates should not only consider the intensity levels of played back sounds, but also the levels of randomisation and frequency of occurrence. In fish and mammalian species, habituation, sensitisation and no difference have all been reported when comparing effects of regular and randomised noise exposure regimes, highlighting the difficulties when trying to extrapolate findings between species and different noise sources (Galhardo et al., 2011; Kastelein et al., 2017; Nedelec, Simpson, et al., 2015; Neo et al., 2014; Prior, 2002).

Whilst the developmental delays reported here at first seem relatively subtle, the corresponding stamina reductions were not. If similar effects were to occur in a natural setting, larvae would spend an additional ~1 day in the water column, vulnerable to predation by planktivores. Those larvae that survived to PL1 stage and settled to the benthos would then face additional predatory challenges due to their impaired escape response stamina. Juvenile shore crabs *Carcinus maenas* exposed to ship noise

playbacks over an eight week period exhibited similar reductions in the frequency and intensity of antipredator responses (Carter et al., 2020).

Unexpectedly, in 2017, mortality was higher in the Ambient group overall when compared to the other sound treatment groups. When viewed per stage, it became clear that an increased mortality at ZI stage was the predominant cause of this overall difference, as significant differences in mortality were not found at ZII and ZIII stages. This mortality rate difference should be treated with caution, as it was not replicated in 2018 experiments.

The release of ROS, if not controlled by antioxidant defence systems, leads to oxidative stress (Abele and Puntarulo, 2004). This is known to occur in marine invertebrates as a result of exposure to environmental toxins, thermal stress and UV light exposure (Donaghy et al., 2015; Lesser, 2006), and more recently as a response to ship noise playbacks in blue mussel *Mytilus edulis* gill tissue (Wale et al., 2019). Wale et al. (2019) attributed the increased TBARs in gill tissue of noise exposed adult *M.edulis* to noise-induced metabolic stress and related oxidative radicals. Evidence of oxidative stress in *N. norvegicus* larvae was not found in this study however. The extent to which metabolism-associated ROS leak across mitochondrial membranes into the cytoplasm remains unclear for most marine invertebrates, both in basal and metabolic stress scenarios, and is likely linked to species life history-strategy and the localised O_2 saturation of typical habitat (Abele and Puntarulo, 2004). As adult *N. norvegicus* lives in muddy burrows prone to hypoxic conditions (Johnson and Johnson, 2013), its larvae may be metabolically adapted to cope well with challenging O_2 scenarios. Chronic (8 day) exposure to oxygen saturation as low as 7% has been shown to have no effect on *N. norvegicus* embryonic survival (Eriksson et al., 2006). It may therefore be the case that noise-induced changes in aerobic respiration do not translate to meaningful changes

in antioxidant defence systems in *N. norvegicus* as its baseline state is sufficient to cope with wide-ranging metabolic demands.

Naïve *N. norvegicus* larvae exposed to playbacks of ship noise responded with an increased OCR which is indicative of increased energy expenditure, either through increased movement or upregulated metabolism. Wale *et al.* (2013) found that shore crabs exposed to playbacks of ship noise (148– 155 dB rms re: 1 µPa (received level)) consume 67% more oxygen compared to those exposed to ambient sound playbacks (108–111 dB rms re: 1 µPa (received level)). Individuals exposed to repeated ambient playbacks consumed more oxygen with each additional playback (likely due to handling stress), whereas those exposed to repeated ship noise playbacks showed no change in oxygen consumption over time. Wale *et al.* (2013) suggested ship noise exposed crabs may become habituated or tolerant over time, thereby counteracting the increased oxygen consumption observed in repeatedly tested ambient sound exposed crabs. In contrast to the aforementioned study and a range of studies concerning vertebrates (Harding et al., 2018; Radford, Lèbre, et al., 2016; Radford, Purser, et al., 2016), our respirometry results do not suggest an increase in tolerance in response to repeated ship noise exposures. If the *N. norvegicus* larvae were to develop tolerance, pre-exposed larvae would be expected to show a reduced OCR increase compared to naïve larvae in response to ship noise, trending towards no change in OCR as the number of ship noise exposures increases (Neo et al., 2014). In actuality, pre-exposed larvae exhibited the opposite response to naïve larvae in response to ship noise, namely a reduced OCR. This demonstrates that rather than tolerating/habituating to repeated ship noise exposures, *N. norvegicus* larvae modify their response strategy. This is strong experimental evidence that crustacean larvae not only respond to noise, but that these responses can change after multiple exposures. An increase in energy expenditure in response to imminent danger is costly to the individual, but clearly advantageous if it

allows for increased predator escape likelihood or maintenance of homeostasis in sub-optimal environmental conditions. However, if a 'danger signal' (in this case ship noise) in fact poses no risk to the individual, *N. norvegicus* larvae may be able to 'learn' this after repeated exposures and modify their response accordingly, thereby conserving energy for other vital functions. In the case of *N. norvegicus* this modified response involves reducing oxygen consumption. A decrease in activity in response to ship noise, whilst likely beneficial in terms of energy conservation, could result in increased predation risk or reduced feeding and growth. This offers an explanation for the eventual developmental delays observed in Occasional group ZIII larvae.

An important conclusion to draw from these results, is that if replicated in the field, *N. norvegicus* larvae in busy shipping areas exposed to multiple instances of ship noise would begin to exhibit the opposite OCR response to each subsequent ship passage than would be predicted by a short-term single exposure experiment. This highlights the vital importance of repeat exposure experiments when studying the impacts of noise pollution sources that animals will likely experience numerous times in the field, otherwise erroneous predictions regarding potential impacts on wild populations could be made. This laboratory-based study allowed this longer-term repeat exposure approach to be undertaken with small planktonic larvae, measuring effects at the level of the individual; an approach that would be highly challenging in the field.

The findings of this study must however be interpreted with care; since ship sounds were produced using an underwater speaker to playback ship sound recordings rather than a real boat, and experiments took place in tanks rather than the free-field sound environment of the open ocean where sounds propagate with minimal interference. Whilst care was taken to match peak sound pressure levels in the experimental tanks to levels likely to be experienced from a passing ship in the field, reflections from tank boundaries can cause small-scale variations in received sound

levels. However, received levels of pressure and particle motion are reported here for reproducibility and our primary aim was to determine for the first time whether repeated exposures to continuous low-frequency noise can affect the key early life stages in a commercially and ecologically important UK invertebrate species. Future research in a field setting would be an interesting avenue for building on our results and determining precise sound thresholds and effect ranges. If continuous low-frequency anthropogenic noise, such as shipping noise, does indeed prolong development, reduce stamina and alter oxygen consumption of early life stage *N. norvegicus* in the field, then not only does this have significant commercial implications for the recruitment to, and productivity of this valuable stock in an environment that is getting progressively louder (Ross, 2005), but it also highlights the need to resolve the extensive knowledge gap relating to the effects of continuous low frequency sound on early life Decapods in general. If stakeholders and decision makers are to assess the economic and environmental consequences (or lack thereof) of introduced anthropogenic noise, this knowledge gap must be addressed.

3.5 - References

Al-Shaeri, M., Ahmed, D., McCluskey, F., Turner, G., Paterson, L., Dyrynda, E.A. and Hartl, M.G. (2013), Potentiating toxicological interaction of single-walled carbon nanotubes with dissolved metals. *Environ Toxicol Chem*, 32: 2701-2710. https://doi.org/10.1002/etc.2365

Abele, D. and Puntarulo, S. (2004). Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *138*(4), 405–415. https://doi.org/10.1016/j.cbpb.2004.05.013

Anderson, S. C., Flemming, J. M., Watson, R. and Lotze, H. K. (2011). Rapid global expansion of invertebrate fisheries: Trends, drivers, and ecosystem effects. *PLoS ONE*, *6*(3), 1–9. https://doi.org/10.1371/journal.pone.0014735

Bejder, L., Samuels, A., Whitehead, H., Finn, H. and Allen, S. (2009). Impact assessment research: Use and misuse of habituation, sensitisation and tolerance in describing wildlife responses to anthropogenic stimuli. *Marine Ecology Progress Series*, *395*, 177–185. https://doi.org/10.3354/meps07979

Borja, Á., Elliott, M., Carstensen, J., Heiskanen, A. S. and van de Bund, W. (2010). Marine management - Towards an integrated implementation of the European marine strategy framework and the water framework directives. *Marine Pollution Bulletin*, *60*(12), 2175–2186. https://doi.org/10.1016/j.marpolbul.2010.09.026

Byrne, M. (2011). Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean. *Oceanography and Marine Biology: An Annual Review*, *49*, 1–42. https://doi.org/10.1016/j.marenvres.2011.10.00

Carter, E. E., Tregenza, T. and Stevens, M. (2020). Ship noise inhibits colour change, camouflage, and anti-predator behaviour in shore crabs. *Current Biology*, *30*(5), R211–R212. https://doi.org/10.1016/j.cub.2020.01.014

Christian, J. R., Mathieu, A., Thomson, D. H., White, D. and Buchanan, R. A. (2003). Effect of Seismic Energy on Snow Crab (*Chionoecetes opilio*). *Environmental Research Funds Report No. 144*, *7 November*, 106.

de Soto, N. A., Delorme, N., Atkins, J., Howard, S., Williams, J. and Johnson, M. (2013). Anthropogenic noise causes body malformations and delays development in marine larvae. *Scientific Reports*, *3*(1), 2831. https://doi.org/10.1038/srep02831

DFO. (2004). Potential Impacts of Seismic Energy on Snow Crab. *DFO Can. Sci. Advis. Sec. Habitat Status Report 2004/003*. https://doi.org/Can. Sci. Advis. Sec. Habitat Status Report 2004/003

Dixon, S., Elliott, M., Ellis, G., Holden, J., Murray, A., Pilkington, J., Reade, S., Williamson, K. and Wintz, P. (2017). UK Sea Fisheries Statistics 2016. *Marine Management Organisation*, 80.

Donaghy, L., Hong, H. K., Jauzein, C. and Choi, K. S. (2015). The known and unknown sources of reactive oxygen and nitrogen species in haemocytes of marine bivalve molluscs. *Fish and Shellfish Immunology*, *42*(1), 91–97. https://doi.org/10.1016/j.fsi.2014.10.030

Eriksson, S. P., Nabbing, M. and Sjöman, E. (2006). Is brood care in *Nephrops norvegicus* during hypoxia adaptive or a waste of energy? *Functional Ecology*, *20*, 1097–1104. https://doi.org/10.1111/j.1365-2435.2006.01182.x

Gajda-Meissner, Z., Matyja, K., Brown, D.M., Hartl, M.G. and Fernandes, T.F. (2020), Importance of Surface Coating to Accumulation Dynamics and Acute Toxicity of

Copper Nanomaterials and Dissolved Copper in *Daphnia magna. Environ Toxicol Chem*, 39: 287-299. https://doi.org/10.1002/etc.4617

Galhardo, L., Vital, J. and Oliveira, R. F. (2011). The role of predictability in the stress response of a cichlid fish. *Physiology and Behavior*, *102*(3–4), 367–372. https://doi.org/10.1016/j.physbeh.2010.11.035

Harding, H. R., Gordon, T. A. C., Hsuan, R. E., Mackaness, A. C. E., Radford, A. N. and Simpson, S. D. (2018). Fish in habitats with higher motorboat disturbance show reduced sensitivity to motorboat noise. *Biology Letters*, *14*(10). https://doi.org/10.1098/rsbl.2018.0441

Harland, E. J., Jones, S. a. S. and Clarke, T. (2005). SEA 6 Technical report: underwater ambient noise. *QINETIQ/S&E/MAC/CR050575*, 18. http://www.offshore-sea.org.uk/site/scripts/consultation_download_info.php?downloadID=150

Hildebrand, J. A. (2009). Anthropogenic and natural sources of ambient noise in the ocean. *Marine Ecology Progress Series*, *395*, 5–20. https://doi.org/10.3354/meps08353

Hill, A. E. (1990). Pelagic dispersal of Norway lobster *Nephrops norvegicus* larvae examined using an advection-diffusion-mortality model. *Marine Ecololgy Progress Series*, *64*, 217–226. https://doi.org/10.3354/meps064217

Johnson, M. L. and Johnson, M. P. (2013). The Ecology and Biology of *Nephrops norvegicus*. In *Advances in Marine Biology* (1st ed., Vol. 64). Elsevier. https://doi.org/10.1017/CBO9781107415324.004

Kastelein, R. A., Jennings, N., Kommeren, A., Helder-Hoek, L. and Schop, J. (2017). Acoustic dose-behavioral response relationship in sea bass (*Dicentrarchus labrax*) exposed to playbacks of pile driving sounds. *Marine Environmental Research*, *130*,

315–324. https://doi.org/10.1016/j.marenvres.2017.08.010

Lesser, M. P. (2006). Oxidative Stress In Marine Environments: Biochemistry and Physiological Ecology. *Annual Review of Physiology*, *68*(1), 253–278. https://doi.org/10.1146/annurev.physiol.68.040104.110001

Ligas, A., Sartor, P. and Colloca, F. (2011). Trends in population dynamics and fishery of *Parapenaeus longirostris* and *Nephrops norvegicus* in the Tyrrhenian Sea (NW Mediterranean): The relative importance of fishery and environmental variables. *Marine Ecology*, *32*(SUPPL. 1), 25–35. https://doi.org/10.1111/j.1439-0485.2011.00440.x

Lordan, C., Power, A. M. and Johnson, M. P. (2013). Chapter Two – Habitat and Ecology of *Nephrops norvegicus*. In *Advances in Marine Biology* (Vol. 64, pp. 27–63). https://doi.org/10.1016/B978-0-12-410466-2.00002-9

MacArthur, R. and Wilson, E. O. (1967). *The Theory of Island Biogeography* (2001 Repri). Princeton University Press.

Merchant, N. D., Fristrup, K. M., Johnson, M. P., Tyack, P. L., Witt, M. J., Blondel, P. and Parks, S. E. (2015). Measuring acoustic habitats. *Methods in Ecology and Evolution*, *6*(3), 257–265. https://doi.org/10.1111/2041-210X.12330

Morley, E. L., Jones, G. and Radford, A. N. (2014). The importance of invertebrates when considering the impacts of anthropogenic noise. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1776), 20132683. https://doi.org/10.1098/rspb.2013.2683

Nedelec, S. L., Radford, A. N., Simpson, S. D., Nedelec, B., Lecchini, D. and Mills, S. C. (2015). Anthropogenic noise playback impairs embryonic development and increases mortality in a marine invertebrate. *Scientific Reports*, *4*, 5891.

https://doi.org/10.1038/srep05891

Nedelec, S. L., Simpson, S. D., Morley, E. L., Nedelec, B. and Radford, A. N. (2015). Impacts of regular and random noise on the behaviour, growth and development of larval Atlantic cod (*Gadus morhua*). *Proceedings of the Royal Society B: Biological Sciences*, *282*, 20151943. https://doi.org/10.1098/rspb.2015.1943

Neo, Y. Y., Seitz, J., Kastelein, R. A., Winter, H. V., ten Cate, C. and Slabbekoorn, H. (2014). Temporal structure of sound affects behavioural recovery from noise impact in European seabass. *Biological Conservation*, *178*, 65–73. https://doi.org/10.1016/j.biocon.2014.07.012

Newland, P. L. and Neil, D. M. (1990). The tail flip of the Norway lobster, *Nephrops norvegicus*: I. Giant fibre activation in relation to swimming trajectories. *Journal of Comparative Physiology A: Sensory, Neural and Behavioral Physiology*, *166*(4), 517–527. https://doi.org/10.1007/BF00192022

Parry, G. D. (1981). The meanings of r-and K-selection. *Oecologia*, *48*(2), 260–264. https://doi.org/10.1007/BF00347974

Payne, R. and Webb, D. (1971). Orientation By Means of Long Range Acoustic Signaling in Baleen Whales. *Annals of the New York Academy of Sciences*, *188*(1), 110–141. https://doi.org/10.1111/j.1749-6632.1971.tb13093.x

Pearson, W. H., Skalski, J. R., Sulkin, S. D. and Malme, C. I. (1994). Effects of seismic energy releases on the survival and development of zoeal larvae of dungeness crab (*Cancer magister*). *Marine Environmental Research*, *38*, 93–113. https://doi.org/10.1016/0141-1136(94)90003-5

Popper, A. N. and Hawkins, A. (2016). *The Effects of Noise on Aquatic Life II* (Vol. 875). https://doi.org/10.1007/978-1-4939-2981-8

Powell, A. and Eriksson, S. P. (2013). Chapter Six – Reproduction: Life Cycle, Larvae and Larviculture. In *Advances in Marine Biology* (Vol. 64, pp. 201–245). https://doi.org/10.1016/B978-0-12-410466-2.00006-6

Prior, H. (2002). Effects of predictable and unpredictable intermittent noise on spatial learning in rats. *Behavioural Brain Research*, *133*(2), 117–124. https://doi.org/10.1016/S0166-4328(01)00466-1

R Core Team. (2017). R: A language and environment for statistical computing URL https://www.R-project.org/. *R Foundation for Statistical Computing, Vienna, Austria*.

Radford, A. N., Lèbre, L., Lecaillon, G., Nedelec, S. L. and Simpson, S. D. (2016). Repeated exposure reduces the response to impulsive noise in European seabass. *Global Change Biology*, *22*(10), 3349–3360. https://doi.org/10.1111/gcb.13352

Radford, A. N., Purser, J., Bruintjes, R., Voellmy, I. K., Everley, K. A., Wale, M. A., Holles, S. and Simpson, S. D. (2016). Beyond a Simple Effect: Variable and Changing Responses to Anthropogenic Noise. In A. N. Popper & A. Hawkins (Eds.), *The Effects of Noise on Aquatic Life II* (pp. 901–907). Springer New York.

Regnault, N. and Lagardere, J.-P. (1983). Effects of ambient noise on the metabolic level of *Crangon crangon* (Decapoda, Natantia). *Marine Ecology Progress Series*, *11*, 71–78. https://doi.org/10.3354/meps011071

Ross, D. (2005). Ship sources of ambient noise. *IEEE Journal of Oceanic Engineering*, *30*(2), 257–261. https://doi.org/10.1109/JOE.2005.850879

Rotllant, G., Charmantier-Daures, M., Charmantier, G., Anger, K. and Sarda, F. (2001). Effects of diet on *Nephrops norvegicus* (L.) larval and postlarval development, growth, and elemental composition. *Journal of Shellfish Research*, *20*(1), 347–352.

Sinclair, M. M. and IIles, T. D. (1989). Population regulation and specification in the oceans. *Journal Du Conseil International Exploration de La Mer*, *45*, 165–175.

Smith, R. S. M. (1987). *The Biology of Larval and Juvenile Nephrops norvegicus (L.) in the Firth of Clyde*. 1–540.

Solan, M., Hauton, C., Godbold, J. A., Wood, C. L., Leighton, T. G. and White, P. (2016). Anthropogenic sources of underwater sound can modify how sediment-dwelling invertebrates mediate ecosystem properties. *Scientific Reports*, *6*(1), 20540. https://doi.org/10.1038/srep20540

Tasker, M. L., Amundin, M., André, M., Hawkins, A. D., Lang, W., Merck, T., Scholik-Schlomer, A., Teilmann, J., Thomsen, F., Werner, S. and Zakharia, M. (2010). MARINE STRATEGY FRAMEWORK DIRECTIVE Task Group 11 Report: Underwater noise and other forms of energy. *JRC European Commission*, *July 2016*, 1–64. papers2://publication/doi/10.2788/87079

Tuck, I. D., Atkinson, R. J. A. and Chapman, C. J. (2000). Population biology of the Norway lobster, *Nephrops norvegicus* (L.) in the Firth of Clyde, Scotland II: Fecundity and size at onset of sexual maturity. *ICES Journal of Marine Science*, *57*, 1227–1239. https://doi.org/10.1006/jmsc.2000.0809

Van der Graaf, A. J., Ainslie, M. A., André, M., Brensing, K., Dalen, J., Dekeling, R. P. A., Robinson, S., Tasker, M. L., Thomsen, F. and Werner, S. (2012). *European Marine Strategy Framework Directive Good Environmental Status (MSFD-GES): Report of the Technical Subgroup on Underwater noise and other forms of energy. February*, 75.

Wale, M. A., Briers, R. A., Hartl, M. G. J., Bryson, D. and Diele, K. (2019). From DNA to ecological performance: Effects of anthropogenic noise on a reef-building mussel. *Science of The Total Environment*, *689*, 126–132.

https://doi.org/10.1016/j.scitotenv.2019.06.380

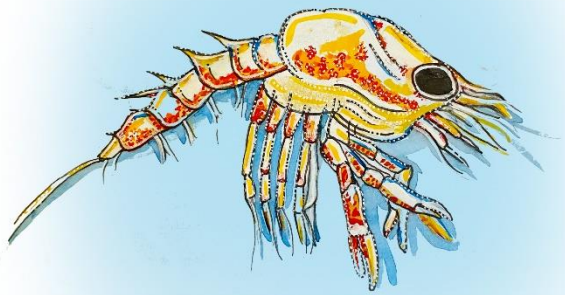
Wale, M. A., Simpson, S. D. and Radford, A. N. (2013a). Size-dependent physiological responses of shore crabs to single and repeated playback of ship noise. *Biology Letters*, *9*(2), 20121194. https://doi.org/10.1098/rsbl.2012.1194

Wale, M. A., Simpson, S. D. and Radford, A. N. (2013b). Noise negatively affects foraging and antipredator behaviour in shore crabs. *Animal Behaviour*, *86*(1), 111–118. https://doi.org/10.1016/j.anbehav.2013.05.001

Williams, R., Wright, A. J., Ashe, E., Blight, L. K., Bruintjes, R., Canessa, R., Clark, C. W., Cullis-Suzuki, S., Dakin, D. T., Erbe, C., Hammond, P. S., Merchant, N. D., O'Hara, P. D., Purser, J., Radford, A. N., Simpson, S. D., Thomas, L. and Wale, M. A. (2015). Impacts of anthropogenic noise on marine life: Publication patterns, new discoveries, and future directions in research and management. *Ocean and Coastal Management*, *115*, 17–24. https://doi.org/10.1016/j.ocecoaman.2015.05.021

Chapter 4

Effects of anthropogenic noise on the early life stage development of *Homarus gammarus*



E. L. Bolger

Chapter 4

Effects of anthropogenic noise on the early life stage development of *Homarus gammarus*

Abstract:

Homarus gammarus (L.) is a commercially and ecologically important fishery species found throughout the eastern Atlantic, with a habitat preference for shallow (<50 m) waters and hard substrate. We now know that anthropogenic noise can negatively affect marine invertebrate behaviour and physiology, but there is currently no information regarding the responses of early life *H. gammarus* to man-made noise. As in chapter 3, newly hatched *H. gammarus* larvae were reared individually in acoustically transparent cups and exposed to three different sound treatments using underwater speakers (n = 83 larvae for each group): 'Busy' treatment, consisting of frequent ship noise playback (totalling 12 hours of noise exposure per 24 hours); 'Occasional' treatment, consisting of less frequent ship noise playback (totalling 3 hours of noise exposure per 24 hours) and 'Ambient' treatment, consisting of constant ambient sound exclusive of any ship noise (Control). The survival and larval stage duration of *H. gammarus* larvae were compared between sound treatments. Ship noise playbacks had no effect on larval stage duration or survival. This highlights the fact that noise impact research findings cannot reliably be extrapolated between species, even those within the same family, as different results were observed with closely related *N. norvegicus* larvae (see chapter 3). Due to low survival rates across all treatments at the third zoeal stage, sample sizes for stage 3 larvae were greatly reduced and follow-on experiments with juvenile lobsters were not possible.

4.1 – Introduction

The European lobster *Homarus gammarus* (L.) is commonly found throughout the eastern Atlantic. Its distributional range extends from north Norwegian fjords Tysfjorden and Nordfoldanorthern, down throughout the coastal regions of western Europe to Morocco in the south (Hayward and Ryland, 2017). There are also resident populations in the Mediterranean Sea and the Black Sea (Triantafyllidis et al., 2005). *H. gammarus* has been observed in depths of up to 150 m but prefers the shallower waters of the continental shelf at depths of <50 m. Adult specimens prefer hard substrate, living in natural crevices in the rock and emerging at night to feed. Adults are solitary and highly territorial. They are opportunistic carrion scavengers, as well as actively preying on small benthic crustaceans, molluscs and polychaetes (National Lobster Hatchery, 2021).

Female *H. gammarus* reach sexual maturity at 5-7 years old, at a total carapace length of 75-85 mm (Lizárraga-Cubedo et al., 2003). Mating usually occurs over the summer months between a hard-shelled male and a newly moulted soft-shelled female. The female can retain the sperm until egg-laying, at which point the eggs are fertilized as they are extruded onto the underneath of the abdomen among the pleopods. The female will carry the eggs for 9-12 months and is colloquially said to be ‘berried’ or a ‘berried hen’ at this stage. As embryos develop and consume the egg yolk, the eggs progressively change in colour from olive-green, to black and finally to an orange-red colour before hatching. Hatching occurs over a period of several nights, when the female will raise her tail and release thousands of planktonic stage 1 larvae (zoeae) into the water column.

The planktonic *H. gammarus* zoeae are opportunistic feeders, consuming both phytoplankton and zooplankton as they drift with ocean currents for approximately 5 – 10 weeks. During this period, they will transition through three morphologically distinct zoeal stages (ZI, ZII and ZIII), with a moult occurring at each stage transition. Larvae undergo a final moult at the end of the 5-10 week period at which point the life-cycle phase of the lobster switches from planktonic zoea to benthic juvenile (Figure 4.3). This transition in life phase is accompanied with a substantial change in morphology, with claws and tail moving from a free hanging ventral position below the cephalothorax, to move upwards in line with the anterior-posterior axis (Figure 4.3). The planktonic period of *H. gammarus* from hatching to juvenile is critical to recruitment to the adult population; it has been estimated that only 0.005% of newly-hatched lobsters survive the planktonic phase to reach the benthic phase and this acts as a bottleneck for population growth (Ellis et al., 2015). Little is known about the early benthic phase of juvenile *H. gammarus*, individuals with a carapace length below 15 mm are rarely observed in the wild, leading to suggestions that they lead sedentary lives in burrows in the sediment for approximately 2 years, before leaving and seeking out appropriate hard substrate for adult life.

H. gammarus is a fished species with a longstanding commercial importance. In 2019, 3,400 tonnes of lobster were landed in the UK with a value of 46.5 million GBP (Marine Management Organisation, 2021). Shellfish species, including *H. gammarus*, are forming an increasingly important component of the fishing economy. Over the last eighty years, pelagic and demersal landings have decreased by 50% and 80% respectively. In contrast landings of shellfish have increased by over 300%, from 32 thousand tonnes to almost 140 thousand tonnes in 2019 (Marine Management Organisation, 2021). This has been driven in part by the lack of landing quotas for shellfish species (excluding *Nephrops norvegicus*). As finfish stocks decreased, and

subsequent management measures restricted opportunities to fish for demersal and pelagic fish, it is likely that the fleet has diversified into the shellfish sector to take advantage of the lack of restrictions on fishing opportunities.

H. gammarus possesses an array of superficial 'hair-fan' receptor organs across the entirety of its outer cuticle, involved in hydrodynamic reception (Laverack, 1962, 1963; Tazaki, 1977). It also has statocyst receptor systems (internal organs to co-ordinate taxis), particularly at the base of the antennules (Cohen, 1955) and chordotonal organs (involved in substrate vibration detection) at the joints of their appendages . All of these sensory systems have been suggested to have the potential for a role in the detection of waterborne acoustic waves (Budelmann, 1992). *H. gammarus* males produce low frequency (100-200 Hz) 'buzzing' sounds during agonistic encounters, by rapidly contracting internal muscles located at the base of the second antennae, causing their carapaces to vibrate. This buzzing has been suggested to be a form of communication, although this is currently unverified (Jézéquel et al., 2020). The rate of buzzing calls produced by *H. gammarus* males during agonistic encounters increases in the presence of shipping noise (Jézéquel et al., 2021), suggesting that these males may be compensating due to the acoustic masking of their buzzing sounds. Unpublished PhD thesis work suggests that adult European lobsters actively avoid a noise source in a tank in both winter and summer, but to a lesser degree in winter, in which the biological drive to seek shelter seemed to override avoidance of acoustic disturbance (Matthew Wale, personal communication). Ship noise playbacks have also been shown to cause increased oxygen consumption and reduced foraging behaviour in other adult decapod crustaceans (Wale et al., 2013a, 2013b). Spiny lobsters *Palinurus elephas* have been documented to increase both locomotion and stress-related haemolymphatic bioindicators such as glucose, total proteins, Hsp70 expression and THC in response to boat noise playbacks in a laboratory setting (Filiciotto et al., 2014).

Given the evidence that adult decapods, including *H. gammarus*, can both detect and be negatively impacted by underwater sound, and given the ship noise playback impacts observed for *N. norvegicus* larvae (see chapter 3 of this thesis), *H. gammarus* larvae were well suited as a candidate species to further explore the potential impacts of anthropogenic noise on early life stage decapods.

The aim of this study was to recreate the sound exposure conditions utilised in chapter 3 to assess the impacts, if any, of ship noise playbacks on the development and survival of newly hatched *H. gammarus* larvae.

4.2 – Materials and methods

4.2.1 - Broodstock

Berried female *H. gammarus* were landed at St Abbs harbour, Scotland (55.898545, -2.128635) in June 2017 and were immediately transported to the St Abbs Marine Station where they were maintained in rectangular tanks (432 L volume) on a continuous flow-through system at ambient seawater temperature. Stocking density was 1 female per tank. The tanks contained a 10 cm layer of sandy substrate and large rocks to allow natural sheltering behaviour. The broodstock was fed cooked mussel *ad libitum* twice weekly, removing any uneaten food after each feeding session. Fine nylon mesh was fitted over tank outflows to ensure any released larvae were retained within the broodstock tank. Tanks were checked daily at 10:00 for hatched stage I zoeae.

4.2.2 – Hatching

On 17th May, one female began the hatching process. Each day, during the period 11:00-12:00, all hatched larvae were transferred to individual rearing chambers in the sound exposure set-up using a wide-mouthed pipette. Hatched ZI larvae on a given day were distributed equally between 3 sound treatment tanks (Busy, Occasional and Ambient). On 2nd day of hatching, all 83 rearing cups in each sound treatment tank were occupied with a single ZI larvae.

4.2.3 – Larval maintenance

Larval maintenance was carried out exactly as described in section 3.2.3.

4.2.4 - Experimental set-up and design

Experimental set-up and design was exactly as described in section 3.2.4.

4.2.5 - Sound recordings and playback design

Sound recordings and playback design were exactly as described in section 3.2.5.

During ship noise playback, there was a sound pressure peak of 115dB re: 1 μPa^2 Hz^{-1} at 747 Hz and 122dB re: 1 μPa^2 Hz^{-1} at 1587 Hz in the rearing cups of the Busy group. There were peaks of 118dB re: 1 μPa^2 Hz^{-1} at 395 Hz; 111dB re: 1 μPa^2 Hz^{-1} at 746 Hz; 115dB re: 1 μPa^2 Hz^{-1} at 915 Hz and 115dB re: 1 μPa^2 Hz^{-1} at 1566 Hz in the rearing cups of the Occasional group. There were peaks of 101dB re: 1 μPa^2 Hz^{-1} at 20 Hz and 70.3dB re: 1 μPa^2 Hz^{-1} at 1565 Hz in the rearing cups of the Ambient group (Figure 4.1).

During ship noise playback, there was a particle acceleration peak of 102dB re: (1 $\mu m\ s^{-2})^2$ Hz^{-1} at 60 Hz, 99dB re: (1 $\mu m\ s^{-2})^2$ Hz^{-1} at 702 Hz and 99dB re: (1 $\mu m\ s^{-2})^2$ Hz^{-1} at 747 Hz in the rearing cups of the Busy group. There were peaks of 102dB re: (1 $\mu m\ s^{-2})^2$ Hz^{-1} at 59 Hz, 98.7dB re: (1 $\mu m\ s^{-2})^2$ Hz^{-1} at 702 Hz and 98.2dB re: (1 $\mu m\ s^{-2})^2$ Hz^{-1} at 747 Hz in the rearing cups of the Occasional group. There were peaks of 88.95dB re: (1 $\mu m\ s^{-2})^2$ Hz^{-1} at 250 Hz and 91.11dB re: (1 $\mu m\ s^{-2})^2$ Hz^{-1} at 600 Hz in the rearing cups of the Ambient group (Figure 4.2).

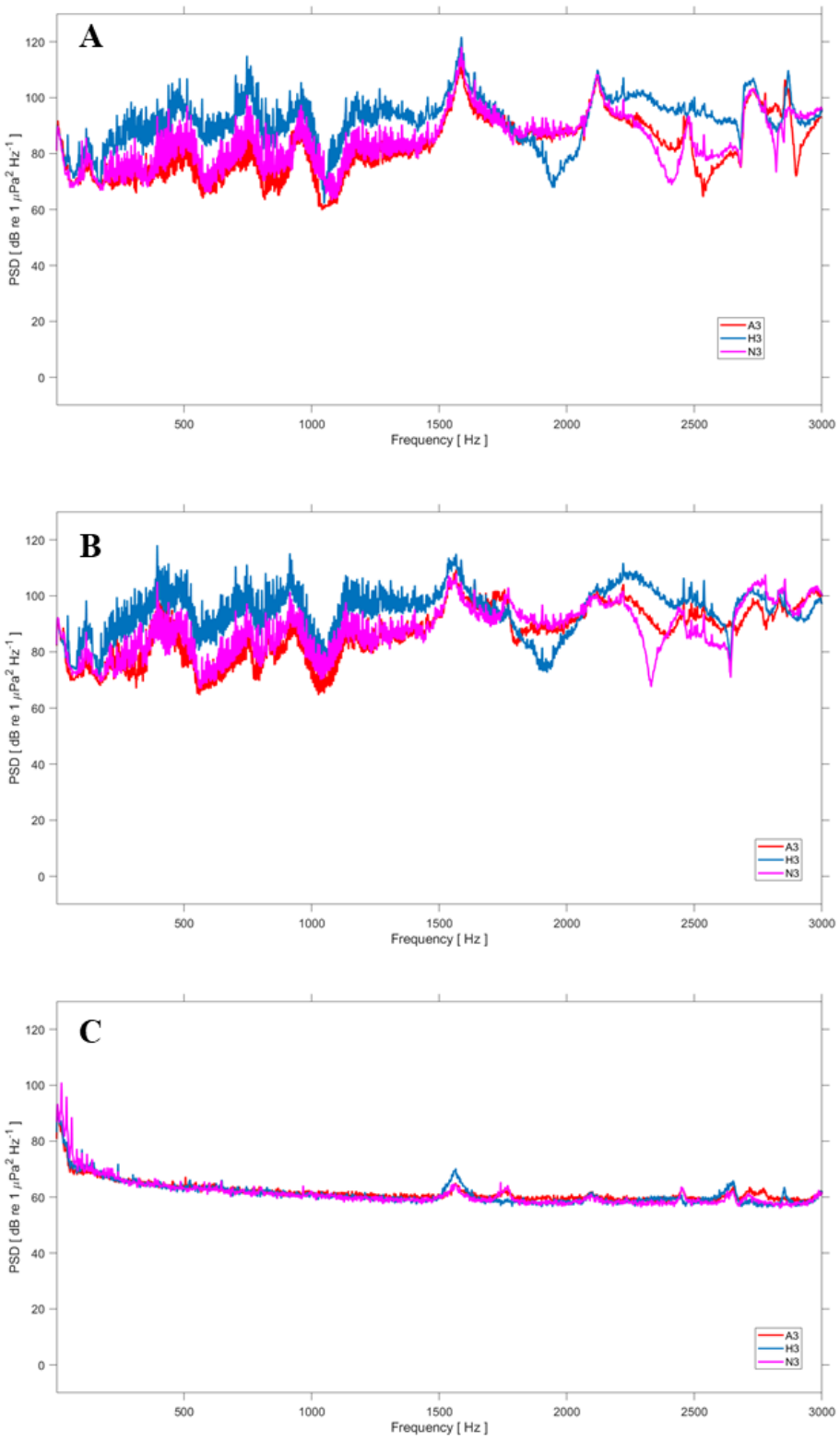


Figure 4.1. Power spectral density plots showing RMS sound pressure levels (dB re: 1µPa^2 Hz^{-1}) received by developing *Homarus gammarus* (L.). Recordings were taken inside rearing cups in **A**) Busy **B**) Occasional and **C**) Ambient treatment groups. In each treatment, recordings were taken directly above the speaker (blue line) and at the furthest cups from the centre moving directly right (pink line) and left (red line). DFT length: 48 kHz, recording lengths 30 s, Hann window length: 1 s.

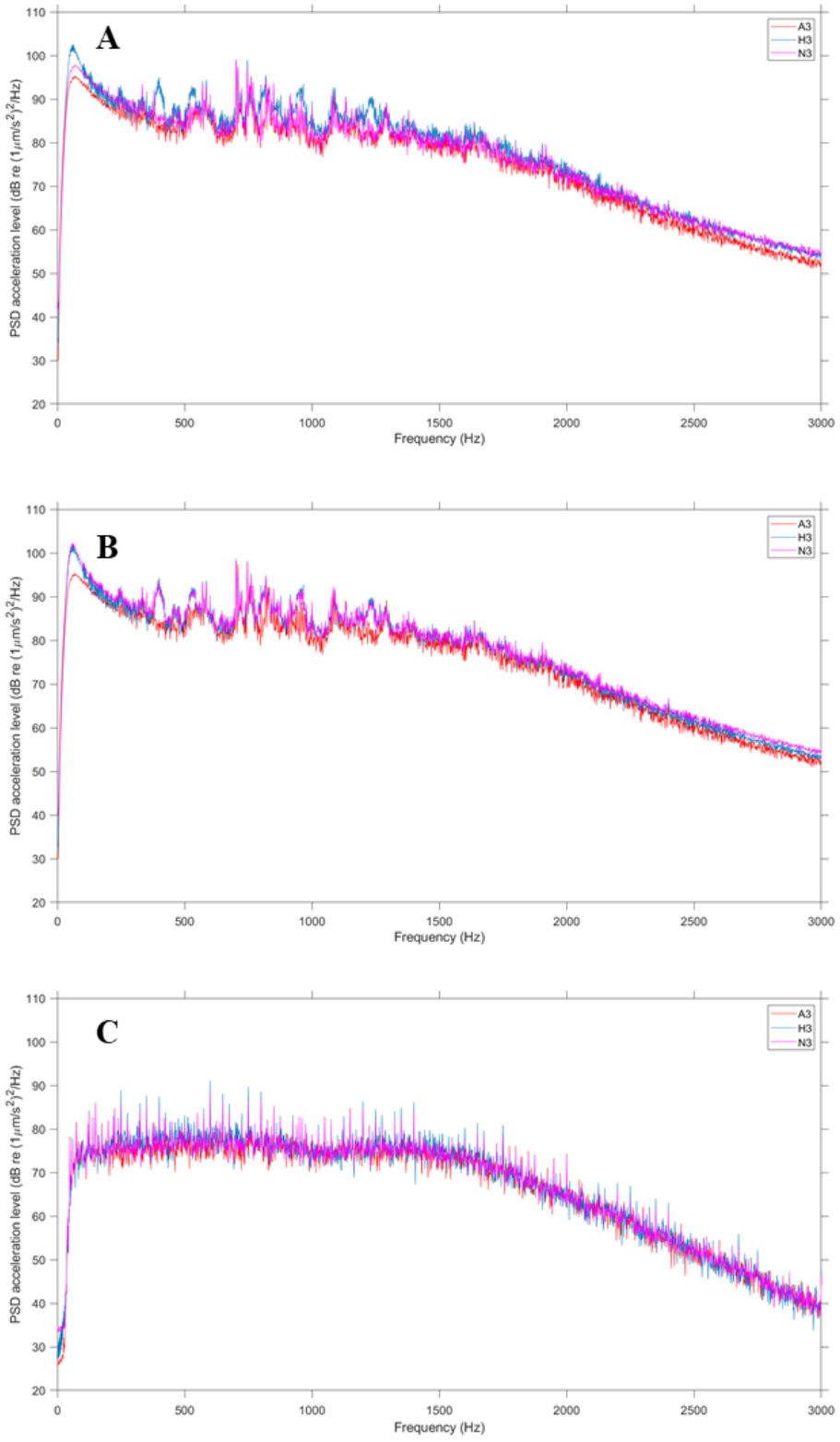


Figure 4.2 Power spectral density plots showing RMS particle acceleration levels (dB re: $(1\mu m\ s^{-2})^2\ Hz^{-1}$) received by developing *Homarus gammarus* (L.). Recordings were taken inside rearing cups in **A**) Busy **B**) Occasional and **C**) Ambient treatment groups. In each treatment, recordings were taken directly above the speaker (blue line) and at the furthest cups from the centre moving directly right (pink line) and left (red line). DFT length: 48 kHz, recording lengths 30 s, Hann window length: 1 s.

4.2.6 - Data collection

4.2.6.1 - Survival and Developmental Stage Duration

Each rearing cup was visually assessed daily for mortality or moulting of the larva. Transitions between stages were clearly observable with the naked eye as there were distinct changes size and morphology and exuviae were clearly visible (Figure 4.3). Briefly, Stage I larvae were characterized by the absence of abdominal appendages (pleopods). Pleopods first appear at stage II but are not functional as swimmerets at this stage, the rostrum also becomes more pronounced. At stage III the pleopods are further developed and uropods develop adjacent to the telson, forming the distinctive 'tail fan' seen in adult lobsters. At stage IV, the chelae and tail reposition from a free hanging ventral position below the cephalothorax, upwards to move in line with the anterior-posterior axis. The antennae are also considerably developed at this stage and project in an anterior direction beyond the chelae.

Mortality was defined as no observable movement of the larvae for 10 seconds followed by a further 10 seconds of no movement following gentle agitation of the rearing cup water.

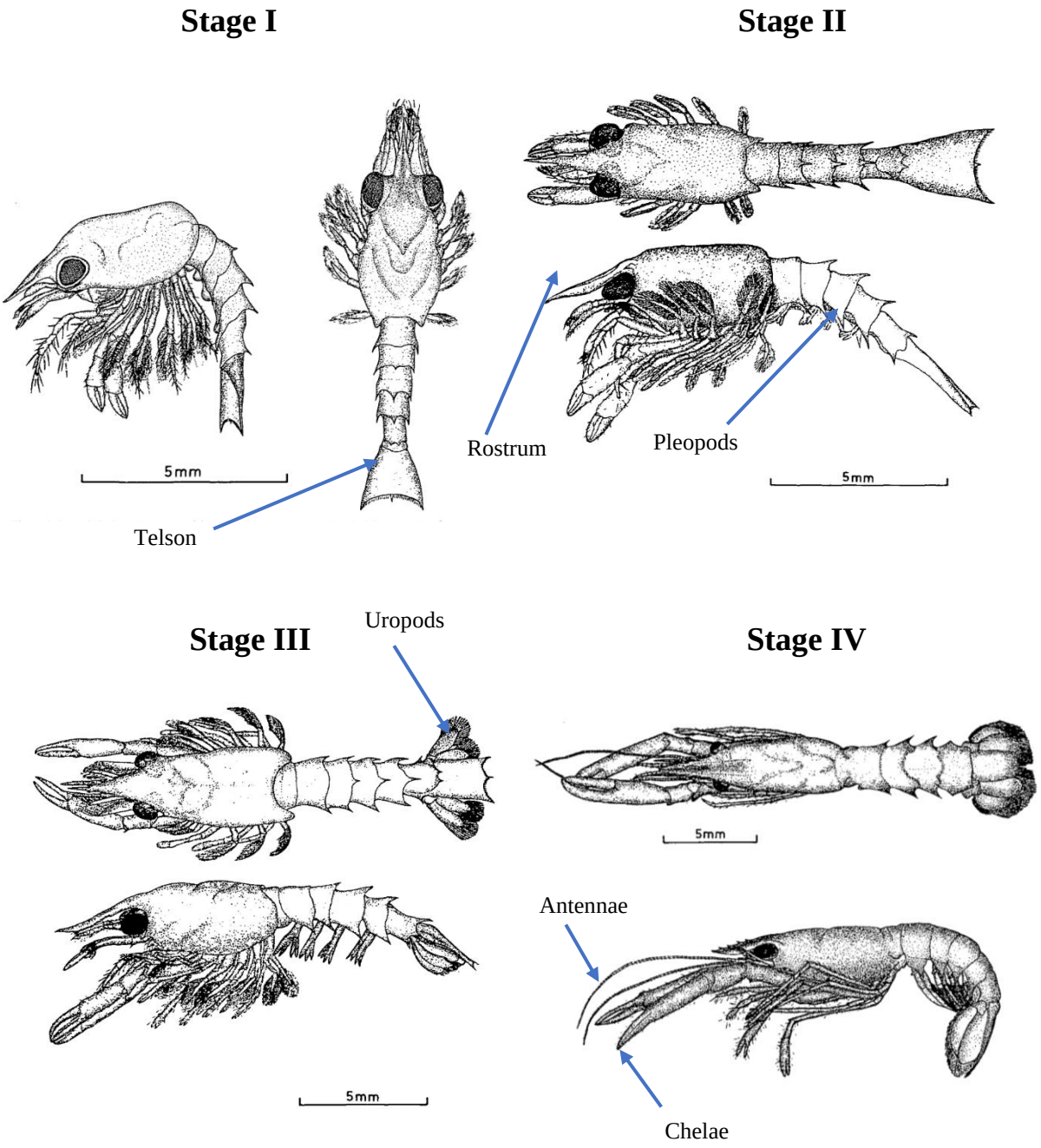


Figure 4.3 Morphology of the zoeal stages I-III and the first juvenile stage (IV) of developing *Homarus gammarus* (L.), adapted from drawings by Nichols and Lawton (1978). Morphological features used to differentiate between stages are labelled.

4.2.7 - Statistical analyses

4.2.7.1 - Developmental Stage Duration

Mean values (± Standard Error (SE)) for the duration (days) of stages I, II, III and overall zoeal development (I + II + III) were calculated for each sound playback group (Busy, Occasional, Ambient) and displayed graphically. As stage duration data did not meet parametric assumptions (assessed using Shapiro-Wilk and Bartlett tests), differences between median duration values were assessed using Kruskal-Wallis rank sum tests followed by post-hoc Wilcoxon rank sum tests with continuity correction.

4.2.7.2 - Survival

Chi-square comparisons were carried out with 2x3 contingency tables with survival (survived and died) as rows and sound playback group (busy, occasional and ambient) as columns to determine whether there is an overall association of sound playback group and survival. Pairwise chi-square tests were also carried out with 2x2 contingency tables for each sound playback group combination to test for survival differences between specific treatment groups.

The time-dependent mortality rates of different groups were determined using a Kaplan-Meier survival analysis in Graphpad Prism 6.01. A post-hoc log-rank (Mantel-Cox) test was used to compare mortality rates between groups.

4.3 - Results

4.3.1 - Developmental Stage Duration

There were no differences in stage duration between sound groups at stage I (Kruskal-Wallis: $X^2_{(2)} = 2.74$, p = 0.25) or stage II (Kruskal-Wallis: $X^2_{(2)} = 2.45$, p = 0.29). There was also no significant difference in duration at ZIII (Kruskal-Wallis: $X^2_{(2)} = 3.04$, p = 0.22), although sample size was small at this stage due to high mortality in the transition from stage III to stage IV (1 stage IV individual remaining in Busy, 4 in Occasional and 6 in Ambient) (Figure 4.4).

4.3.2 - Survival

No significant differences were detected in survival between sound groups at stage I ($X^2_{(2)} = 2.05$, p = 0.36), stage II ($X^2_{(2)} = 2.29$, p = 0.32) or stage III ($X^2_{(2)} = 3.60$, p = 0.17). A Chi-squared test was not conducted for differences in total larval duration (hatching to stage IV) due to the low number of individuals that successfully completed larval development (1 stage IV individual remaining in Busy, 4 in Occasional and 6 in Ambient) (Figure 4.5, Figure 4.6). It was possible to compare survival over total larval development using fitted Kaplan-Meier curves and a Mantel-Cox log-rank test, which was marginally insignificant ($\chi^2_{(2)} = 5.63$, df = 2, p = 0.06) (Figure 4.7).

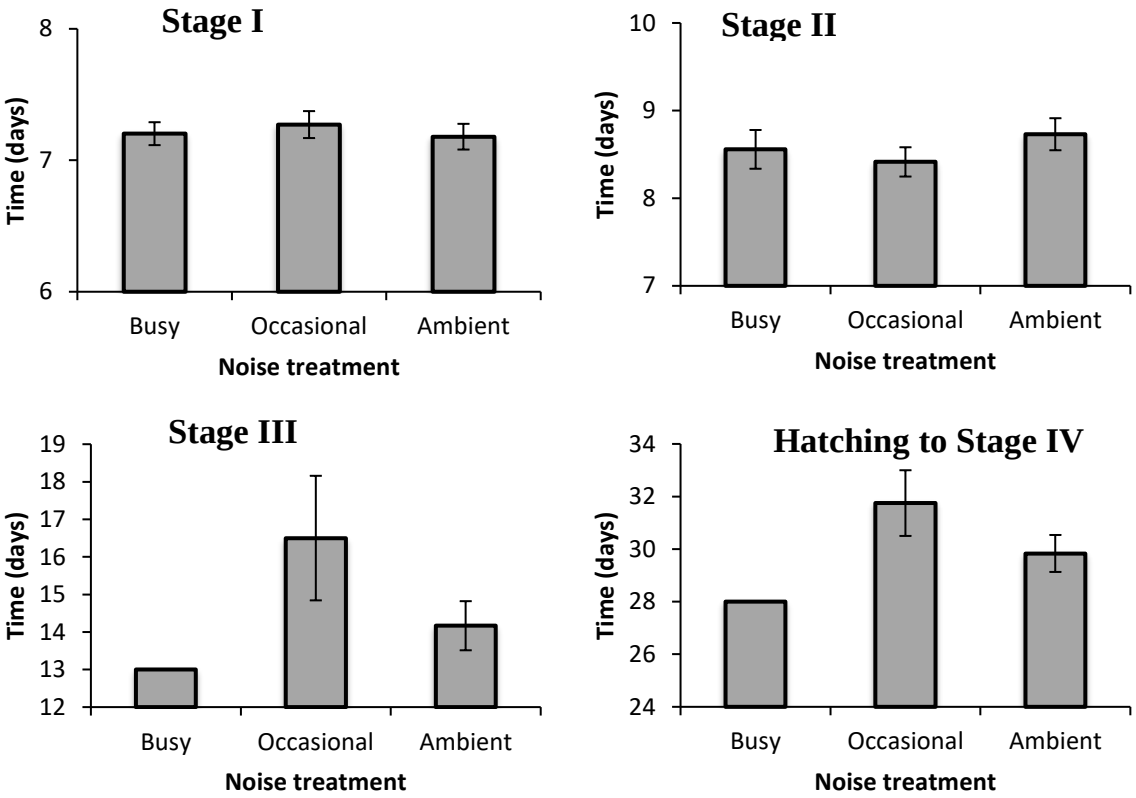


Figure 4.4 Mean (± SE) duration (days) of each planktonic zoeal stage (I-III) and hatching to stage IV of *Homarus gammarus* (L.) in response to different ship sound playback treatments. All groups were statistically homogeneous ($p > 0.05$), calculated using a Kruskall-Wallis test.

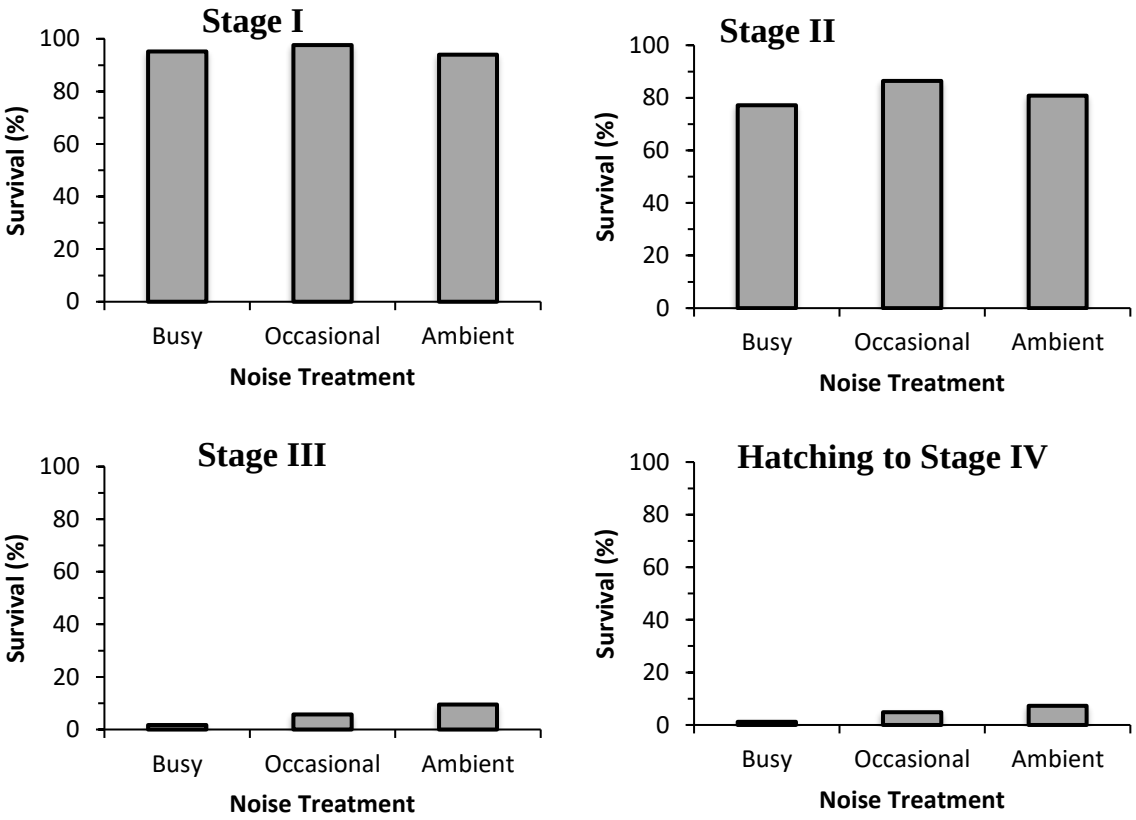


Figure 4.5 Survival (%) of *Homarus gammarus* (L.) from hatching to juvenile (stage IV) and at each planktonic zoeal stage (I - III) in response to different ship sound playback treatments. Equal letters indicate statistically homogeneous groups ($p > 0.05$) calculated using Chi-square tests.

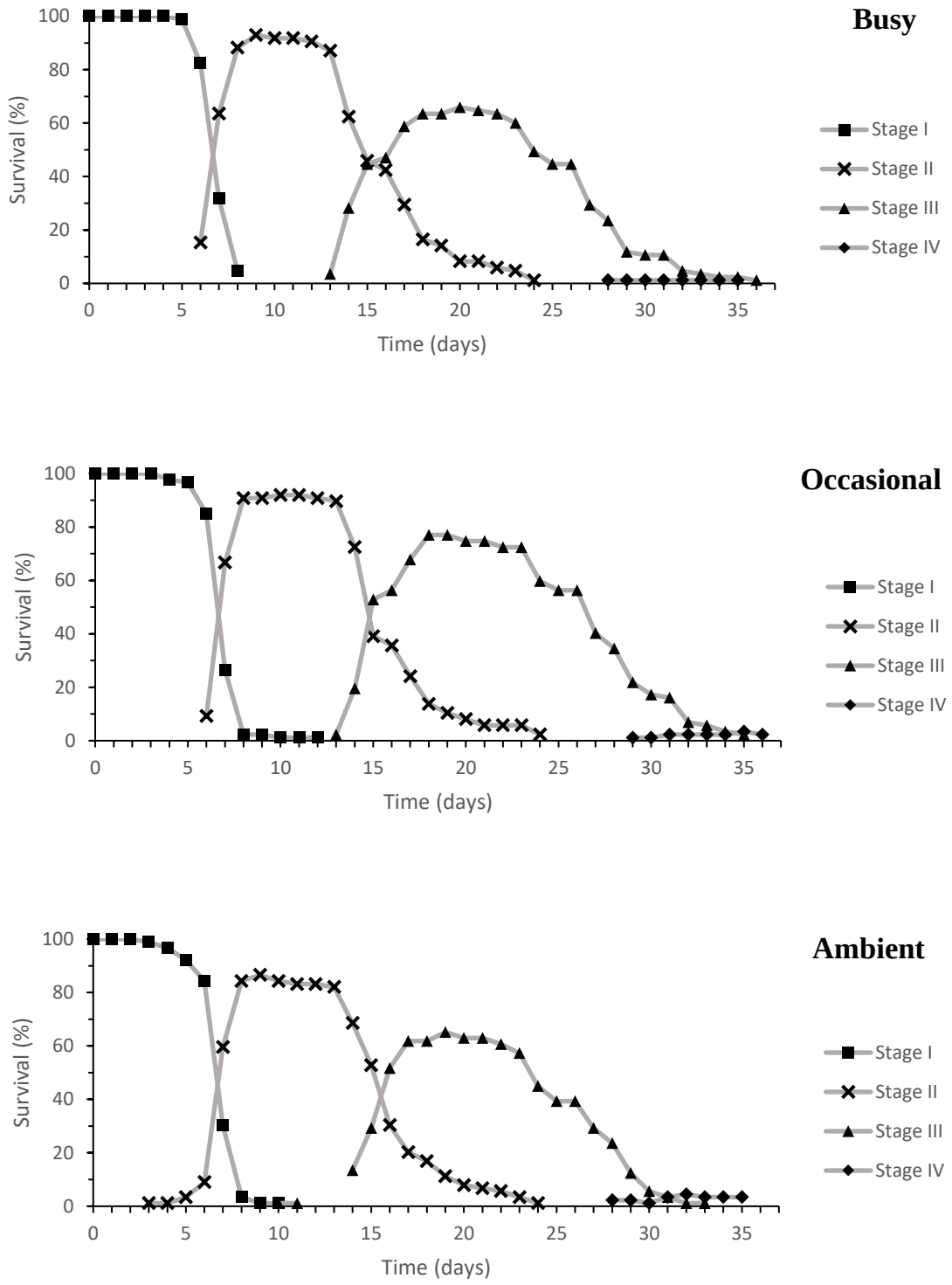


Figure 4.6 Survival (%) and development of *H.gammarus* larvae through successive zoeal stages from hatching to juvenile (ZI-stage IV) in different ship sound playback treatments. (*n* initial = 83 larvae per treatment, % values post-ZI relate to the initial *n* = 83). Data displayed until 37 days post hatching when no surviving larvae remained.

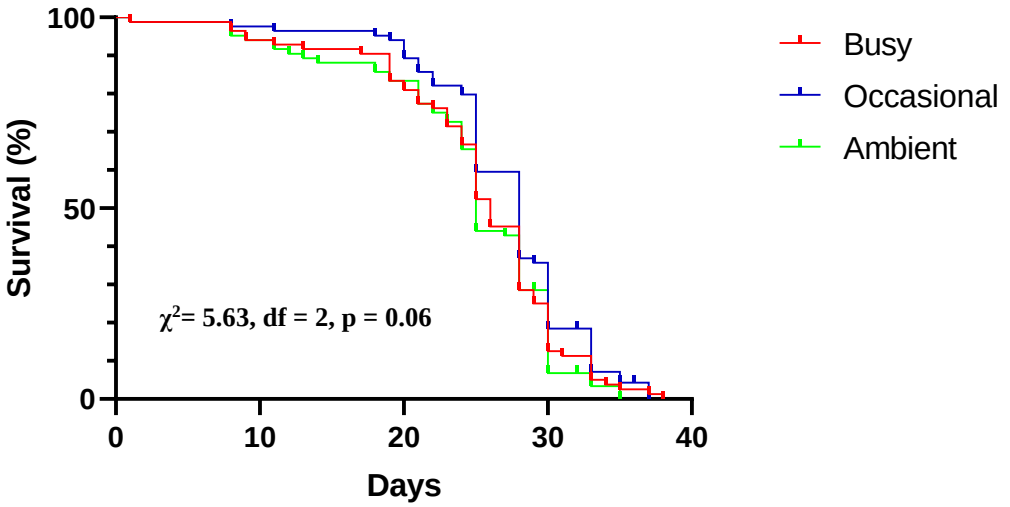


Figure 4.7 Kaplan-Meier survival curves of *Homarus gammarus* (L.) from hatching to juvenile stage IV in response to different ship sound playback treatments. % values refer to initial n=83 larvae in each sound group. Results of a log-rank test for difference between curves is displayed.

4.4 – Discussion

This study is the first to investigate the effects of anthropogenic noise on larval *H. gammarus*. No effects on larval survival or developmental duration were found, in contrast to what was observed for *N. norvegicus* larvae (see chapter 3).

It was hypothesised that as *H. gammarus* and *N. norvegicus* are fellow members of the family Nephropidae (known as true lobster or clawed lobsters), the delays in development of *N. norvegicus* larvae in response to ship noise playbacks (chapter 3) would be mirrored in *H. gammarus* larvae in this study. The larvae of *H. gammarus* and *N. norvegicus* have similar early-life strategies; both have three planktonic zoeal stages and the morphological changes that characterise the transition between stages are shared by both species (e.g. appearance of pleopods at stage II and uropods at stage III). Further, the larvae of both species have similar modes of zooplankton predation, namely the opportunistic grasping of zooplankton that drift into close proximity with the chelae (personal observations). Given these similarities it seemed reasonable to hypothesise that any evolved sound or vibration responsive receptor systems would perform similar functions in the planktonic stages of each species when exposed to the same sound exposure.

The fact that *H. gammarus* larvae did not respond to noise in the same way as *N. norvegicus* is an indication that anthropogenic noise impacts observed in marine invertebrates cannot reliably be extrapolated between ostensibly similar species, which is an issue for environmental managers and decision makers due to the dearth of species tested for sensitivity to anthropogenic noise. The disparity between *H.* gammarus and *N. norvegicus* could either be due to differences in physiology, behaviour or sensory systems. Adults of these species have distinct behaviours and life-history strategies, with *N. norvegicus* digging complex burrow systems in muddy sediment (Katoh et al., 2013), whilst *H. gammarus* lives in close association with hard substrate (Hooper and

Austen, 2014). These differences in habitat and strategy will of course entail different behaviours and physiological adaptations. Burrow-dwelling *N. norvegicus* necessarily have high hypoxia tolerances for example (Eriksson et al., 2006, 2013). Very little is known about the behaviour of the larvae of either species in the field and studies in laboratories struggle to create the conditions for 'natural' behaviours to manifest (Ellis et al., 2015; Powell and Eriksson, 2013). More work on the mechanism of impacts of noise on *N. norvegicus* may in turn elucidate the reasons for the lack of impact in *H. gammarus.*

In this study, the low survival of larvae in all treatments as they transitioned from stage III to stage IV is noteworthy, since it may have masked any changes in both stage duration and survival that may or may not have been produced by the noise exposure. Noise impacts were not observed in *N. norvegicus* until zoea stage II, if impacts manifest later in *H. gammarus* (e.g. stage III or stage IV) these changes will have been missed. The low success rate for larval development seen here is in line with other studies, which have reported larval survival rates (hatching to stage IV) as low as 1.4% (Daniels et al., 2010). Future studies may have to sacrifice the fine control and observational opportunities that individual rearing allows for and instead trial communal rearing at a larger scale to increase the number of surviving larvae if further work is to be done on *H. gammarus* larvae from hatching to stage III onwards.

4.5 – References

Budelmann, B. U. (1992). Hearing in Crustacea. In *The Evolutionary Biology of Hearing* (pp. 131–139). Springer-Verlag.

Cohen, M. J. (1955). The function of receptors in the statocyst of the lobster *Homarus americanus*. *The Journal of Physiology*, *130*(1), 9–34. https://doi.org/https://doi.org/10.1113/jphysiol.1955.sp005389

Daniels, C. L., Merrifield, D. L., Boothroyd, D. P., Davies, S. J., Factor, J. R. and Arnold, K. E. (2010). Effect of dietary *Bacillus* spp. and mannan oligosaccharides (MOS) on European lobster (*Homarus gammarus* L.) larvae growth performance, gut morphology and gut microbiota. *Aquaculture*, *304*(1–4), 49–57. https://doi.org/10.1016/j.aquaculture.2010.03.018

Ellis, C. D., Hodgson, D. J., Daniels, C. L., Boothroyd, D. P., Bannister, R. C. A. and Griffiths, A. G. F. (2015). European lobster stocking requires comprehensive impact assessment to determine fishery benefits. *ICES Journal of Marine Science*, *72*, i35–i48. https://doi.org/10.1093/icesjms/fsu196

Eriksson, S. P., Hernroth, B. and Baden, S. P. (2013). Chapter Five – Stress Biology and Immunology in *Nephrops norvegicus*. In *Advances in Marine Biology* (Vol. 64, pp. 149–200). https://doi.org/10.1016/B978-0-12-410466-2.00005-4

Eriksson, S. P., Nabbing, M. and Sjöman, E. (2006). Is brood care in *Nephrops norvegicus* during hypoxia adaptive or a waste of energy? *Functional Ecology*, *20*, 1097–1104. https://doi.org/10.1111/j.1365-2435.2006.01182.x

Filiciotto, F., Vazzana, M., Celi, M., Maccarrone, V., Ceraulo, M., Buffa, G., Stefano, V. Di, Mazzola, S. and Buscaino, G. (2014). Behavioural and biochemical stress responses of *Palinurus elephas* after exposure to boat noise pollution in tank.

Marine Pollution Bulletin, *84*(1–2), 104–114. https://doi.org/10.1016/j.marpolbul.2014.05.029

Hayward, P. J., & Ryland, J. S. (Eds.). (2017). *Handbook of the Marine Fauna of North-West Europe* (2nd ed.). Oxford University Press. https://doi.org/10.1093/acprof:oso/9780199549443.001.0001

Hooper, T. and Austen, M. (2014). The co-location of offshore windfarms and decapod fisheries in the UK: Constraints and opportunities. *Marine Policy*, *43*, 295–300. https://doi.org/10.1016/j.marpol.2013.06.011

Jézéquel, Y., Bonnel, J. and Chauvaud, L. (2021). Potential for acoustic masking due to shipping noise in the European lobster (*Homarus gammarus*). *Marine Pollution Bulletin*, *173*, 112934. https://doi.org/10.1016/j.marpolbul.2021.112934

Jézéquel, Y., Coston-Guarini, J., Chauvaud, L. and Bonnel, J. (2020). Acoustic behaviour of male European lobsters (*Homarus gammarus*) during agonistic encounters. *Journal of Experimental Biology*, *223*(4). https://doi.org/10.1242/jeb.211276

Katoh, E., Sbragaglia, V., Aguzzi, J. and Breithaupt, T. (2013). Chapter Three – Sensory Biology and Behaviour of *Nephrops norvegicus*. In *Advances in Marine Biology* (Vol. 64, pp. 65–106). https://doi.org/10.1016/B978-0-12-410466-2.00003-0

Laverack, M. S. (1962). Responses of cuticular sense organs of the lobster, *Homarus vulgaris* (crustacea)—II. Hair-fan organs as pressure receptors. *Comparative Biochemistry and Physiology*, *6*(2), 137–145. https://doi.org/https://doi.org/10.1016/0010-406X(62)90160-3

Laverack, M. S. (1963). Responses of cuticular sense organs of the lobster, *Homarus*

vulgaris (crustacea)—III. Activity invoked in sense organs of the carapace. *Comparative Biochemistry and Physiology*, *10*(4), 261–272. https://doi.org/https://doi.org/10.1016/0010-406X(63)90227-5

Lizárraga-Cubedo, H. A., Tuck, I., Bailey, N., Pierce, G. J. and Kinnear, J. A. M. (2003). Comparisons of size at maturity and fecundity of two Scottish populations of the European lobster, *Homarus gammarus*. *Fisheries Research*, *65*(1–3), 137–152. https://doi.org/10.1016/j.fishres.2003.09.012

Marine Management Organisation. (2021). *UK Sea Fisheries Annual Statistics Report 2019*. https://www.gov.uk/government/statistics/uk-sea-fisheries-annual-statistics-report-2019

National Lobster Hatchery. (2021). *Biology of the European lobster, Homarus gammarus*. www.nationallobsterhatchery.co.uk

Powell, A. and Eriksson, S. P. (2013). Chapter Six – Reproduction: Life Cycle, Larvae and Larviculture. In *Advances in Marine Biology* (Vol. 64, pp. 201–245). https://doi.org/10.1016/B978-0-12-410466-2.00006-6

Tazaki, K. (1977). Nervous responses from mechanosensory hairs on the antennal flagellum in the lobster, *Homarus gammarus* (L.). *Marine Behaviour and Physiology*, *5*, 1–18.

Triantafyllidis, A., Apostolidis, A. P., Katsares, V., Kelly, E., Mercer, J., Hughes, M., Jørstad, K. E., Tsolou, A., Hynes, R. and Triantaphyllidis, C. (2005). Mitochondrial DNA variation in the European lobster (*Homarus gammarus*) throughout the range. *Marine Biology*, *146*(2), 223–235. https://doi.org/10.1007/s00227-004-1435-2

Wale, M. A., Simpson, S. D. and Radford, A. N. (2013a). Size-dependent physiological

responses of shore crabs to single and repeated playback of ship noise. *Biology Letters*, *9*(2), 20121194. https://doi.org/10.1098/rsbl.2012.1194

Wale, M. A., Simpson, S. D. and Radford, A. N. (2013b). Noise negatively affects foraging and antipredator behaviour in shore crabs. *Animal Behaviour*, *86*(1), 111–118. https://doi.org/10.1016/j.anbehav.2013.05.001

Chapter 5

Effects of anthropogenic noise on the oxygen consumption rate of *Loligo forbesii* embryos

E. L. Bolger

Chapter 5

Effects of anthropogenic noise on the oxygen consumption rate of *Loligo forbesii* embryos

Abstract:

Cephalopods are keystone marine species that play important roles as both predators and prey in coastal and oceanic ecosystems. We now know that anthropogenic noise can negatively impact adult cephalopod behaviour and damage statocyst organs, but no study to date has explored the responses of pre-hatching embryos to noise. This study investigated whether playbacks of ship motor noise affect the physiology of embryonic veined squid *Loligo forbesii*. Acute ship noise playbacks affected the oxygen consumption of developing *L. forbesii* embryos in a stage-dependent way, with stage-27 embryos increasing oxygen consumption in response to noise, while stage-29 embryos showed no significant changes in response to noise (regardless of whether they had experienced noise pre-exposure or were naïve to ship noise playbacks). These findings suggest that noise playbacks are sufficient to elicit a stress response in cephalopod embryos, but that this response may be masked by the upregulated metabolic environment found in late-stage embryos immediately prior to hatching. Noise-induced increases in embryonic energy consumption may have negative implications for the efficiency of the conversion of egg yolk to body growth and subsequent survival probabilities for post-hatching larvae. Given the results reported here, further work exploring this possibility is now warranted.

The results presented in the following chapter form part of the manuscript "Diele, K., Bolger, E., Fusi, M., Rueckert, S., Scott, K., Wale, M., Harsanyi, P. Effects of Shipnoise Playbacks on Squid Embryogenesis and Hatchlings, *Loligo forbesii*. In Prep."

The above cited study was a multi-disciplinary investigation with research input from various researchers. The manuscript draft contains a condensed version of the results reported in this chapter, which I have written. For the work presented in this thesis chapter K.D and E.B conceived the respirometry component of the study; K.D., P.H., M.W. and E.B. designed the egg exposure setup; E.B. set up and analysed the egg exposure acoustics, conducted all respirometry work; performed the respirometry statistical analysis; and wrote the respirometry methods and results section. All work below is my own work (e.g. introduction and discussion sections are my own words and do not form part of the manuscript in preparation.)

5.1 - Introduction

Squids have been given more attention in the anthropogenic noise impact research field than some other invertebrate groups, given their high mobility, complex behaviour, clearly visible startle responses, and commercial and ecological importance. There is strong evidence that high amplitude sound can cause both behavioural changes and mechanical damage to adult squid. Startle responses (inking) and behavioural changes (increased movement) have been observed in the southern reef squid (*Sepioteuthis australis*) exposed to seismic airgun blasts above 147 dB re 1 μPa^2.s (sound exposure level) in the field (Fewtrell and Mccauley, 2012). The long fin squid (*Doryteuthis pealeii*) responds to pure tones of 80 to 1000 Hz with increased inking or jetting behaviour, with the type and severity of response dependent on frequency and sound level (Mooney et al., 2016). The fact that behaviour such as inking, typically understood

as an anti-predator adaptation (Messenger and Hanlon, 2018), alters according to sound frequency and intensity is suggestive of a relative loudness concept to sound perception in squid. Pile driving playbacks also reduce the number of prey caught by *D. pealeii* in aquaria (Jones et al., 2021), demonstrating that noise can impact on squid behaviour as an apparent 'distraction', even if not loud enough to elicit startle responses.

Adult squid possess 10 parallel 'epidermal lines' of ciliated sensory cells that run in a longitudinal direction over the head and onto the arms (Arnold and Williams-Arnold, 1980; Sundermann, 1983). These epidermal lines are roughly analogous to lateral lines of fish and are sensitive to water oscillations (B. Budelmann and Bleckmann, 1988).

The most likely candidate sensory system for hearing in cephalopods however are the statocysts. These are fluid filled sacs with a denser (often calcium carbonate) statolith contained within. The inertia of the dense statolith in comparison to the surrounding sac means that movements of the animal's body cause relative movements of the statolith in the opposite direction and resultant stimulation of sensory hair cells that line the interior of the surrounding sac. Whilst this mechanism is used for gravity and equilibrium perception, it could also detect particle vibrations caused by passing sound waves (B. U. Budelmann, 1992).

Sound sweeps from 50–400 Hz with peak sound pressure levels of 175 dB re 1 µPa cause lesions in the statocysts of four Mediterranean Sea cephalopod species: *Sepia officinalis*, *Octopus vulgaris*, *Loligo vulgaris* and *Illex condietii* (Solé, Lenoir, Durfort, López-Bejar, Lombarte, van der Schaar, et al., 2013). These lesions would likely impair the movement and behaviour of the individuals affected.

Whilst there is a growing evidence base for the ability of anthropogenic noise to alter behaviour and cause damage to statocysts of adult squid, the effects of noise on cephalopod embryos, their development and any carry-over effects from embryogenesis

to the paralarvae is unknown for all cephalopod species. Embryos and prelarvae of coastal squid of the genus Loligo (including the study species below) develop inside egg strands attached to hard substrate. Egg masses consist of numerous finger-like capsules or 'strings' that are attached to hard substrate by females one by one (Oesterwind et al., 2010). The veined squid *Loligo forbesii* (Steenstrup, 1857) predominantly spawns in Scottish waters over the period December to June, peaking in March, but some mature specimens can be found there throughout the year, and egg masses are reported by fishermen up to September (Laptikhovsky et al., 2021). It is thought that there are distinct summer and winter spawning populations (Pierce et al., 1994). Embryonic development occurs for approximately one month (Rosa et al., 2012), a substantial proportion (~8%) of the short-lived squid's lifespan with spawning often occurring one year after hatching and death following immediately afterwards (Pierce et al., 1994). During this period, developing embryos have an obligate sessile existence, unable to move away from any localised stressors. The embryos and larvae of many invertebrates are likely more vulnerable to environmental stressors than the more robust adults, and stressor induced mortality at this key stage is considered to be a bottleneck for population recruitment (Byrne, 2011). It is important therefore to consider the potential for noise to impact squid embryogenesis if we are to gain insight into how noise impacts cephalopods across their life-cycles, thereby working towards a holistic understanding of noise impacts at the population, community and ecosystemic level.

This study investigated whether acute playbacks of ship motor sound affect the oxygen consumption of embryonic veined squid *Loligo forbesii*. The effects of noise playback exposures on naïve and pre-exposed embryos were also compared to explore whether any tolerance, habituation or sensitisation effects of noise playbacks occurred over repeated exposures.

5.2 – Materials and methods

5.2.1 – Experimental setup and husbandry

Loligo forbesii egg strand clusters were deposited overnight on the ropes of lobster creels in the Berwickshire Marine Reserve (North Sea, Scottish East coast) at a water depth of ~15-35 m. Egg clusters were carefully removed from the ropes and immediately transported to St Abbs Marine Station on 03/08/2019.

Upon arrival at the marine station, squid egg strands were placed in acoustically transparent free-hanging plastic bags (7 L volume) inside two 675 L (1040 x 1000 x 650 mm) holding tanks with continuous flow-through of natural seawater (Figure 5.1). Each tank contained a free-hanging underwater speaker (Clark Synthesis AQ339; effective frequency range 20 – 17,000 Hz, Clark Synthesis Inc., Littleton, CO, USA.) situated at the bottom of the tank facing upwards (Figure 5.1). This study utilised two water bath tanks: One played back ship-noise, whilst the other played back silence (control). Noise from inflowing water was avoided using subsurface inflow pipes avoid and tanks were isolated from any surrounding vibrations with a layer of neoprene rubber situated between the tank bases and the floor. Water parameters, measured daily with a WTW Multi 3430 multiparameter meter (Xylem Analytics, Weilheim, Germany), remained relatively constant throughout the experiment, with salinity at 34-35 ppt, temperature at 14-15 ^{o}C and pH 7.8-8.2. A total of four acoustically transparent bags were placed in each treatment tank, with 15 eggstrands from 2 separate egg clusters at the start of the experiment (t_0) on 03/08/2019. Each bag was filled with UV-sterilized and filtered seawater. Constant aeration was achieved by wooden airstones in the bags. The clear roof of the Aquarium area provided a natural photoperiod with 15 h light and 9 h darkness. Each tank was covered with a shade net to reduce light intensity (maximum 300 lx). Water quality was maintained by daily 50% water change.

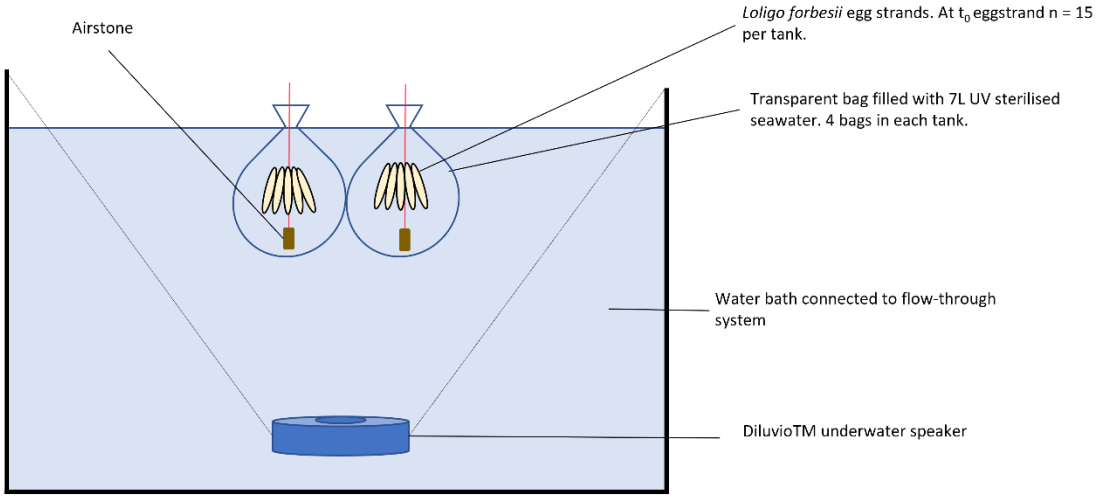


Figure 5.1 Experimental set-up for exposure of *Loligo forbesii* (Steenstrup, 1857) egg strands to ship noise playbacks. The water bath tank had dimensions 1600 x 1030 x 690 mm a was connected to a continuous flow through system. Rearing bags were filled with aerated UV sterilised seawater and had dimensions of 250 mm H x 200 mm W. There were 4 rearing bags in each (noise and control) water bath tank. The Diluvio underwater speaker was free hanging, 100 mm above the tank base and 490 mm below the support tray. Both the rearing bags and the underwater speaker were centrally located on the x-y plane of the tank base. At t = 0, there were 15 egg strands in each sound exposure tank (noise and control).

5.2.2 - Sound recordings and playback design

Sound playback track preparation and subsequent in-tank playback recordings were conducted as in section 3.2.9.

Received sound pressure levels at the position of the exposure peaked at 132-136 dB re 1 μPa^2Hz^{-1} for ship noise playbacks and 104–108 dB re 1 μPa^2Hz^{-1} for control exposures (Figure 5.2A), as measured in PAMGuide (Merchant et al., 2015). Particle acceleration peaked at 159-162 dB re 1 $(\mu ms^{-2})^2$ Hz^{-1} for ship noise playbacks, and

153-157dB re 1 $(\mu ms^{-2})^2$ Hz^{-1} for control exposures (Figure 5.2B), as measured in paPAM (Nedelec et al., 2016).

Squid egg strands in the noise exposure tank were exposed repeatedly to a total of two hours of low frequency ship noise playbacks in every four-hour period (interspersed with silent sound playbacks). The control tank received constant silent sound playbacks. For more details on playback structuring, see the 'Occasional' and 'Ambient' sound treatments utilised in section 3.2.9.

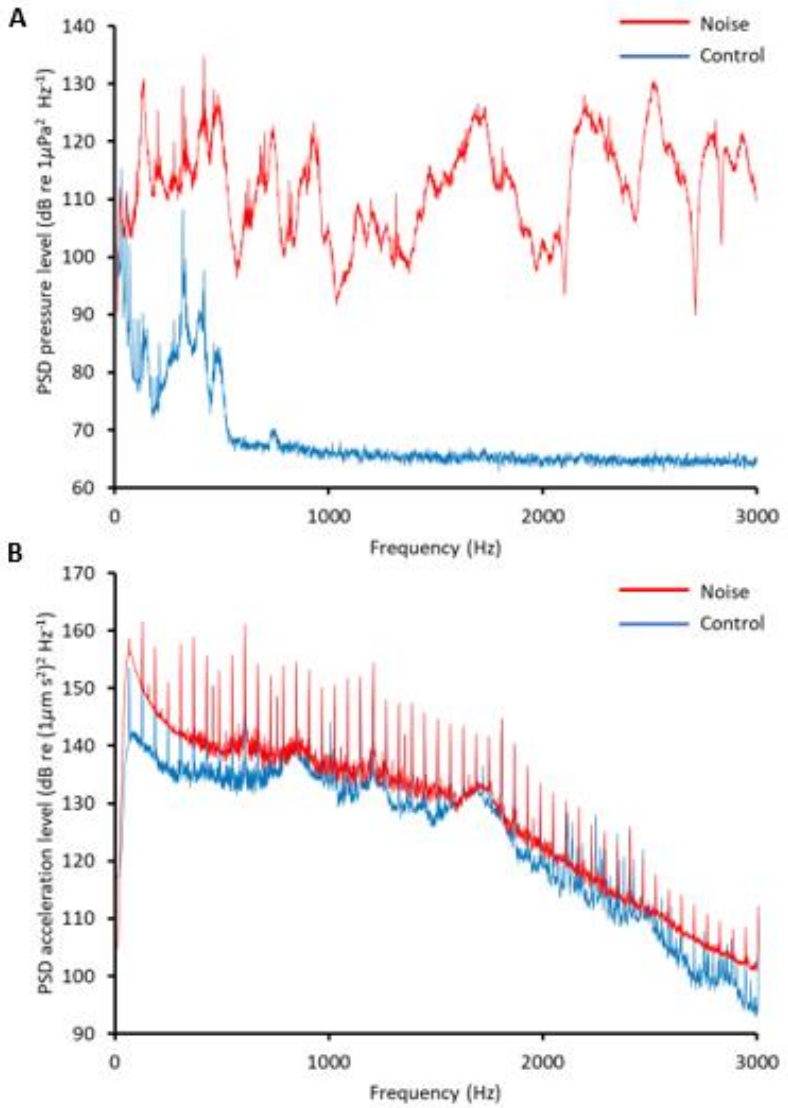


Figure 5.2 Power spectral density plots showing RMS levels of **A)** pressure (dB re: 1 μPa^2 Hz^{-1}) and **B)** particle acceleration (dB re: (1 µm s^{-2})^2 Hz^{-1}) received by developing *Loligo forbesii* (Steenstrup, 1857) egg strands. Recordings were taken inside the plastic bags used to contain the egg strands. DFT length: 48 kHz, recording lengths 30 s, Hann window length: 1 s.

5.2.3 – Respirometry

Respirometry trials were run on 30/08/2019 and 05/09/2019, to test the effects of acute ship noise playback exposure on the oxygen consumption of individually separated stage-27 and stage-29 (respectively) squid eggs. Eggs were staged following the protocol devised by (Arnold, 1965). Briefly, at stage 27 the secondary cornea begins to cover the eye and eyes move freely in the orbit. Early dorsal chromatophores are also evident. At stage 29, pigmentation of ink sac occurs, and the arms are equal in length to the yolk sac. Stage 29 is the stage immediately prior to hatching.

These experiments were conducted on eggs that were 'naïve' with respect to ship noise playbacks (dissected from egg strands in control conditions that had never before received ship noise playbacks). Prior to the respirometry, they were reared in intact egg strands (Figure 5.1). At stage 27 (newly dissected naïve eggs), 12 eggs were subjected to ship noise playback, whilst 12 received silence playback. At stage 29 (newly dissected naïve eggs), 20 eggs were subjected to ship noise playback, whilst 19 received silence playback.

Follow-on respirometry trials were run to test for effects of noise pre-exposure on the oxygen consumption responses of stage 29 eggs subjected to subsequent acute noise exposures. 10 naïve and 9 pre-exposed eggs were subjected to acute ship noise playbacks, 10 naïve and 10 pre-exposed eggs were subjected to acute silence playback.

Egg oxygen consumption was measured using the Unisense microrespirometry system (Unisense, 2021). Eggs were dissected from the egg strands and placed individually in 2 ml glass respirometry chambers filled with UV sterilized seawater (temperature 14 ^{O}C, salinity 35 ppt). The chambers were situated in a larger waterbath tank (dimension 1000 mm x 500 mm x 400 mm).

Each egg rested upon a steel gauze mesh, above a magnetic stirrer to prevent stratification of dissolved O_2 in the chamber. The eggs were acclimatised for 10 minutes, and then subjected to 20 minutes of either silence or ship noise playbacks. O_2 levels in each chamber were measured using a microrespirometry probe (Unisense 2019) at t = 0,10 and 20 minutes. Each experimental run had 3 experimental chambers (containing one egg each) and 1 empty 'blank' chamber, used to correct for any background microbial oxygen consumption.

Sound playback was produced using an AQ Sub Aqua-30 speaker connected to a Pioneer A-10-K amplifier and Lenovo Thinkpad laptop. Ship and ambient sound recordings used were as in section 5.2.2. Received sound pressure levels at the position of the exposure peaked at 118 dB re 1 μPa^2Hz^{-1} for ship noise playbacks and 94 dB re 1 μPa^2Hz^{-1} for control exposures (Figure 5.3A) as measured in PAMGuide (Merchant et al., 2015). Particle acceleration peaked at 99-102 dB re 1 $(\mu ms^{-2})^2$ Hz^{-1} for ship noise playbacks, and 88.5 -90.5 dB re 1 $(\mu ms^{-2})^2$ Hz^{-1} for control exposures (Figure 5.3B), as measured in paPAM (Nedelec et al., 2016).

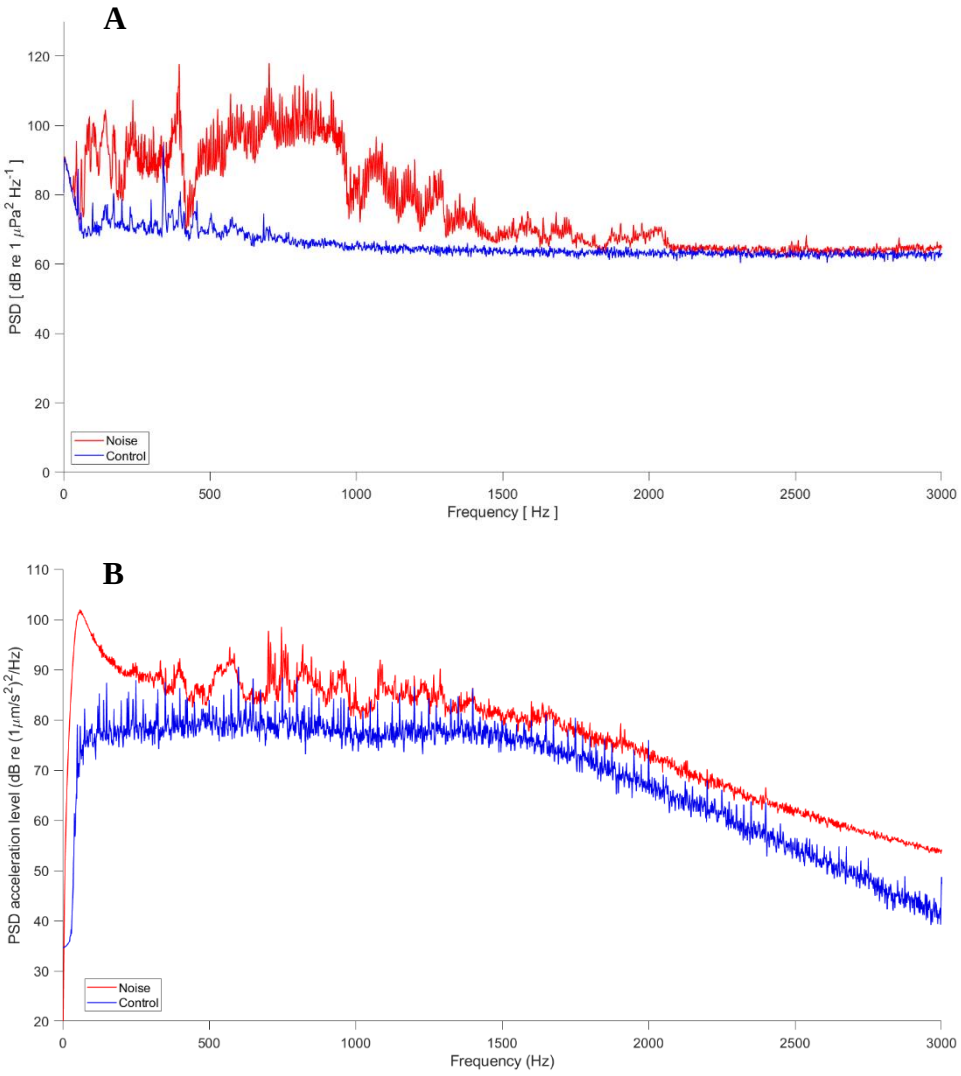


Figure 5.3 Power spectral density plots showing RMS levels of **A)** sound pressure (dB re: 1 µPa^2 Hz^{-1}) and **B)** particle acceleration (dB re: (1 µm s^{-2})^2 Hz^{-1}) received by *Loligo forbesii* (Steenstrup, 1857) eggs in respirometry trials. DFT length: 48 kHz, recording lengths 30 s, Hann window length: 1 s.

5.2.4 - Statistical analyses

Oxygen consumption rate (µMol L^{-1} h^{-1}) for each respiration chamber was calculated using a linear regression. R^2 values were greater than 0.9 for all trials analysed. The consumption rate of blank chambers was subtracted from experimental chambers of the same run to account for background levels of O_2 consumption attributable to microorganisms. Final consumption rate values were expressed per unit of egg wet weight, giving µMol L^{-1} h^{-1} g^{-1}. Following tests for homogeneity of variance and normal distribution, one-way ANOVA tests were used to test for differences in the mean oxygen consumption of ship noise exposed and control eggs. For the follow-on trials testing for the effects of acute sound exposure *and* noise pre-exposure on oxygen consumption, 2-way ANOVAs were conducted. All statistical analyses were carried out in R statistics package version 3.2.4 (R Core Team, 2017)

5.3 - Results

5.3.1 - Naïve eggs

Stage 27 eggs (newly dissected from egg strands) consumed significantly more oxygen when subject to ship noise playbacks (ANOVA, $F_{(1,22)} = 4.393$, $p = 0.048$). Ship noise exposed eggs consumed an average 88.14 ± 10.38 µmol/L/h/g, whilst control eggs consumed 58.29 ± 9.75 µmol/L/h/g (Figure 5.4).

In contrast, there was no significant effect of ship noise playbacks on the oxygen consumption of stage 29 eggs (ANOVA, $F_{(1,37)} = 0.001$, $p = 0.972$). Ship noise exposed eggs consumed an average 123.61 ± 14.32 µmol/L/h/g, whilst control eggs consumed 123.61 ± 10.61 µmol/L/h/g (Figure 5.5).

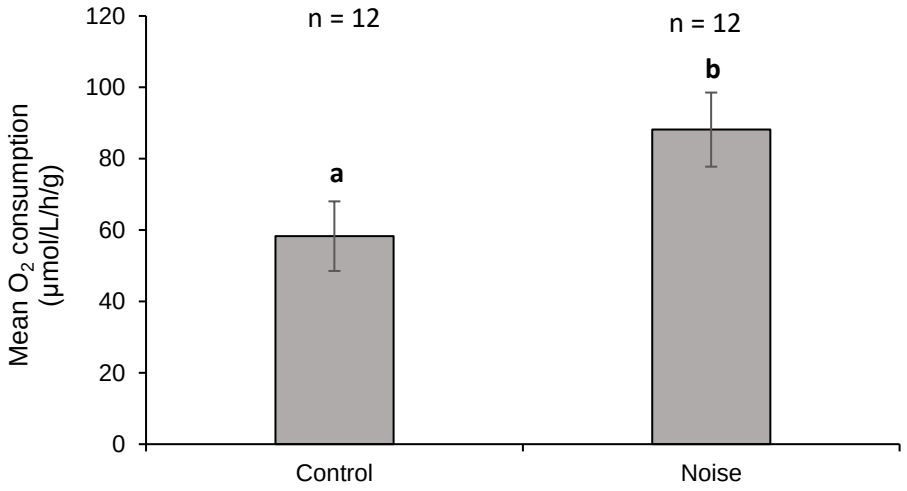


Figure 5.4 Mean (±SE) O_2 consumption (µmol/L/h/g) of naïve stage-27 squid eggs in response to acute ship noise and control playbacks. Equal letters indicate statistically homogenous groups.

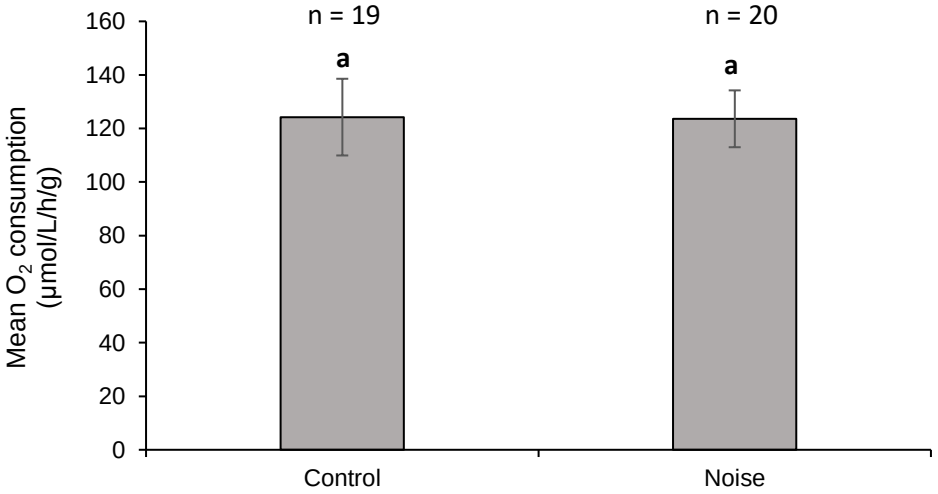


Figure 5.5 Mean (±SE) O_2 consumption (µmol/L/h/g) of naïve stage-29 squid eggs in response to acute ship noise and control playbacks. Equal letters indicate statistically homogenous groups.

5.3.2 - Pre-exposure trial

There was no significant effect of noise pre-exposure (2-way ANOVA, $F_{(1,35)}$ = 0.095, p = 0.76) or acute noise exposure (2-way ANOVA, $F_{(1,35)}$ = 0.0016, p = 0.97) on the oxygen consumption of individually cup-reared stage-29 eggs. There was also no interaction between these two factors (2-way ANOVA, $F_{(1,35)}$ = 0.16, p = 0.69).

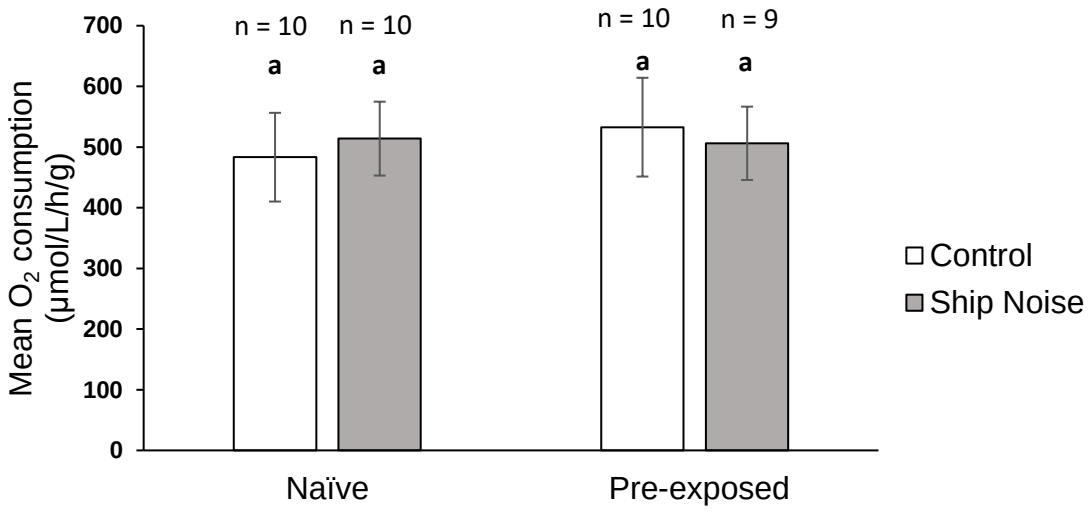


Figure 5.6 Mean (±SE) O_2 consumption (µmol/L/h/g) of naïve and pre-exposed stage-29 squid eggs in response to either ship noise or silence playbacks.

5.4 - Discussion

This study evidences that cephalopod embryos can be negatively affected by noise. Ship noise playbacks increased the oxygen consumption of developing *L. forbesii* embryos in a stage-dependent way.

The increased oxygen consumption of naïve (having never received experimental ship noise playbacks previously) stage-27 *L. forbesii* embryos is indicative of increased metabolism, which can often be a result of, in simple colloquial terms, a 'fight-or-flight' response. This is a neuroendocrine response, whereby in reaction to sensory cues that signify the presence of danger such as a predator (e.g. an approaching shadow or a predator-specific olfactory cue), an animal increases energy consumption in preparation for avoidance. Whilst stage-27 *L. forbesii* are pre-hatching and therefore unable to exhibit behaviours such as moving away from predators, they are morphologically well-developed; loliginid squids already possess the statocysts involved in adult audio perception as early as 15 days after egg laying (Arnold, 1965; Rocha and Guerra, 1999; Villanueva, 2000). The highly-complex environments that these animals inhabit mean that they experience a wide range of stressors and stimuli, with each complex combination of the aforementioned signalling an overall net probability of risks and rewards, or dangers and 'desirables'. This suite of information has driven the evolution of a wide array of sensory systems and diverse responses to endogenous and exogenous (internal and environmental) stimuli and stressors. It may therefore be an oversimplification to attribute the increased oxygen consumption of stage-27 *L. forbesii* in response to ship noise playbacks to a 'fight-or-flight' response at this stage, but the literature does support a link between low frequency noise and anti-predator responses in cephalopods, with adult squid increasing inking and jetting (both stereotypical anti-predator behaviours (Messenger and Hanlon, 2018)) in response to both impulsive and continuous low frequency sound (Fewtrell and Mccauley, 2012; Mooney et al., 2016).

By stage-29, the oxygen consumption of *L. forbesii* was no longer observed to be affected by the ship noise playbacks utilised in this study, regardless of whether they have been exposed to the playbacks throughout their development or not. Whilst the lack of an effect on pre-exposed stage-29 individuals could be attributed to habituation to noise exposure, when taken with the fact that naïve stage-29 individuals are also unaffected by noise playbacks, it is more likely that a playback-independent process is driving the change in noise response from stage-27 to stage-29. Stage-29 is defined as the final stage before hatching, at which point embryos are fully developed and could hatch at any moment (Arnold, 1965). It may be the case that the physiological processes involved in the preparation for a physically challenging and energetically costly hatching process have been activated at stage-29, and any physiological impacts of ship noise playbacks are masked in an already upregulated metabolic environment. Squid hatching is preceded by an increase in mantle contractions and fin movement of the embryo (Lee et al., 2009) and the metabolic rate of *L. vulgaris* more than doubles after hatching for example (Pimentel et al., 2012). This is also supported by the higher O_2 consumption observed in all treatments at stage-29 compared to stage-27 in this study.

This study has shown that squid embryos respond to acute ship noise playbacks with elevated oxygen consumption, but that this effect is stage dependent. Stage-27 embryos are affected, whilst stage-29 individuals are not. Future work could explore oxygen consumption responses of earlier stage embryos. It would be hypothesised that no responses to noise playbacks would be observed until after formation of the statocyst organs and this would give further weight to the growing body of evidence that statocysts are used by cephalopods for audio perception (e.g. (Kaifu et al., 2008; Packard et al., 1990). If squid embryos increase their metabolic rate (which the increased O_2 consumption reported here indicates) in response to environmental noise characteristic of ship noise in the field, then the efficiency of the consumption and

conversion of egg yolk to body growth may be altered. If maintained for a substantial period of embryonic development, this could affect embryonic growth rates and the energy reserves available in yolk sacs to newly hatched squid, a variable which is critical to the survival of squid to reproductive age (Vidal et al., 2006). At the population level, this could lead to changes in recruitment to the adult population. Given that there is now evidence for: 1) acute effects of noise on both adult and embryonic squid, and 2) the ability of noise to damage squid statocysts, a question arises as to whether exposure to low-frequency sounds characteristic of anthropogenic sources such as shipping during the embryonic development of squid such as *L. forbesii* modifies the subsequent behavioural responses to underwater sound in adulthood. If noise induced changes to statocyst functioning, as observed in adults (Solé, Lenoir, Durfort, López-Bejar, Lombarte, Van Der Schaar, et al., 2013), also occur during early development, this may also affect the ability of post-larval squid to move and orient themselves in the water column. Answering these questions will be an important task for future studies that wish to understand how increasingly-noisy environments may impact squid populations over their entire life cycle.

Further response parameters such as embryonic growth, developmental duration and success are reported in the manuscript that this work also forms a part of, but as this additional work was not carried out by EB it is not reported here.

5.5 – References

Arnold, J. M. (1965). Normal Embryonic Stages of the Squid , *Loligo pealii* (Lesueur). *Biological Bulletin*, *128*(1), 24–32.

Arnold, J. M. and Williams-Arnold, L. D. (1980). Development of the Ciliature Pattern on the Embryo of the Squid *Loligo pealei* : A Scanning Electron Microscope Study. *Biological Bulletin*, *159*(1), 102–116.

Budelmann, B. and Bleckmann, H. (1988). A lateral line analogue in cephalopods: water waves generate microphonic potentials in the epidermal head lines of Sepia and Lolliguncula. *Journal of Comparative Physiology A*, *164*, 1–5.

Budelmann, B. U. (1992). Hearing in Nonarthropod Invertebrates. In *The Evolutionary Biology of Hearing* (pp. 141–155). Springer-Verlag.

Byrne, M. (2011). Impact of ocean warming and ocean acidification on marine invertebrate life history stages: Vulnerabilities and potential for persistence in a changing ocean. *Oceanography and Marine Biology: An Annual Review*, *49*, 1–42. https://doi.org/10.1016/j.marenvres.2011.10.00

Fewtrell, J. L. and Mccauley, R. D. (2012). Impact of air gun noise on the behaviour of marine fish and squid. *Marine Pollution Bulletin*, *64*(5), 984–993. https://doi.org/10.1016/j.marpolbul.2012.02.009

Jones, I. T., Peyla, J. F., Clark, H., Song, Z., Stanley, J. A. and Mooney, T. A. (2021). Changes in feeding behavior of longfin squid (*Doryteuthis pealeii*) during laboratory exposure to pile driving noise. *Marine Environmental Research*, *165*, 105250. https://doi.org/10.1016/j.marenvres.2020.105250

Kaifu, K., Akamatsu, T. and Segawa, S. (2008). Underwater sound detection by cephalopod statocyst. *Fisheries Science*, *74*(4), 781–786. https://doi.org/10.1111/j.1444-2906.2008.01589.x

Laptikhovsky, V., Cooke, G., Barrett, C., Lozach, S., MacLeod, E., Oesterwind, D.,

Sheerin, E., Petroni, M., Barnwall, L., Robin, J. P., Allcock, L. and Power, A. M. (2021). Identification of benthic egg masses and spawning grounds in commercial squid in the English Channel and Celtic Sea: *Loligo vulgaris* vs *L. forbesii*. *Fisheries Research*, *241*, 106004. https://doi.org/10.1016/j.fishres.2021.106004

Lee, P. N., Callaerts, P. and de Couet, H. G. (2009). The embryonic development of the hawaiian bobtail squid (*Euprymna scolopes*). *Cold Spring Harbor Protocols*, *4*(11), 1–10. https://doi.org/10.1101/pdb.ip77

Merchant, N. D., Fristrup, K. M., Johnson, M. P., Tyack, P. L., Witt, M. J., Blondel, P. and Parks, S. E. (2015). Measuring acoustic habitats. *Methods in Ecology and Evolution*, *6*(3), 257–265. https://doi.org/10.1111/2041-210X.12330

Messenger, J. B., & Hanlon, R. T. (Eds.). (2018). Defence. In *Cephalopod Behaviour* (2nd ed., pp. 97–147). Cambridge University Press. https://doi.org/10.1017/9780511843600.007

Mooney, T. A., Samson, J. E., Schlunk, A. D. and Zacarias, S. (2016). Loudness-dependent behavioral responses and habituation to sound by the longfin squid (*Doryteuthis pealeii*). *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *202*(7), 489–501. https://doi.org/10.1007/s00359-016-1092-1

Nedelec, S. L., Campbell, J., Radford, A. N., Simpson, S. D., Merchant, N. D. and Fisher, D. (2016). Particle motion: the missing link in underwater acoustic ecology. *Methods in Ecology and Evolution*, *7*(7), 836–842. https://doi.org/10.1111/2041-210X.12544

Oesterwind, D., ter Hofstede, R., Harley, B., Brendelberger, H. and Piatkowski, U. (2010). Biology and meso-scale distribution patterns of North Sea cephalopods. *Fisheries Research*, *106*(2), 141–150. https://doi.org/10.1016/j.fishres.2010.06.003

Packard, A., Karlsen, H. E. and Sand, O. (1990). Low frequency hearing in

cephalopods. *Journal of Comparative Physiology A*, *166*, 501–505.

Pierce, G. J., Boyle, P. R., Hastie, L. C. and Key, L. (1994). The life history of *Loligo forbesi* (Cephalopoda: Loliginidae) in Scottish waters. *Fisheries Research*, *21*(1–2), 17–41. https://doi.org/10.1016/0165-7836(94)90094-9

Pimentel, M. S., Trübenbach, K., Faleiro, F., Boavida-Portugal, J., Repolho, T. and Rosa, R. (2012). Impact of ocean warming on the early ontogeny of cephalopods: A metabolic approach. *Marine Biology*, *159*(9), 2051–2059. https://doi.org/10.1007/s00227-012-1991-9

R Core Team. (2017). *R: A Language and Environment for Statistical Computing*. https://www.r-project.org/

Rocha, F. and Guerra, A. (1999). Age and growth of two sympatric squid *Loligo vulgaris* and *Loligo forbesi*, in Galician waters (north-west Spain). *Journal of the Marine Biological Association of the United Kingdom*, *79*(4), 697–707. https://doi.org/10.1017/S002531549800085X

Rosa, R., Pimentel, M. S., Boavida-Portugal, J., Teixeira, T., Trübenbach, K. and Diniz, M. (2012). Ocean warming enhances malformations, premature hatching, metabolic suppression and oxidative stress in the early life stages of a keystone squid. *PLoS ONE*, *7*(6). https://doi.org/10.1371/journal.pone.0038282

Solé, M., Lenoir, M., Durfort, M., López-Bejar, M., Lombarte, A., van der Schaar, M. and André, M. (2013). Does exposure to noise from human activities compromise sensory information from cephalopod statocysts? *Deep Sea Research Part II: Topical Studies in Oceanography*, *95*, 160–181. https://doi.org/10.1016/j.dsr2.2012.10.006

Solé, M., Lenoir, M., Durfort, M., López-Bejar, M., Lombarte, A., Van Der Schaar, M. and André, M. (2013). Does exposure to noise from human activities compromise sensory information from cephalopod statocysts? *Deep-Sea Research Part II:*

Topical Studies in Oceanography, *95*, 160–181. https://doi.org/10.1016/j.dsr2.2012.10.006

Sundermann, G. (1983). The fine structure of epidermal lines on arms and head of postembryonic *Sepia officinalis* and *Loligo vulgaris* (Mollusca, Cephalopoda). *Cell and Tissue Research*, *232*(3), 669–677. https://doi.org/10.1007/BF00216437

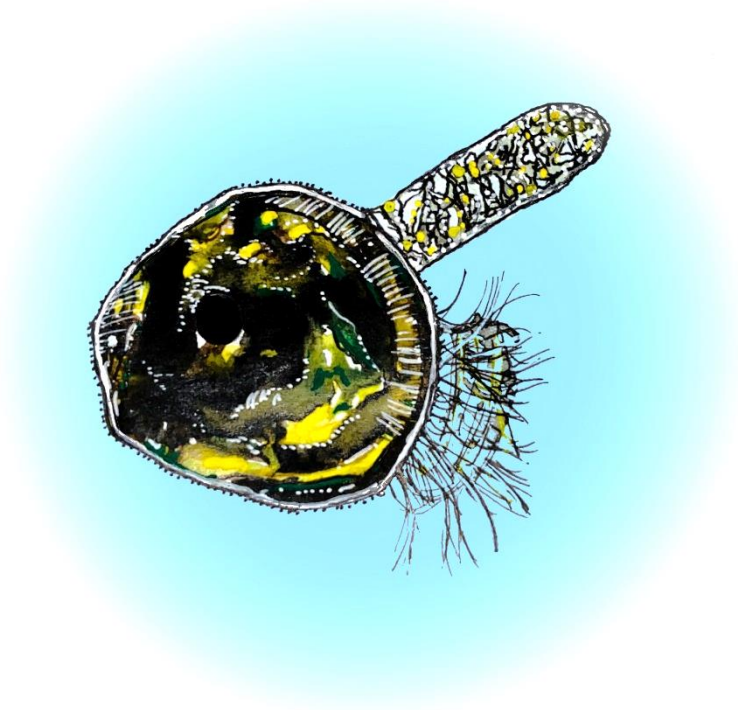
Unisense. (2021). *Unisense MicroRespiration System*. https://www.unisense.com/MicroRespiration_System

Vidal, É. A. G., DiMarco, P. and Lee, P. (2006). Effects of starvation and recovery on the survival, growth and RNA/DNA ratio in loliginid squid paralarvae. *Aquaculture*, *260*(1–4), 94–105. https://doi.org/10.1016/j.aquaculture.2006.05.056

Villanueva, R. (2000). Differential increment-deposition rate in embryonic statoliths of the loliginid squid *Loligo vulgaris*. *Marine Biology*, *137*(1), 161–168. https://doi.org/10.1007/s002270000323

Chapter 6

Effects of anthropogenic noise on the survival and settlement behaviour of European oyster *Ostrea edulis* larvae



E. L. Bolger

Chapter 6

Effects of anthropogenic noise on the survival and settlement behaviour of European oyster *Ostrea edulis* larvae

Abstract:

There is a currently a concerted effort across the North-East Atlantic to restore once-abundant European flat oyster *Ostrea edulis* (L.) reefs to their historical extent and functionality. As eastern oyster (*Crassostrea virginica*) larvae are known to alter their settlement behaviour in response to underwater sound, it was hypothesised that anthropogenic noise may also alter *O. edulis* settlement behaviour. In this laboratory study we presented settlement-competent *O. edulis* pediveligers with two substrate types, namely bio-film stone (desirable) and bare plastic substrate (non-desirable) and two sound playback treatments, namely 'Shipping noise' and 'Ambient'. Shipping noise consisted of a 15 min playback of ship noise at a random timepoint within every 2 hours (thereby emulating a busy shipping lane), whilst Ambient consisted of a constant playback of underwater sound from a harbour with no ships nearby**.** Ship noise playbacks had no impact on larval settlement rate and success, regardless of settlement substrate. However, the larval survival and active swimming behaviour was reduced by ship noise when suitable settlement substrate was absent. These findings have implications for restoration efforts, where larvae from restored reefs may have reduced dispersal potential over noisy shipping lanes with little suitable settlement substrate.

6.1 - Introduction

The European flat oyster *Ostrea edulis* (L.) once formed extensive beds across the North-East Atlantic (Figure 6.1). These reefs covered large parts of the English Channel, many estuarine areas of the British coast and ~20% of the Dutch part of the North Sea floor (over 25,000 km^2) (Olsen, 1883). These once abundant beds contributed to food security, by providing a cheap and readily available source of protein to coastal communities since pre-historic times (Gɒnther, 1897; Pogoda, 2019). During the 19th century, with the invention of steam-powered trawlers, *O. edulis* began to be heavily commercially exploited to meet high demand (Pogoda, 2019), with approximately 700 million oysters consumed in London alone during 1864 (Philpots, 1890). Consistent overexploitation combined with habitat degradation, water pollution and disease led to major declines in *O. edulis* throughout its range and functional extinction in Belgian, German and Dutch waters (Airoldi and Beck, 2007; Gercken and Schmidt, 2014; Pogoda, 2019).

In addition to food provision, these biogenic reefs would have performed a key ecological role. The complex three-dimensional structure of oyster reefs provides habitat, shelter, food, larval settlement substrate and spawning ground for a wide range of associated flora and fauna, with some of commercial importance (Grabowski and Peterson, 2007; Möbius, 1877). This biogenic hard-substrate reefs provided a rich and distinct habitat type in a North Sea otherwise dominated by soft sediment (Christianen et al., 2018) and were thus considered to be biodiversity hotspots (Möbius, 1877) . As filter-feeding bivalves, oysters also capture plankton and particles of organic matter from the water column, thereby increasing localised water clarity and quality and mitigating against algal blooms (Coen et al., 2007). Particles that are not eaten are deposited as pseudofaeces which enriches surrounding sediment and contributes to organic nitrate and organic carbon fixation and removal from the water column (Coen et

al., 2007; Fodrie et al., 2017; Lee et al., 2020). These critical ecosystem functions and services, combined with the potential for flood/erosion defence if coastally located, give oyster reefs an estimated economic value (excluding harvesting for consumption) of $5500–$99,000 per hectare per year (Grabowski et al., 2012).

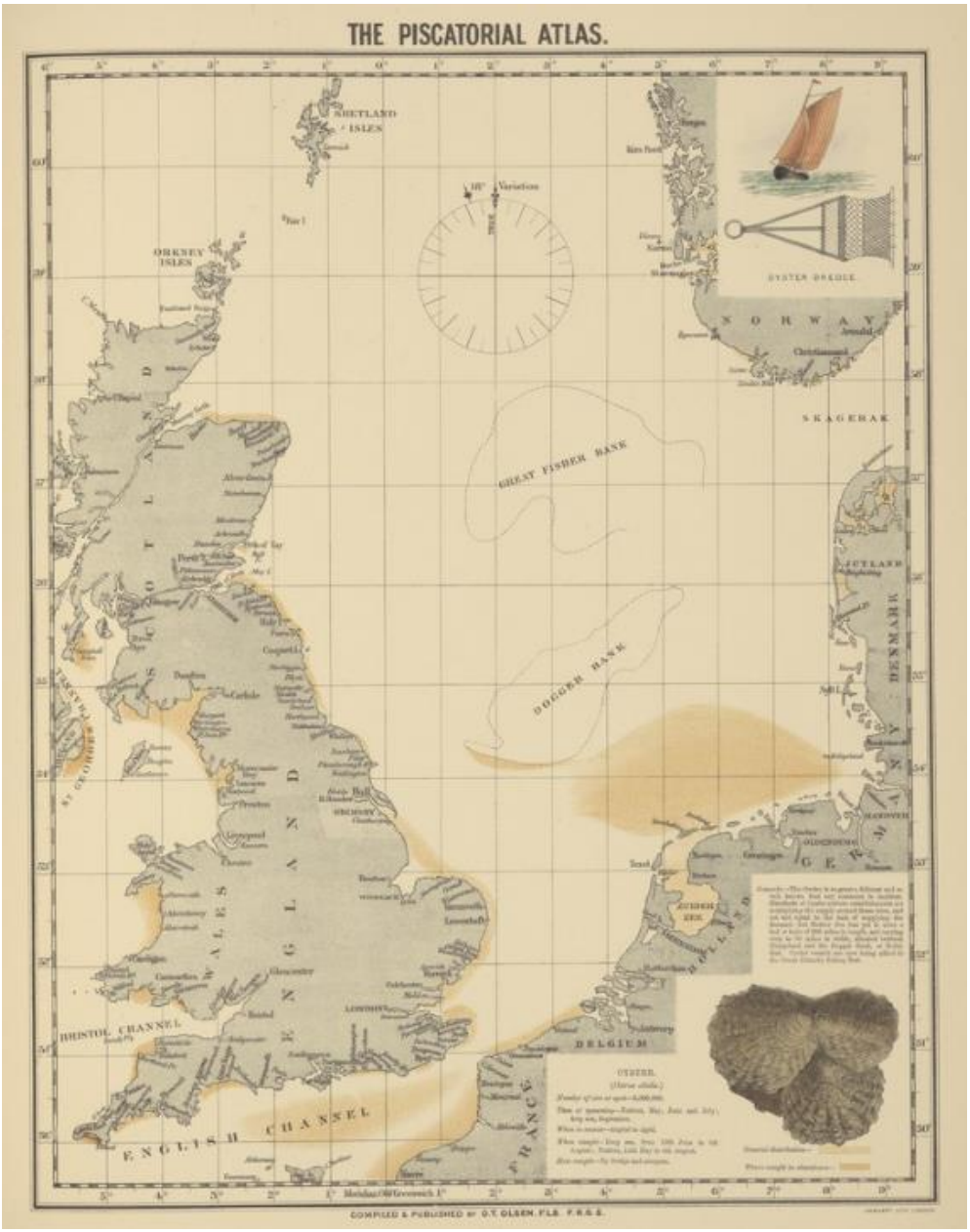


Figure 6.1 Piscatorial Atlas Map showing the spatial distribution of European Flat Oyster *Ostrea edulis* (L.) in the North Sea in 1883 (Olsen, 1883).

Today, the temperate biogenic reefs once formed by *O. edulis* rank among some of the most threatened habitat types globally (Airoldi and Beck, 2007; Gercken and Schmidt, 2014). For this reason, remaining *O. edulis* reefs are now protected under the EU Flora–Fauna–Habitat Directive (FFH Directive, 92/43/EWG) (Council Directive 92/43/EEC of 21 May 1992 on the Conservation of Natural Habitats and of Wild Fauna and Flora, 1992), the Marine Strategy Framework Directive (Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 Establishing a Framework for Community Action in the Field of Marine Environmental Policy (Marine Strategy Framework Directive), 2008) and are listed as a 'Threatened and Declining species' by the OSPAR convention for the Protection of the Marine Environment of the North-East Atlantic (The North-East Atlantic Environment Strategy-Strategy of the OSPAR Commission for the Protection of the Marine Environment of the North-East Atlantic 2010–2020, 2010). To meet their conservation commitments, OSPAR/EU member states have adopted national legislation/policy to not only conserve existing *O. edulis* reefs, but to restore them to their previously occupied ranges (MMO, 2019; Pogoda, 2019; Pogoda et al., 2019; Smaal et al., 2015). These concerted efforts aim to meet conservation objectives, restore pre-existing ecosystem functions and benefit from the associated ecosystem services (Pogoda et al., 2019).

The long-term success of re-introduced *O. edulis* beds is dependent, in part, on the successful recruitment and ongrowing of conspecific larvae. *O. edulis* larvae can prolong their planktonic phase if no suitable settlement sites are present, or locatable (Rodriguez-Perez et al., 2019). An understanding of the factors influencing the 'choice' of settlement location and the subsequent success of settling larvae will allow the identification of suitable sites for restoration and the preparation of these sites to maximize larval settlement. We now know that bivalves can detect and alter their behaviour in response to underwater sound, for example adult Pacific oyster *Crassostrea*

gigas detects sound within the range 10 to <1000 Hz and respond with transient valve closures (Charifi et al., 2017). Coincidentally, 10 to <1000 Hz is the frequency range dominated by shipping noise in most contemporary marine environments (Slabbekoorn et al., 2010). Underwater sound can also influence the settlement rate of Eastern oyster *Crassostrea virginica* larvae (Lillis et al., 2015) and shipping noise playbacks have been shown to cause stress and alter feeding behaviour of the adult blue mussel *Mytilus edulis* (Wale et al., 2019), but there is currently no information on the potential positive, or negative, impacts of underwater sound on *O. edulis* settlement. Whilst sound could guide the larvae and facilitate gregarious settlement, ever-increasing anthropogenic noise, particularly in the busy shipping area of the North Sea, may mask settlement facilitating natural sounds or directly alter larval behaviour.

In this study we presented settlement-competent *O. edulis* pediveligers with two substrate types, namely bio-film stone (desirable) and bare plastic (non-desirable), in combination with two sound playback treatments, namely 'Shipping noise' and 'Ambient'. Shipping noise consisted of a 15 min playback of ship noise at a random timepoint within every 2 hours (thereby emulating a busy shipping lane), whilst Ambient consisted of a constant playback of underwater sound from a harbor with no ships nearby. We hypothesized that ship noise playbacks would act as a stressor, reducing settlement rate and survival and that this effect would be greater in the presence of a non-desirable substrate type. Here we present the results, propose potential avenues for future research and discuss the implications for *O. edulis* restoration efforts in the North-East Atlantic.

6.2 – Material and methods

6.2.1 - Broodstock and larval rearing

Adult oysters were collected from the Danish Limfjord (N 56.832309, E 8.903373) and transported back to the Danish Shellfish Centre (DSC) where they were held in 25 L flow-through broodstock tanks and allowed to spawn naturally. Released larvae were transferred to 15 L flow-through upweller tanks at an initial concentration of ~10 larvae/mL and raised at 20 ^{o}C in 1 µm filtered seawater (Figure 6.2). Both broodstock adults and larvae were fed a microalgae mixture, namely *Chaetoceros muelleri*, *Tisochrisys lutea* and *Pavlova gyrans* at a ratio of 14:3:3 respectively. Microalgal feed was maintained at a constant concentration of ~100 cells/µL using a dosing system set to dispense food solution at 6 mL min^{-1}.

Figure 6.2 15L upwelling larval rearing tank on the flow-through system with continuous feed dosing system (green).

A subsample of approximately 20 larvae were visually inspected daily using a confocal microscope. After approximately 10 days, larvae had reached the pediveliger stage, typified by the presence of a dark pigmented 'eyespot' and foot, indicating settlement-competency and ability to metamorphose to a spat (Figure 6.3). At this point, the oyster larvae were transferred to the experimental system.

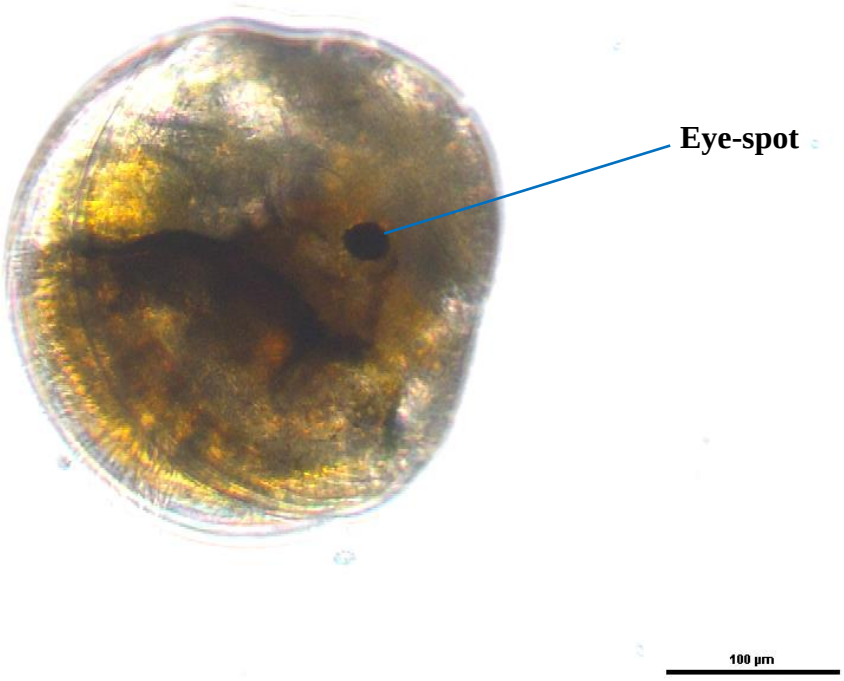


Figure 6.3 *Ostrea edulis* larva at the mature pediveliger stage – indicated by the presence of a dark pigmented 'eye-spot'.

6.2.2 - Experimental design

Pediveliger larvae were transferred to 16 ml wells within 6-well plates (4 larvae per well) and subjected to 4 distinct treatments in a 2-way factorial design, combining 2 sound treatment levels (ship noise or ambient sound playback) and 2 settlement substrate treatment levels (biofilm stone or bare plastic) (Table 6.1). Well plates were semi-submerged in large 70 litre water-bath tanks which acted to maintain temperature at 20 ^{o}C using an IKS temperature control system (https://www.iks-aqua.com/html/engl/aquastar.php) and to provide sound exposure using an upward facing free-floating underwater speaker situated 30 cm below the well plates (Figure 6.6). A total of 4 tanks were used (2 ship noise and 2 ambient playback tanks). Each playback tank contained 4 6-well plates, with 12 of the total wells per tank. Each tank contained filtered seawater with a biofilm stone and 12 wells contained filtered seawater only (bare-plastic settlement substrate) (Figure 6.7). The positioning of each substrate type within the well plates was randomised. Larvae were maintained at 20 ^{o}C under a natural light-dark cycle.

The biofilm stone treatment consisted of filtered seawater and a marine stone of 1–1.25 cm diameter covered by a natural biofilm. The stones were collected from Nykøbing Bugt adjacent to the DSC at a depth of ~1 m. Only stones with a continuous monochromatic light-green covering of biofilm across the entire upper surface were selected. Both *C. gigas* and *O. edulis* are found in the biofilm stone collection area.

6.2.3 - Sound recordings and playback

Using the Ableton Live Suite software and an M-Audio M-Track Quad, 2 separate audio channels were played from a single Lenovo Thinkpad laptop (Playback sample rate: 96 kHz). Each audio track output from the M-Track Quad was routed through a Pioneer A-10(K) Stereo Amplifier (2 x 50 W) amplifier to an AQ Sub-aqua 30 speaker (Frequency Response 100 Hz – 17 kHz). The two sound playback groups were composed of a combination of ambient and ship noise recordings from three major UK ports ((A) Gravesend, (B) Plymouth and (C) Portsmouth), originally recorded by Sophie Nedelec (University of Exeter) and Irene Voellmy (University of Bristol) (Wale et al., 2013a, 2013b). At each location, during a single day, recordings were made of both ambient sound and the sound generated by a single passing ship at approximately 200 m distance. The ships recorded at each location were: (A) Gravesend: Rio de la Plata, a 286 m long, 64730 tonne container ship, (B) Plymouth: Bro Distributor, a 146 m long, 14500 tonne LPG tanker and (C) Portsmouth: Commodore Goodwill, a 126 m long, 5215 tonne ferry. Ship travel speed was relatively low and remained approximately constant for the duration of recordings (<10 knots). For hydrophone and recorder specifications and calibration information, see: (Wale et al., 2013a, 2013b).

The ‘ambient’ sound group received playbacks of the ambient underwater sound recordings made by Wale *et al.* (2013), this group served as the control in this experiment. Ambient sound, rather than silence was chosen in order to ensure that any differences observed in ship noise playback groups compared to the ambient group were solely due to the ship sound component of the playback. As there were 3 recording locations, 3 ambient tracks (2 hrs each) were used in the treatment and cycled between randomly for the duration of the experiment. The ‘ship noise’ group received playbacks of 15 minutes of ship noise (including a 1-minute fade-in and fade-out), interspersed within 1h45 of corresponding ambient sound recording from the same location, repeated

for the duration of the experiment. The positioning of the 15-minute ship noise block within this 2 hour period could occur at 0 mins, 30 mins, 60 mins, 105 mins. These 4 ship/ambient combinations were created for each recording location (A,B and C) giving a total of 12 possible 2-hour sound playback files for the ship noise group; these 12 playback files were cycled between randomly (Figure 6.4). All experimental tracks were compiled in Audacity 2.1.3 (http://audacity.sourceforge.net/) and exported as 24-bit WAV files for playback.

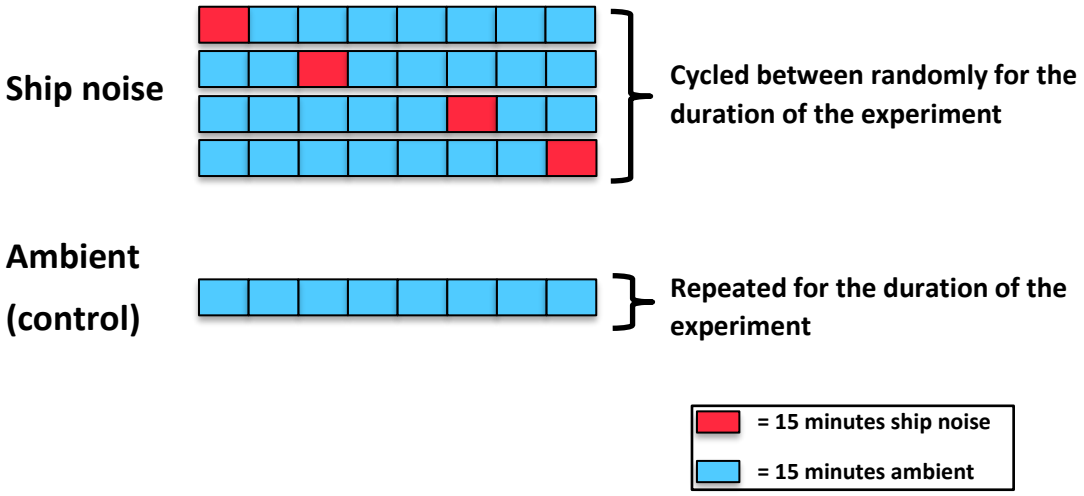


Figure 6.4 The sound playback exposure regime received by *Ostrea edulis* (L.) pediveligers larvae in ship noise and ambient treatment groups. Sound options were cycled between randomly for the duration of the experiment.

The power spectral density (PSD) levels received by the pediveligers were measured in the space taken up by the well plates (30 cm above the AQ Sub-aqua 30 speaker), prior to beginning the experiment. Recordings were made using a HTI-94-SSQ hydrophone (sensitivity of -165 dB re: 1 V/µPa) with a frequency response of 2 Hz to 30 kHz, routed to a calibrated Roland R-26 digital recorder at a sample rate of 96 kHz. Sound recordings were stored as 24-bit WAV files. The recorder was calibrated using a signal generator and oscilloscope. Each recording was 30 s in length.

Power spectral density graphs were produced in Matlab R2017a using the PAMGuide (Merchant et al., 2015) for pressure and the paPAM software package for particle acceleration. PSD plots were produced from 30 second recordings. The Hann window type was used, with window length of 1 second, 50% window overlap and Discrete Fourier Transformation length of 48 kHz. Root mean square (rms) sound levels were used for comparisons. Ambient group sound recordings were taken whilst ship noise playback was occurring in Busy and Occasional tanks confirm there was no sound transference between tanks.

During ship noise playback, there was a pressure peak of 120-124dB re: 1 μPa^2 Hz^{-1} in both tanks at 395 Hz, with sound levels varying according to frequency component, but remaining at approximately 100dB re: 1 μPa^2 Hz^{-1} across the frequency range of 0-3000 Hz (Figure 6.5). Sound levels remained at approximately 63dB re: 1 μPa^2 Hz^{-1} across the 0-3000 Hz frequency range in the ambient sound tanks (Figure 6.5). Particle acceleration peaked at 99dB (1 µm $s^{-2})^2$ Hz^{-1} at 375 Hz and 102.5dB (1 µm $s^{-2})^2$ Hz^{-1} at 2282 Hz in ship noise tanks. Particle acceleration remained relatively constant at 75dB from 0 to 1600 Hz before gradual declining in intensity at higher frequencies (figure 6.5).

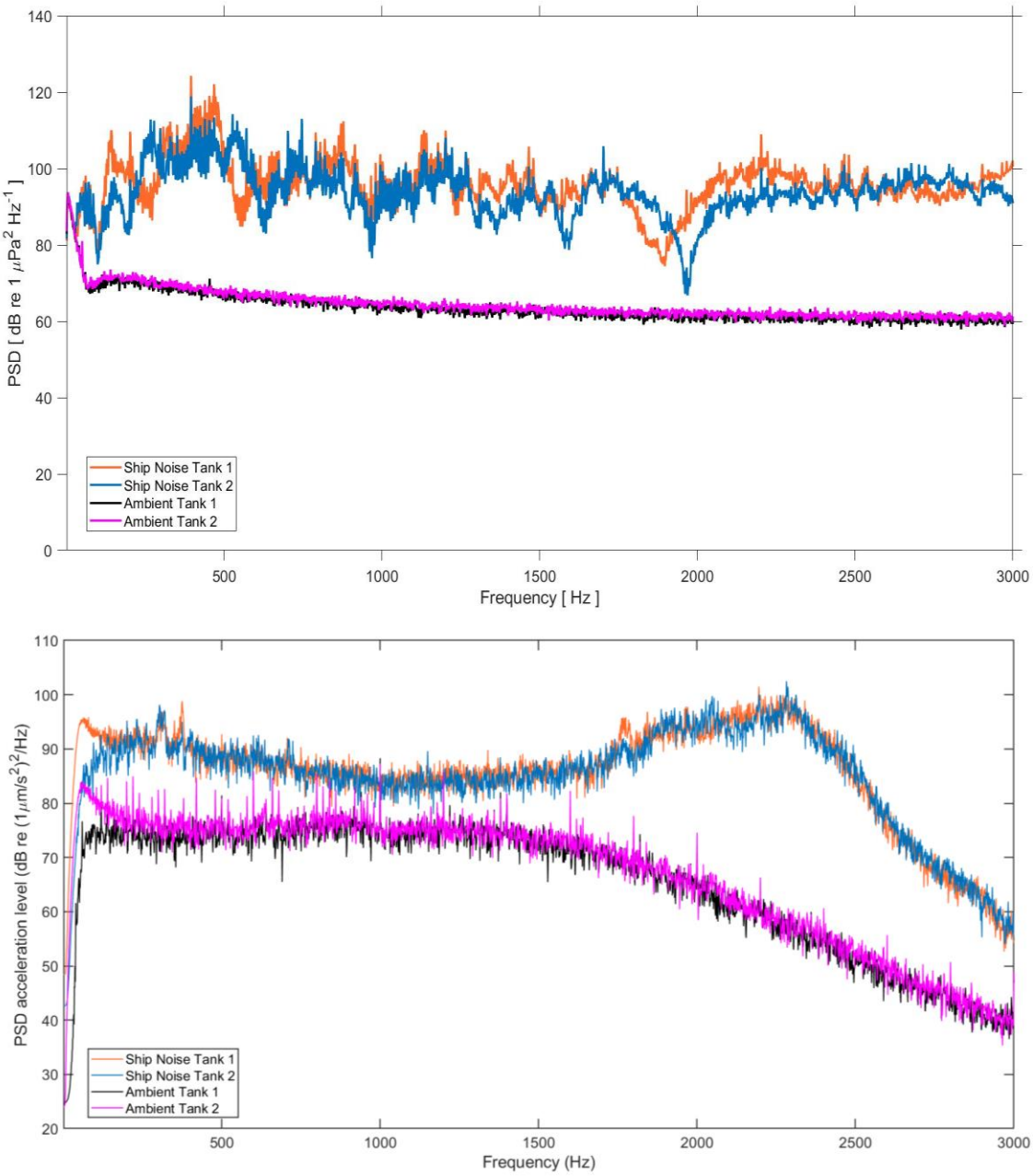


Figure 6.5 Power spectral density plots showing RMS levels of **A)** pressure (dB re: 1 µPa^2 Hz^{-1}) and **B)** particle acceleration (dB re: (1 µm s^{-2})^2 Hz^{-1}) received by *Ostrea edulis* (L.) pediveligers. Recordings were taken at the position of experimental well plates in ship noise and ambient playback tanks (2 tanks per sound treatment). In each treatment, recordings were taken directly above the speaker DFT length: 48 kHz, recording lengths 30 s, Hann window length: 1 s.

6.2.4 - Data collection

As pediveligers matured late-evening, the experiment was set-up and observations began the next morning, 10 h after set-up, at which point no settlement had yet occurred. Thereafter, approximately every 2.5 hours the behaviour and settlement status of experimental pediveligers was visually assessed using a Teslong Digital USB Microscope 10-200X magnification waterproof USB microscope in-situ (https://www.amazon.co.uk/Microscope-Teslong-Multi-function-Magnification-Waterproof/dp/B01K1V23XY), except during sleep breaks where intervals were longer. The observational process was repeated until the end of the experiment at t = 96.75 hours. The settlement status of each larvae was recorded (settled vs. non-settled) and the behaviour of non-settled pediveligers categorised into 'active' (swimming), 'inactive' (stationary) and 'foot searching'. 'Foot searching' is a stereotypical behaviour of mature pediveligers attempting to identify a suitable settlement location and involves the use of an extended foot to drag the pediveliger along the substrate (Rodriguez-Perez et al., 2019). If it was not possible to locate a larva during an observation (e.g. due to its positioning under the substrate), its settlement status and behaviour was recorded as 'unknown'.

Dead larvae could be differentiated from inactive larvae due to a combination of prolonged inactivity and the loss of all green/brown colouration from their bodies, transitioning to a distinctive 'milky' appearance. At the end of the experiment, all biofilm stones were upturned and carefully inspected for any attached settled larvae that may have hitherto been unable to be observed without disturbing the substrate.

All 'unknown' designations were retroactively corrected to either 'settled' or 'non-settled' status once a subsequent observation provided the validation for this correction e.g. an 'unknown' larvae that is subsequently observed to be actively

swimming will be assigned a 'non-settled' status to replace all previous 'unknown' designations. 'Unknown' designations were not able to be corrected for non-settled behaviour however, so were censored from the behavioural analysis for that timepoint.

6.2.5 - Statistical analyses

The time taken for larvae to settle was compared between treatment groups using a non-parametric Kaplan-Meier survival analysis (Kaplan and Meier, 1958). Briefly, the probability of a larvae remaining non-settled at time t_j, $S(t_j)$, is calculated from: (1) $S(t_{j-1})$, the probability of being non-settled at t_{j-1}, (2) n_j, the number of larvae non-settled just before t_j and (3) d_j number of settlement events at t_j, using the following equation:

$$S(t_j) = S(t_{j-1})\left(1 - \frac{d_j}{n_j}\right)$$

where $t_0 = 0$ and $S(0) = 1$. As the Kaplan-Meier estimator calculates the probability of the event of interest *not* happening $S(t_j)$, the estimator was inversed to gain the probability of settlement at time t : 1- $S(t_j)$. Larvae that remained non-settled at the end of the experiment (t = 96.75 hours) were marked as 'censored', as were larvae that died over the course of the experiment, thereby removing their contribution to subsequent probability estimator calculations. Both survival and settlement data was analysed in this way. Survival curves were compared between treatment groups using a log-rank test, testing against the null hypothesis that survival curves are identical in the overall populations. All survival analysis was performed in Graphpad Prism 7.00 (Graphpad Software, 2020).

Differences in behaviour of non-settled pediveligers ('active' (swimming), 'inactive' (stationary) and 'foot searching') were tested using Chi-square contingency tables.

Table. 6.1 Sound exposure and substrate treatments used to study the effects of ship noise playbacks on *Ostrea edulis* (L.) settlement in different settlement substrate suitability contexts.

		Substrate type	Initial number of pediveligers
Sound playback group	Ambient	Biofilm stone	48
		Bare plastic	48
	Ship Noise	Biofilm stone	48
		Bare plastic	48

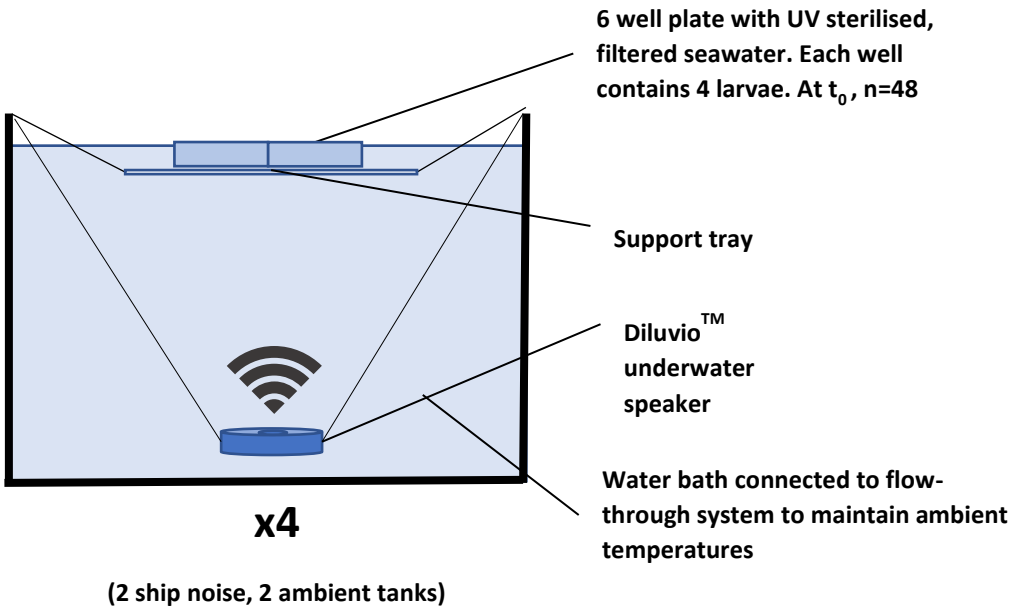


Figure 6.6 Experimental set-up for the rearing and sound exposure of *Ostrea edulis* (L.) pediveligers. External dimensions: 750mm L x 550mm W x 400mm H and volume: 150 litres. Well plate support tray dimensions were 400 x 400mm. The Diluvio underwater speaker centrally located, facing upwards, and free-hanging 50mm above the tank base and 325mm below the support tray. Each tank contained 4 6-well plates located centrally, directly above the centre of the speaker cone. There were 4 tanks in total (2 noise and 2 control).

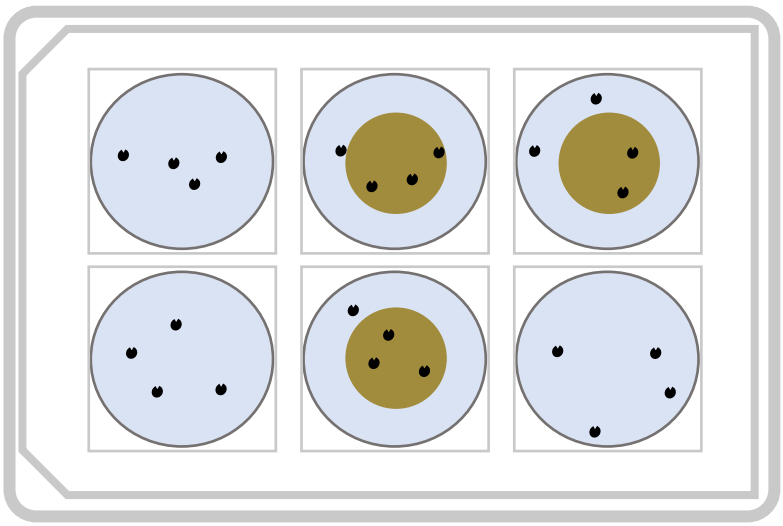


Figure 6.7 Diagram of a 6-well *Ostrea edulis* pediveliger settlement plate with four larvae per well and 2 settlement substrate types. Each plate had 3 wells containing filtered seawater with a biofilm stone and 3 wells with filtered seawater only (bare plastic). Substrate type was assigned to wells at random. Eight plates were assigned to each sound exposure tank, giving a total of Image not to scale.

6.3 – Results

6.3.1 – Larval settlement

There were clear differences in the cumulative number of larvae that settled during the experiment between substrate types (Log-rank test, $\chi2=122.7$, df=3, $p < 0.0001$), with 77-81% of larvae having settled in the biofilm stone wells by the end of the experiment, compared to only 0-0.04% in the filtered seawater (bare-plastic) wells (Figure 6.8). Pediveligers began to settle rapidly in the biofilm treatment after ~40 hours and passed the 50% settlement threshold after 69 h when exposed to ambient sound playback, and after 71 h when exposed to ship noise playbacks. However, the nature of sound playbacks had no significant effect on settlement rate in both bare-plastic and biofilm stone treatments (Log-rank test, $\chi2=1.896$, df=1, $p = 0.17$ and Log-rank test, $\chi2=0.01735$, df=3, $p = 0.90$ respectively) (Figure 6.8).

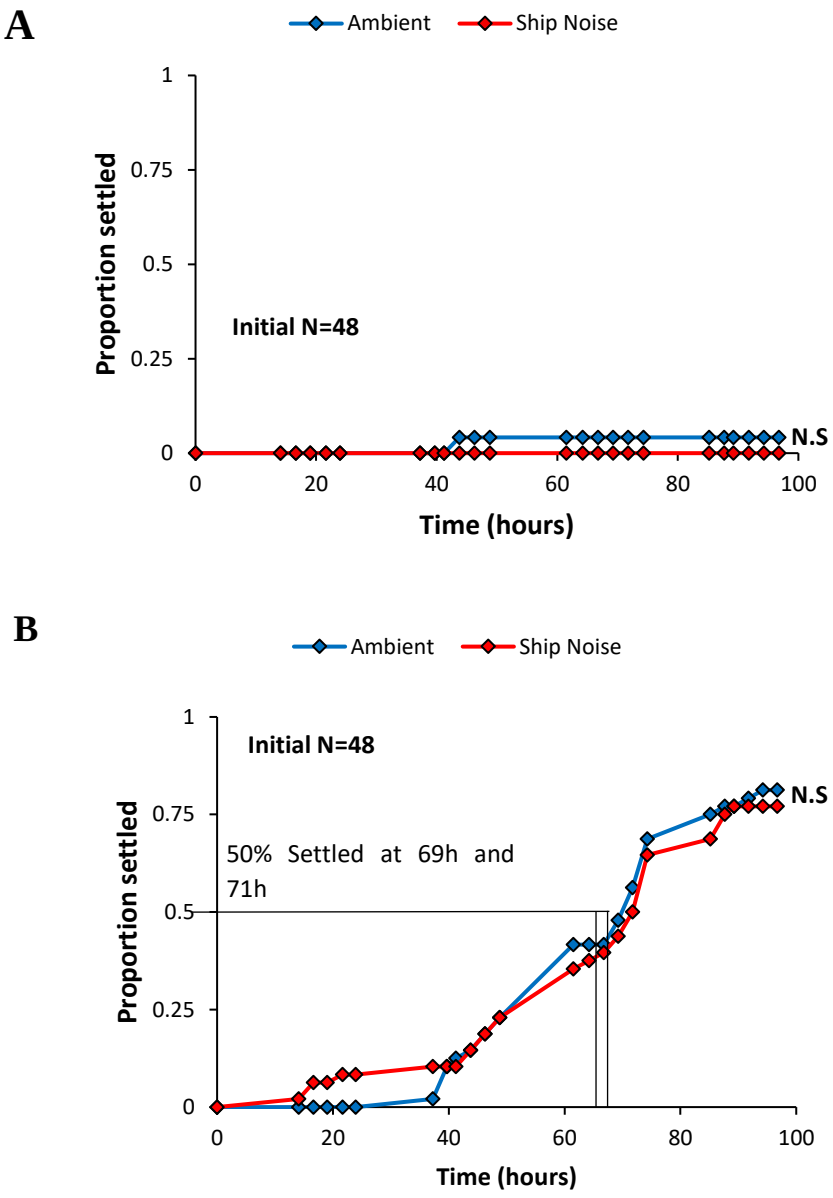


Figure 6.8 Cumulative proportion of *Ostrea edulis* pediveligers that settled over the 97 h experiment in **A)** Bare-plastic and **B)** Biofilm stone treatments. All treatment wells contained filtered seawater. Blue curves received ambient sound playback, red curves were exposed to ship noise playbacks. **N.S** indicates a non-significant logrank test for difference in Kaplan-Meier survival curves.

6.3.2 – Non-settled larval survival

All larvae that settled and metamorphosed remained alive at the end of the experiment. Of the pediveligers that remained non-settled in the biofilm stone treatments, 98% survived when exposed to ambient playbacks, compared to 92% exposed to ship noise playbacks. However, differences between the two respective Kaplan-Meier survival curves were not significant (Log-rank test, $\chi 2=1.949$, df=1, p = 0.16) (Figure 6.9B). In contrast, in the two bare-plastic substrate treatments ship noise playbacks caused a significant reduction in survival probability, compared to ambient playbacks (Log-rank test, $\chi 2=4.322$, df=1, p = 0.038) (Figure 6.9A). A total of 90% of non-settled pediveligers survived for the entire 96.75 hour experimental duration in bare-plastic wells whilst exposed to ambient sound playback, whereas only 73% survived under ship noise playback conditions. The survival of ship noise exposed pediveligers began to decline more rapidly after approximately 60 hours of experimental time (Figure 6.9A).

6.3.3 – Non-settled larval behaviour

In bare plastic wells, non-settled pediveligers spent 36% more time inactive in the ship noise exposure treatment, compared to larvae in the ambient sound playback group in bare-plastic wells ($\chi^2_{(1, 2001)} = 264.5$; $p < 0.001$) (Figure 6.10A). This behavioural difference was mirrored in the presence of biofilm stone substrate, with larvae spending 20% more time inactive when exposed to ship noise playbacks compared to ambient playback ($\chi^2_{(1, 1333)} = 64.82$ $p < 0.001$) (Figure 6.10B). Foot-search behaviour was rarely observed, only in biofilm stone treatments and was unaffected by sound playback ($\chi^2_{(1, 1333)} = 1.17$ $p = 0.28$) (Figure 6.10B).

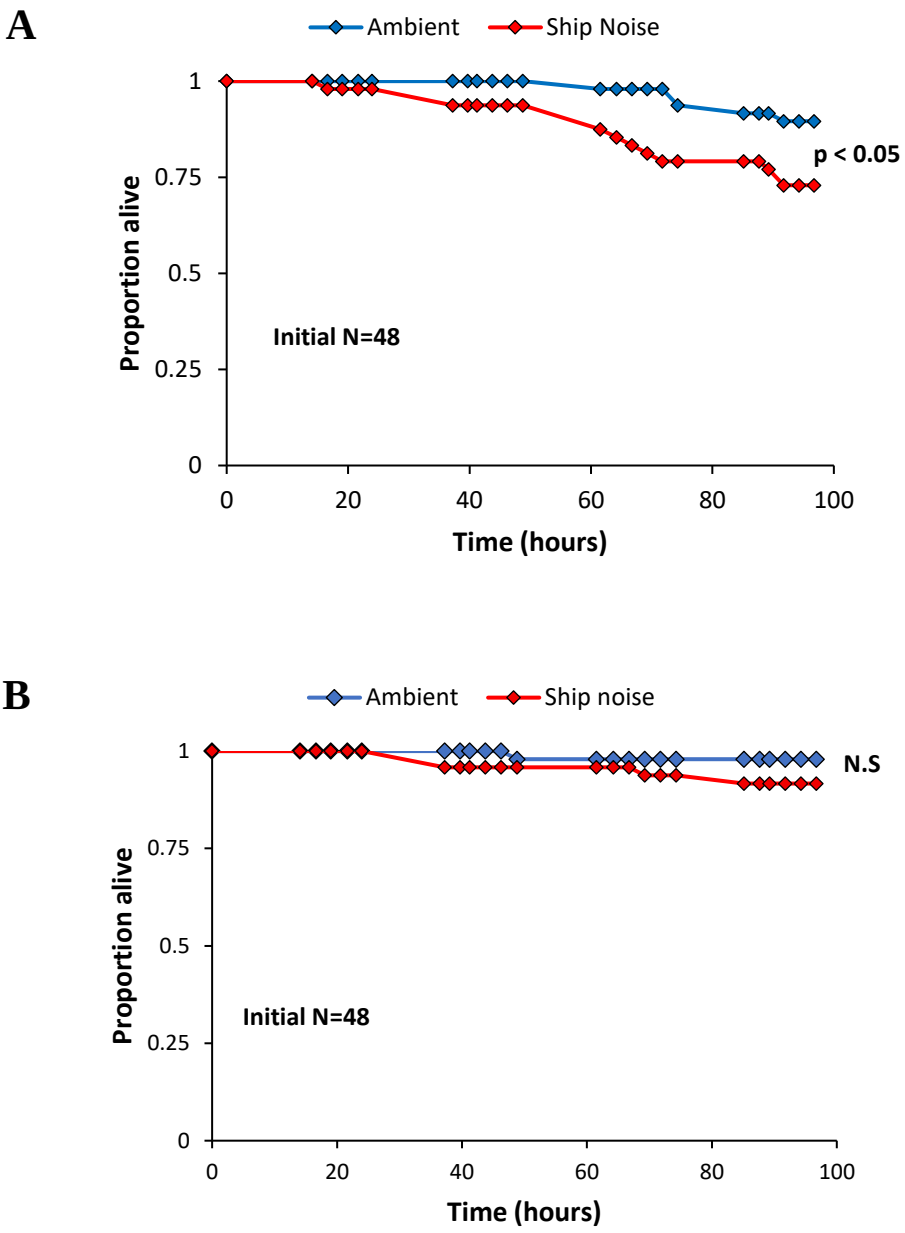


Figure 6.9. Proportion of non-settled *Ostrea edulis* pediveligers that survived over the 97 hour experiment in **A)** Bare-plastic and **B)** Biofilm stone treatments. All treatment wells contained filtered seawater. Blue curves received ambient sound playback, red curves were exposed to ship noise playbacks. **N.S** indicates a non-significant logrank test for difference in Kaplan-Meier survival curves.

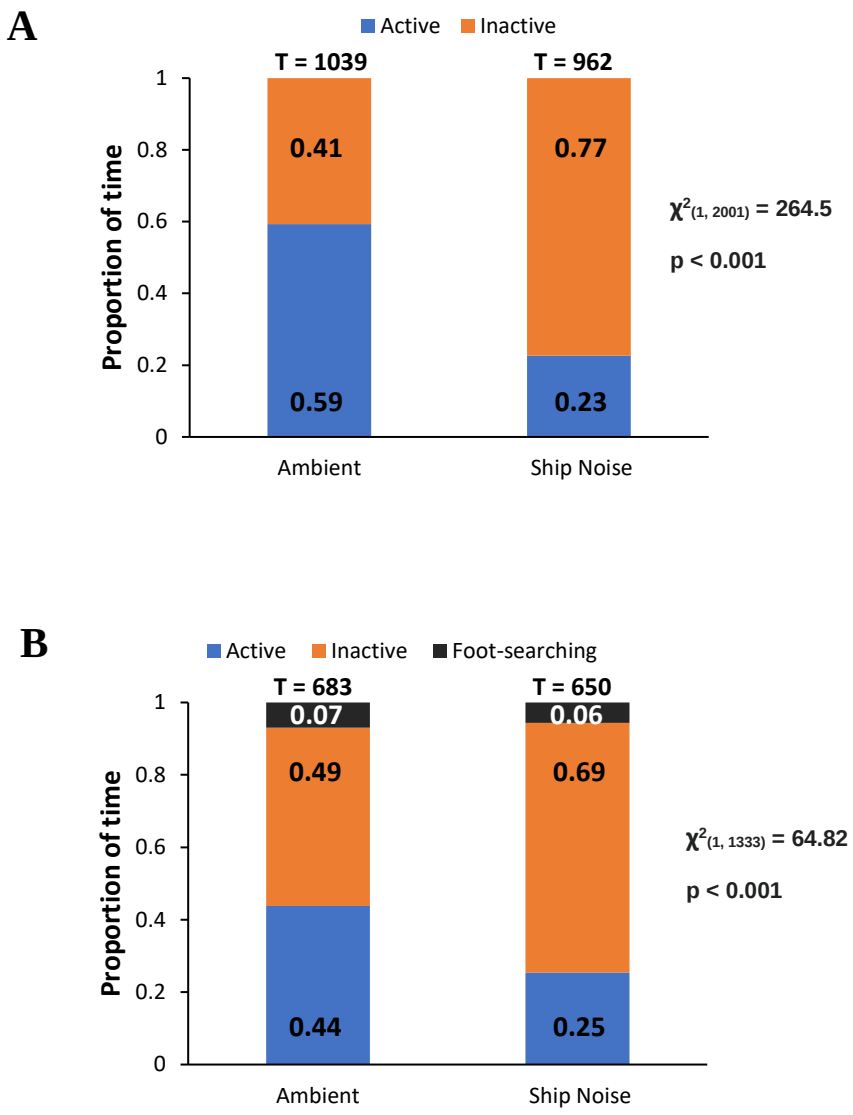


Figure 6.10. Proportion of time that non-settled *Ostrea edulis* pediveligers were observed displaying each of three behavioural categories: Active, inactive and foot-searching in **A)** Bare-plastic and **B)** Biofilm stone substrate treatments. The observed behaviour is displayed for both sound playback treatments: Ambient and Ship Noise. T=total number of behavioural observations in each treatment. Behavioural data was only possible to record for observable non-settled pediveligers. 'Unknown' larvae that were not seen in a given observational timepoint are therefore censored from the analysis. Chi-squared test results for differences in behaviour between sound groups are included.

6.4 - Discussion

The aim of this study was to assess whether additional anthropogenic noise, characteristic of ship noise (the most prevalent marine noise source globally (Andrew et al., 2002; Frisk, 2012)) has the potential to influence *Ostrea edulis* larval settlement, behaviour and survival. The majority of pediveligers successfully settled in response to the presence of relevant biofilm, but not in bare-plastic treatments, as hypothesised. This substrate dependent settlement rate remained unaffected by additional anthropogenic noise playbacks, suggesting that *O. edulis* restoration projects in busy shipping areas should see recruitment unaffected by additional noise, provided they achieve a critical mass of suitable settlement cues, co-locating with a supply of settlement competent pediveligers. However, the effects of ship noise playbacks on pediveliger behaviour and survival in the absence of suitable settlement substrate is a potential concern for the successful transport of larvae through noisy substrate-poor areas and may have impacts on long-distance transport and connectivity between *O. edulis* populations. Many areas of the Northeast Atlantic, such as the English Channel, Norwegian Trench, port surroundings, offshore installations and shipping channels have a median annual broadband (63-4000 Hz) shipping noise pressure levels exceeding 120dB re 1 μPa (Farcas et al., 2020). The oyster larvae in this study were exposed to similar broadband levels, peaking at 120-124dB re 1 μPa, so the sound exposures used are relevant to real-world conditions.

This is the first study to our knowledge to assess the effects of additional anthropogenic noise, characteristic of ship noise, on the settlement behaviour of oyster. There are, however, demonstrated effects of natural oyster reef associated sound on Eastern oyster, *C. virginica* settlement (Lillis et al., 2015). The distinct soundscape associated with *C. virginica* reefs is likely due to associated sound producing fish

species (e.g. Oyster toadfish *Opsanus tau* and croaker spot *Leiostomus xanthurus*) and snapping shrimp, rather than the presence of the oysters themselves ((Lillis et al., 2014)). Due to the scarcity of *O. edulis* across its natural range ((Airoldi and Beck, 2007; Gercken and Schmidt, 2014)), locating an existing reef with a great enough size and density to promote the residence of associated biophonic fauna would be challenging, but if a field recording of an *O. edulis* reef can be obtained and verified to be sonically distinct from surrounding non-reef areas, future research of this sound on settlement behaviour may be warranted.

Biofilm stone was the only substrate to induce significant settlement in this study, with 77-81% of pediveligers settling, compared to 0-0.04% on bare-plastic over a 96.75 hour period. Here our results closely align with work carried out by Rodriguez-Perez *et al.* (2019), who found that 81% of *O. edulis* larvae settled on biofilm stone whilst 0% settled on bare-plastic. We have therefore further shown that habitat-relevant biofilms, taken from areas known to harbour adult oyster (both *C. gigas* and *O. edulis* in this case) are sufficient to produce good settlement. Biofilms are complex, multispecies assemblages that vary in their composition according to age and location (Hadfield, 2011). As *O. edulis* larvae settle gregariously, attractive biofilms are likely to contain markers that either signal, or are characteristic of, the presence of conspecifics (Unabia and Hadfield, 1999). The settlement inducing effect of biofilm on oyster larvae is known to depend on cues from specific bacterial taxa present within the film (Campbell et al., 2011), and therefore the suitability of biofilms as a settlement substrate may vary according to the site and age/stability of biofilms present. The exact components of biofilm that cause a settlement inducing effect for *O. edulis* have not yet been determined (Rodriguez-Perez et al., 2019). There is extensive literature on the settlement inducing effects of biofilm for a wide range of taxa (Bao et al., 2007; Simith et al., 2013, 2017) and a range of bacteria-associated chemicals are known to increase

the settlement of *O. edulis* (Mesías-Gansbiller et al., 2013). We have shown here that additional environmental anthropogenic noise, characteristic of ship noise, is insufficient to alter pediveliger settlement rate in the presence of suitable biofilm and in the absence of natural reef sounds. The lack of effect could be attributed to either of the following causes: (1) *O. edulis* larval settlement behaviour is not meaningfully affected by underwater sound of the type presented in this study or (2) The strength of the settlement induction effect of suitable biofilm masks any noise-induced behavioural changes that may have otherwise impacted settlement. The implication for *O. edulis* restoration projects in the North Sea, is that there is currently no evidence that settlement-competent pediveligers that find themselves in close association with suitable substrate will not attach and metamorphose at rates independent of shipping noise levels in the area. Restoration project managers that can secure important settlement parameters (e.g. hydrodynamic regime, critical mass of suitable settlement substrate (biofilms or conspecifics), minimal fishing disturbance of the seabed (Gercken and Schmidt, 2014)) and a reliable supply of *O. edulis* pediveligers to site, do not currently need to consider any settlement effects of localised levels of boating traffic noise at a prospective restoration site as an additional factor, based on current evidence. A note of caution is that we did not consider natural reef sound in this study, due to the current scarcity of sufficiently dense *O. edulis* beds to sample audio from. Unlike other oyster species (e.g. Lillis et al. (2015)), it is still unknown whether *O. edulis* reefs produce a distinct soundscape and whether this is used as settlement cue for larvae. If this is the case, then it is possible that shipping noise could mask this auditory settlement cue (Clark et al., 2009).

Whilst no effect of ship noise playbacks on settlement rate was observed, there were clear effects of sound playbacks on larval swimming behaviour and survival of the larvae that had not settled by the end of the experiment (in the bare-plastic treatment).

90% of the non-settled pediveligers survived for the 96.75-hour experimental duration in the treatment group with bare-plastic substrate and ambient sound playback, whilst only 73% survived in bare-plastic wells under ship noise playback conditions, and ship noise exposed pediveligers' survival declined more rapidly after approximately 60 hours of experimental time. *O. edulis* larvae can delay metamorphosis in the absence of favourable settlement conditions (Cole and Jones, 1939; Cole and Knight-Jones, 1949; Rodriguez-Perez et al., 2019), exemplified by the fact that the majority of non-settled larvae survived the duration of this experiment. This ability to delay metamorphosis, in favour of encountering more suitable substrate elsewhere, is likely to be adaptive, providing fitness benefits in *O. edulis*' recent evolutionary history (Pawlik, 1992). However, since the distribution range and population density of *O. edulis* has so drastically contracted over recent history (in evolutionary terms) (Airoldi and Beck, 2007; Gercken and Schmidt, 2014; Thurstan et al., 2013), it is not clear in the present-day context whether this behaviour continues to confer fitness benefits. For the settlement-delaying strategy to be more successful than indiscriminate settling, it is necessary that individual survival remains sufficiently high during the delay phase, thereby outweighing the cost of increased mortality during the delay (e.g. through increased exposure time to predators) with the increased fitness prospects of surviving larvae that settle in chemically desirable locations (high densities of conspecifics and associated biofilms). Given that some restoration projects observe large inter-annual variation in recruitment success, despite receiving good pediveliger supply and making efforts to optimise settlement substrate on-site (Korringa, 1946; Low et al., 2007), the significant increase in mortality of ship-noise exposed non-settled larvae after 60 hours has the potential to further shift the balance towards settlement failure in a scenario where larvae are dispersing through busy shipping areas with little suitable settlement substrate.

Our results, if replicable in the field with real-world shipping noise, suggest that busy shipping lanes and the noise they produce in the North Sea may act to limit both: 1) dispersal distance of *O. edulis* pediveliger larvae and 2) settlement success in noisy areas with low densities of adult *O. edulis* or suitable biofilm. It should be noted that this study focused only on pediveliger larvae – as it is the key stage at which 'settlement decisions' are made. Given the impacts of noise playbacks observed at this stage, further work considering the effects of underwater sound on earlier larval stages and post-settlement *O. edulis* is warranted. If noise induced impacts occur earlier in development, this may have further implications for long distance *O. edulis* transport dynamics.

The data generated in this study underline the importance of good connectivity between different *O. edulis* restoration projects. Ideally, the European *O. edulis* restoration effort should be working towards a scenario where a given restored oyster bed is not only able to successfully seed itself with enough spat to maintain and expand the bed, but is also able to export planktonic larvae to adjacent restored populations (with those populations in turn providing larvae to other populations) – thereby securing a continuous network of *O. edulis* across the North Sea and beyond, with good gene-connectivity between sub-populations within the network. Gene-connectivity is a vital component of sessile shellfish restoration (Gaffney, 2006); As previously scarce *O. edulis* beds are restored, likely from hatchery-reared spat, there is a danger of producing oyster beds with high genetic homogeneity, as hatchery techniques tend to reduce allelic richness and heterozygosity (Lallias et al., 2010). This low genetic diversity could result in populations that are more susceptible to present-day and future stressors such as predation, fishing mortality, disease and increasing temperatures. This is of particular concern in a scenario where restoration projects reintroduce oysters to their previous extent using hatchery stocks, forming oyster beds comprising millions of individuals

with comparatively small effective population sizes. In combination with selectively engineering genetic diversity/desirable phenotypic traits into hatchery reared *O. edulis* spat for restoration projects, well thought-out spatial distribution of restoration projects will be needed to promote gene-flow between restored beds in a scenario where high levels of shipping noise and low levels of suitable substrate may limit larval dispersal distances.

Where successful recruitment of *O. edulis* larvae from offsite beds is uncertain due to geographical or environmental factors, establishment of a self-recruiting population is necessary for long term viability of a reintroduced population. Self-recruitment of larvae is an important mechanism for sessile bivalve populations to persist through time (Paris and Cowen, 2004). This mechanism is of particular importance to the gregarious *O. edulis* due to its reproductive life history strategy. *O. edulis* is a protandrous, alternating hermaphrodite, with successive sex changes throughout its life span. The timing of sex changes varies between individuals according to temperature and food availability within a population (Mann, 1979), which can lead to deviation from a 1:1 sex ratio. A male to female ratio as high as 6:1 has been recorded in wild populations (Eagling et al., 2018; Kamphausen et al., 2011). A skewed sex ratio of this magnitude can lead to scarcity of one gamete type acting as a limiting factor for successful fertilisation. Further, whilst functional male *O. edulis* are broadcast spawners, functional females use their inhalant siphon to capture sperm within the mantle cavity where eggs are brooded for up to 15 days prior to release. This viviparous female strategy places greater importance on the close proximity of male *O. edulis* to provide sperm to adjacent females than in oviparous broadcast spawners such as the Pacific oyster *Crassostrea gigas* (Thunberg, 1793) (Helmer et al., 2019). Taken together, these life history characteristics have been proposed to cause *O. edulis* to

suffer from Allee effects, where individual oysters in a bed that is below a critical size and density are likely to experience reduced fitness (through inability to fertilise gametes), leading to reduced recruitment to the bed as a whole (Low et al., 2007). For long-term persistence, an oyster bed must therefore be of a sufficient density to enable successful broadcast fertilisation and be of a sufficient bed size and oyster density to retain released larvae for settlement upon the parental bed. Recent evidence shows that *O. edulis* larvae favour close association with the substrate, as opposed to swimming in the water column or at the surface, at least in laboratory tanks with the absence of water currents (Rodriguez-Perez et al., 2020). This behaviour indicates a larval preference for limited dispersal as the close association with the substrate would increase the chances of entrapment and retention of larvae within the complex 3D structuring of a natal oyster bed, leading to subsequent self-recruitment to the parent population. However, in most prospective reintroduction sites, current velocities are likely to override any demersal preferences of the larvae, with current speeds of >15 cm s^{-1} thought to be sufficient to resuspend and disperse larvae from the seabed (Jonsson et al., 1991).

Our results, showing reduced activity and survival of pediveligers in noisy conditions in the absence of suitable settlement substrate, further emphasize the need for any reintroduced oyster population to achieve the critical size and density required to retain released larvae for self-recruitment. This presents a dilemma for restoration projects where the number of oysters available for reintroduction will likely be limited by cost or supply: A small, dense *O. edulis* bed is likely to achieve good fertilisation rates, but may lose much of its larvae to the prevailing seabed currents. A larger, less dense bed will provide more suitable settlement substrate over a wider area (allowing for greater drift of larvae with currents) but will suffer reduced fertilisation rates in turn.

The key knowledge gap for *O. edulis* restoration efforts in the North Sea is therefore: what size and density of an oyster bed is required to reach the critical mass

required for larval retention and self-recruitment? Estimates for success have ranged from 800,000 to 10,000,000 oysters (Korringa, 1946; Low et al., 2007), but the true value will vary from site to site. A greater understanding of (1) larval dispersal (ideally models incorporating both larval behaviour and hydrodynamics) and (2) how the 3D structuring of a dense oyster bed may reduce current flow at the boundary layer thereby promoting larval retention, will allow restoration projects to assess the long-term feasibility of *O. edulis* reintroduction/restoration. Our results give further urgency to addressing this knowledge gap as it may be more prudent to first focus on promoting larval retention and self-recruitment in restored *O. edulis* beds before working towards inter-population larval exchange networks, e.g. in a North Sea environment, where anthropogenic noise may be acting to alter larval behaviour and survival during dispersal. The results presented here should provide the impetus for further work on different sound sources, noise levels, life stages and follow-on field experiments to further build a picture of how, why and when noise impacts *O. edulis* larvae and to what extent these effects are of concern to international *O. edulis* restoration efforts.

6.5 - References

Airoldi, L. and Beck, M. W. (2007). Loss, status and trends for coastal marine habitats of Europe. *Oceanography and Marine Biology: An Annual Review*, *45*, 345–405. https://doi.org/10.1201/9781420050943.ch7

Andrew, R. K., Howe, B. M., Mercer, J. A. and Dzieciuch, M. A. (2002). Ocean ambient sound: Comparing the 1960s with the 1990s for a receiver off the California coast. *Acoustics Research Letters Online*, *3*(2), 65–70. https://doi.org/10.1121/1.1461915

Bao, W. Y., Satuito, C. G., Yang, J. L. and Kitamura, H. (2007). Larval settlement and metamorphosis of the mussel *Mytilus galloprovincialis* in response to biofilms. *Marine Biology*, *150*(4), 565–574. https://doi.org/10.1007/s00227-006-0383-4

Campbell, A. H., Meritt, D. W., Franklin, R. B., Boone, E. L., Nicely, C. T. and Brown, B. L. (2011). Effects of age and composition of field-produced biofilms on oyster larval setting. *Biofouling*, *27*(3), 255–265. https://doi.org/10.1080/08927014.2011.560384

Charifi, M., Sow, M., Ciret, P., Benomar, S. and Massabuau, J.-C. (2017). The sense of hearing in the Pacific oyster, *Magallana gigas*. *Plos One*, *12*(10), e0185353. https://doi.org/10.1371/journal.pone.0185353

Christianen, M. J. A., Lengkeek, W., Bergsma, J. H., Coolen, J. W. P., Didderen, K., Dorenbosch, M., Driessen, F. M. F., Kamermans, P., Reuchlin-Hugenholtz, E., Sas, H., Smaal, A., van den Wijngaard, K. A. and van der Have, T. M. (2018). Return of the native facilitated by the invasive? Population composition, substrate preferences and epibenthic species richness of a recently discovered shellfish reef with native European flat oysters (*Ostrea edulis*) in the North Sea. *Marine Biology Research*, *14*(6), 590–597. https://doi.org/10.1080/17451000.2018.1498520

Clark, C. W., Ellison, W. T., Southall, B. L., Hatch, L., Van Parijs, S. M., Frankel, A. and Ponirakis, D. (2009). Acoustic masking in marine ecosystems: Intuitions, analysis, and implication. *Marine Ecology Progress Series*, *395*, 201–222. https://doi.org/10.3354/meps08402

Coen, L. D., Brumbaugh, R. D., Bushek, D., Grizzle, R., Luckenbach, M. W., Posey, M. H., Powers, S. P. and Tolley, S. G. (2007). Ecosystem services related to oyster restoration. *Marine Ecology Progress Series*, *341*, 303–307. https://doi.org/10.3354/meps341303

Cole, H. A. and Jones, E. W. K. (1939). Some observations and experiments on the setting behaviour of larvae of *Ostrea edulis*. *ICES Journal of Marine Science*, *14*(1), 86–105. https://doi.org/10.1093/icesjms/14.1.86

Cole, H. A. and Knight-Jones, E. W. (1949). The setting behavior of larvae of the European oyster, *Ostrea edulis* L. and its influence on methods of cultivation and spat collection. *Fish. Invest., Lond. Ser. II*, *17*, 1–39. http://ci.nii.ac.jp/naid/10016123291/en/

Council Directive 92/43/EEC of 21 May 1992 on the Conservation of Natural Habitats and of wild Fauna and Flora, 7 (1992).

Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 Establishing a Framework for Community Action in the Field of Marine Environmental Policy (Marine Strategy Framework Directive), 19 (2008).

Eagling, L. E., Ashton, E. C., Jensen, A. C., Sigwart, J. D., Murray, D. and Roberts, D. (2018). Spatial and temporal differences in gonad development, sex ratios and reproductive output, influence the sustainability of exploited populations of the European oyster, *Ostrea edulis*. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *28*(2), 270–281. https://doi.org/10.1002/aqc.2855

Farcas, A., Powell, C. F., Brookes, K. L. and Merchant, N. D. (2020). Validated shipping noise maps of the Northeast Atlantic. *Science of the Total Environment*, *735*, 139509. https://doi.org/10.1016/j.scitotenv.2020.139509

Fodrie, F. J., Rodriguez, A. B., Gittman, R. K., Grabowski, J. H., Lindquist, N. L., Peterson, C. H., Piehler, M. F. and Ridge, J. T. (2017). Oyster reefs as carbon sources and sinks. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1859). https://doi.org/10.1098/rspb.2017.0891

Frisk, G. V. (2012). Noiseonomics: The relationship between ambient noise levels in the sea and global economic trends. *Scientific Reports*, *2*(1), 2–5. https://doi.org/10.1038/srep00437

Gaffney, P. M. (2006). The role of genetics in shellfish restoration. *Aquatic Living Resources*, *19*(3), 277–282. https://doi.org/10.1051/alr:2006028

Gɒnther, R. T. (1897). The Oyster Culture of the Ancient Romans. *Journal of the Marine Biological Association of the United Kingdom*, *4*(4), 360–365. https://doi.org/10.1017/S0025315400005488

Gercken, J. and Schmidt, A. (2014). Current Status of the European Oyster (*Ostrea edulis*) and Possibilities for Restoration in the German North Sea. *Report for the Federal Agency for Nature Conservation*, 1–96.

Grabowski, J. H., Brumbaugh, R. D., Conrad, R. F., Keeler, A. G., Opaluch, J. J., Peterson, C. H., Piehler, M. F., Powers, S. P. and Smyth, A. R. (2012). Economic Valuation of Ecosystem Services Provided by Oyster Reefs. *BioScience*, *62*(10), 900–909. https://doi.org/10.1525/bio.2012.62.10.10

Grabowski, J. H. and Peterson, C. H. (2007). *Restoring oyster reefs to recover ecosystem services in Cuddington, K., Byers, JE, Wilson, WG, and Hastings, A.,*

editors. Ecosystem Engineers: Plants to Protists. Academic Press, Burlington, MA, USA.

Graphpad Software. (2020). *GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com*.

Hadfield, M. G. (2011). Biofilms and Marine Invertebrate Larvae: What Bacteria Produce That Larvae Use to Choose Settlement Sites. *Annual Review of Marine Science*, *3*(1), 453–470. https://doi.org/10.1146/annurev-marine-120709-142753

Helmer, L., Farrell, P., Hendy, I., Harding, S., Robertson, M. and Preston, J. (2019). Active management is required to turn the tide for depleted *Ostrea edulis* stocks from the effects of overfishing, disease and invasive species. *PeerJ*, *2019*(2), 1–26. https://doi.org/10.7717/peerj.6431

Jonsson, P. R., Andre, C. and Lindegarth, M. (1991). Swimming behaviour of marine bivalve larvae in a flume boundary- layer flow: evidence for near-bottom confinement. *Marine Ecology Progress Series*, *79*(1–2), 67–76. https://doi.org/10.3354/meps079067

Kamphausen, L., Jensen, A. and Hawkins, L. (2011). Unusually high proportion of males in a collapsing population of commercially fished oysters (*Ostrea edulis*) in the Solent, United Kingdom. *Journal of Shellfish Research*, *30*(2), 217–222. https://doi.org/10.2983/035.030.0204

Kaplan, E. L. and Meier, P. (1958). Nonparametric estimation from incomplete samples. *J. of the ASA*, *73*(282), 457–481. https://web.stanford.edu/~lutian/coursepdf/KMpaper.pdf

Korringa, P. (1946). The decline of natural oyster beds. *Basteria*, *10*, 36–41.

Lallias, D., Boudry, P., Lapègue, S., King, J. W. and Beaumont, A. R. (2010). Strategies

for the retention of high genetic variability in European flat oyster (*Ostrea edulis*) restoration programmes. *Conservation Genetics*, *11*(5), 1899–1910. https://doi.org/10.1007/s10592-010-0081-0

Lee, H. Z. L., Davies, I. M., Baxter, J. M., Diele, K. and Sanderson, W. G. (2020). Missing the full story: First estimates of carbon deposition rates for the European flat oyster, *Ostrea edulis*. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *30*(11), 2076–2086. https://doi.org/10.1002/aqc.3402

Lillis, A., Bohnenstiehl, D. R. and Eggleston, D. B. (2015). Soundscape manipulation enhances larval recruitment of a reef-building mollusk. *PeerJ*, *3*, e999. https://doi.org/10.7717/peerj.999

Lillis, A., Eggleston, D. B. and Bohnenstiehl, D. R. (2014). Estuarine soundscapes: Distinct acoustic characteristics of oyster reefs compared to soft-bottom habitats. *Marine Ecology Progress Series*, *505*, 1–17. https://doi.org/10.3354/meps10805

Low, P. J., Moore, P. G., Smith, I. P. and Hannah, F. (2007). Conservation status of the native oyster (*Ostrea edulis*) in Scotland. *Scottish Natural Heritage Commissioned Report No. 251 (ROAME No. F02AA408)*. http://ethos.bl.uk/OrderDetails.do?uin=uk.bl.ethos.436359

Mann, R. (1979). Some Biochemical and Physiological Aspects of Growth and Gametogenesis in *Crassostrea gigas* and *Ostrea edulis* Grown at Sustained Elevated Temperatures. *Journal of the Marine Biological Association of the United Kingdom*, *59*(1), 95–110. https://doi.org/10.1017/S0025315400046208

Merchant, N. D., Fristrup, K. M., Johnson, M. P., Tyack, P. L., Witt, M. J., Blondel, P. and Parks, S. E. (2015). Measuring acoustic habitats. *Methods in Ecology and Evolution*, *6*(3), 257–265. https://doi.org/10.1111/2041-210X.12330

Mesías-Gansbiller, C., Silva, A., Maneiro, V., Pazos, A., Sánchez, J. L. and Pérez-Parallé, M. L. (2013). Effects of chemical cues on larval settlement of the flat oyster (*Ostrea edulis* L.): A hatchery approach. *Aquaculture*, *376–379*, 85–89. https://doi.org/10.1016/j.aquaculture.2012.11.022

MMO. (2019). *Identifying sites suitable for marine habitat restoration or creation. A report produced for the Marine Management Organisation by ABPmer and AER, MMO Project No: 1135*. 93.

Möbius, K. A. (1877). *Die Auster und die Austernwirthschaft*. Verlag von Wiegandt, Hemple & Parey.

Olsen, O. T. (1883). *The Piscatoral Atlas of the North Sea, English and St. Georges Channels* (Vol. 44). O. T. Olsen.

The North-East Atlantic Environment Strategy-Strategy of the OSPAR Commission for the Protection of the Marine Environment of the North-East Atlantic 2010–2020, 1 (2010).

Paris, C. B. and Cowen, R. K. (2004). Direct evidence of a biophysical retention mechanism for coral reef fish larvae. *Limnology and Oceanography*, *49*(6), 1964–1979. https://doi.org/10.4319/lo.2004.49.6.1964

Pawlik, J. R. (1992). Chemical ecology of the settlement of benthic marine invertebrates. *Oceanography and Marine Biology: An Annual Review*, *30*, 273–335.

Philpots, J. R. (1890). *Oysters and all about them*. London: John Richardson & Co.

Pogoda, B. (2019). Current Status of European Oyster Decline and Restoration in Germany. *Humanities*, *8*(1), 9. https://doi.org/10.3390/h8010009

Pogoda, B., Brown, J., Hancock, B., Preston, J., Pouvreau, S., Kamermans, P.,

Sanderson, W. and Nordheim, H. Von. (2019). *Living Resources The Native Oyster Restoration Alliance (NORA) and the Berlin Oyster Recommendation : bringing back a key ecosystem engineer by developing and supporting best practice in Europe*.

Rodriguez-Perez, A., James, M., Donnan, D. W., Henry, T. B., Møller, L. F. and Sanderson, W. G. (2019). Conservation and restoration of a keystone species: Understanding the settlement preferences of the European oyster (*Ostrea edulis*). *Marine Pollution Bulletin*, *138*, 312–321. https://doi.org/10.1016/j.marpolbul.2018.11.032

Rodriguez-Perez, A., Sanderson, W. G., Møller, L. F., Henry, T. B. and James, M. (2020). Return to sender: The influence of larval behaviour on the distribution and settlement of the European oyster *Ostrea edulis*. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *30*(11), 2116–2132. https://doi.org/10.1002/aqc.3429

Simith, D. de J. de B., Abrunhosa, F. A. and Diele, K. (2013). Chemical induction in mangrove crab megalopae, *Ucides cordatus* (Ucididae): Do young recruits emit metamorphosis-triggering odours as do conspecific adults? *Estuarine, Coastal and Shelf Science*, *131*, 264–270. https://doi.org/10.1016/j.ecss.2013.07.015

Simith, D. de J. de B., Abrunhosa, F. A. and Diele, K. (2017). Metamorphosis of the edible mangrove crab *Ucides cordatus* (Ucididae) in response to benthic microbial biofilms. *Journal of Experimental Marine Biology and Ecology*, *492*, 132–140. https://doi.org/10.1016/j.jembe.2017.01.022

Slabbekoorn, H., Bouton, N., van Opzeeland, I., Coers, A., ten Cate, C. and Popper, A. N. (2010). A noisy spring: The impact of globally rising underwater sound levels on fish. *Trends in Ecology and Evolution*, *25*(7), 419–427. https://doi.org/10.1016/j.tree.2010.04.005

Smaal, A. C., Kamermans, P., van der Have, T. M., Engelsma, M. and Sas, H. J. W. (2015). Feasibility of Flat Oyster (*Ostrea edulis* L.) restoration in the Dutch part of the North Sea. *IMARES Wageningen UR*, *February*, 1–58.

Thurstan, R. H., Hawkins, J. P., Raby, L. and Roberts, C. M. (2013). Oyster (*Ostrea edulis*) extirpation and ecosystem transformation in the Firth of Forth, Scotland. *Journal for Nature Conservation*, *21*(5), 253–261. https://doi.org/10.1016/j.jnc.2013.01.004

Unabia, C. R. C. and Hadfield, M. G. (1999). Role of bacteria in larval settlement and metamorphosis of the polychaete *Hydroides elegans*. *Marine Biology*, *133*(1), 55–64. https://doi.org/10.1007/s002270050442

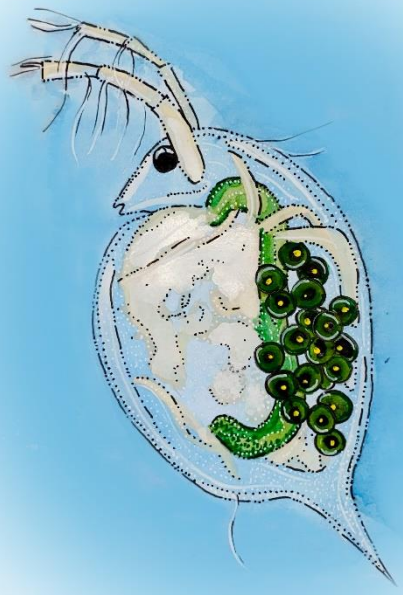
Wale, M. A., Briers, R. A., Hartl, M. G. J., Bryson, D. and Diele, K. (2019). From DNA to ecological performance: Effects of anthropogenic noise on a reef-building mussel. *Science of The Total Environment*, *689*, 126–132. https://doi.org/10.1016/j.scitotenv.2019.06.380

Wale, M. A., Simpson, S. D. and Radford, A. N. (2013a). Size-dependent physiological responses of shore crabs to single and repeated playback of ship noise. *Biology Letters*, *9*(2), 20121194. https://doi.org/10.1098/rsbl.2012.1194

Wale, M. A., Simpson, S. D. and Radford, A. N. (2013b). Noise negatively affects foraging and antipredator behaviour in shore crabs. *Animal Behaviour*, *86*(1), 111–118. https://doi.org/10.1016/j.anbehav.2013.05.001

Chapter 7

Multi-generational effects of anthropogenic noise on the water flea *Daphnia magna*



E. L. Bolger

Chapter 7

Multi-generational effects of anthropogenic noise on the water flea *Daphnia magna*

Abstract:

There is growing evidence that acute anthropogenic noise can negatively impact aquatic invertebrates. However, whether the exposure of parents to noise affects the responses of offspring, and how this is modified over generations is an important consideration when thinking about how noisy environments may impact populations and ecosystems in the long term. This laboratory study utilised underwater speakers to expose the water flea *Daphnia magna* (L.) to playbacks of boat noise or ambient lake sound (control) over a period of four generations and key life history parameters (time-till-maturity, reproductive output, survival, and time-till-starvation) were compared between sound groups. Boat noise playbacks caused significant developmental delays and reduced reproductive output compared to ambient sound controls, but only after *two* generations of repeated exposure. This highlights the importance of long-term studies of this type, as an acute noise study observing a single generation would likely have concluded that boat noise impacts do not meaningfully impact *D. magna.* Fourth generation individuals reared in control conditions after three generations of boat noise exposure retained a reduced reproductive output, demonstrating that noise impacts can be transmitted from parent to offspring and persist for at least one generation. These findings suggest that anthropogenic noise impacts on aquatic invertebrates require consideration at a multigenerational time scale. *D. magna* has been demonstrated to be a good candidate for future epigenetic noise impact studies. Further investigation of the mechanisms underlying these impacts, and their transferral to offspring is needed.

7 - Introduction

7.1 - Daphnia magna

Daphnia or the 'water flea' is a freshwater planktonic crustacean genus belonging to the order Cladocera. The body of *Daphnia* species (spp.) is enclosed by a chitinous carapace which has a fold down the midline, giving a bivalved appearance. *Daphnia* spp. have 7 pairs of appendages which are, from anterior to posterior: Antennules (sensory antennae); antennae (Large second antennae used for locomotion), maxillae; mandibles, 5 thoracic appendage pairs (Figure 7.1). The thoracic appendages form an apparatus for feeding and respiration, whilst the large branching antennae are used for swimming.

Daphnia spp. are found in almost all types of standing water, from lakes to ponds, on every continent. They are primary consumers of phytoplankton, although as generalists they can opt to feed on benthic detritus, giving an advantage over obligate pelagic filter feeders where drivers of planktonic community structure are variable. *Daphnia* spp. grazing has a greater impact on phytoplankton abundance than any other zooplankton in lake systems (Lampert et al., 1986; Sarnelle, 1993) and is the primary driver of seasonal increases in lake water transparency, known as 'clear-water' phases, resulting from shifts in the producer-consumer dynamics (Hairston et al., 2005; Sarnelle, 2005). *Daphnia* spp. are also a key food source for secondary consumers, their high P content making them highly nutritious (Elser et al., 2000) and a preferred prey source. (Drenner et al., 1978; Hambright and Hall, 1992). The combined roles of greatest primary consumer, nutrient cycler and source of food to secondary consumers give *Daphnia* spp. a uniquely important ecological linkage role in many lake ecosystems and they have been described as 'keystone species' for this reason (Ives et al., 1999). *Daphnia* spp. possess a range of phenotypic adaptations ranging from morphological to physiological to behavioural that modify the strength the of interaction

in each of their ecological roles (e.g consumer vs prey species) and these phenotypic traits show both developmental plasticity and heritability ((Cousyn et al., 2001)).

Daphnia magna (Straus, 1820) is the largest herbivorous cladoceran, with adults reaching 4- 5 mm total length. It is widely distributed across the Northern Hemisphere. Under favourable conditions, it reproduces via cyclical parthenogenesis; females produce a clutch of diploid clonal female eggs after every moult if food availability permits. These eggs hatch in the brood chamber, located dorsally beneath the carapace (Figure 7.1). The hatched embryos develop internally for ~3 days (at 20 ^{o}C) before being released by the mother as juvenile daphniids. Male offspring are only produced in response to low temperature, short daylength, crowding and low food density (Kleiven et al., 1992). Increasing numbers of male neonates coincides with the production of haploid ephippial 'resting eggs' which require fertilisation before being shed on the subsequent moult and deposited on the sediment. Thus, the sex ratio and reproductive mode (asexual to sexual) in *D. magna* is environmentally determined.

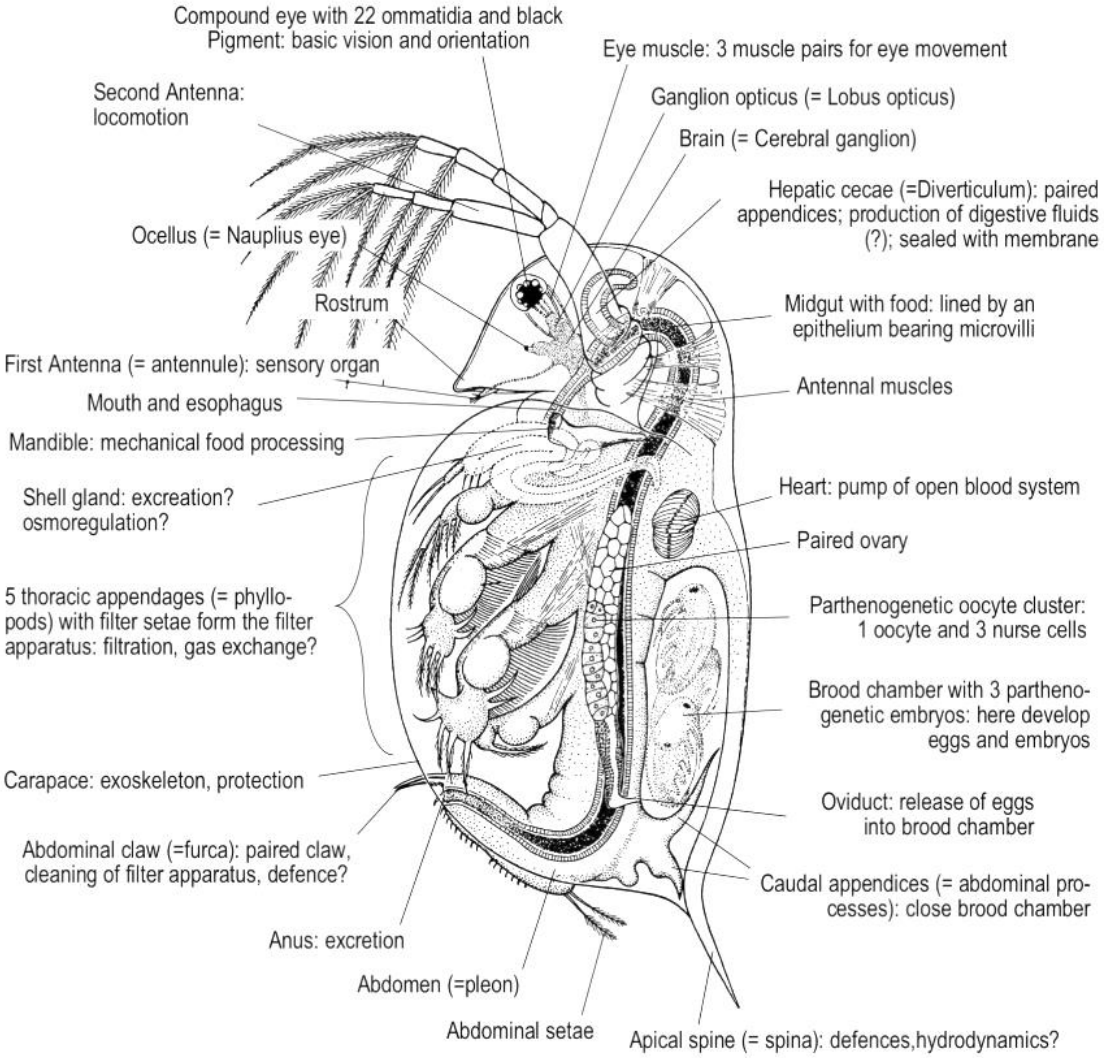


Figure 7.1 Morphology of an adult female *Daphnia magna* (L.). Image taken from Ebert (2005).

7.2 - Daphnia magna as a model organism

D. magna has a relatively short lifespan (7-8 weeks at 20 ^{O}C) and typically reaches maturity in 5-10 days (at 20 ^{O}C), allowing for entire lifespan analyses of stressor effects to be carried out under controlled laboratory conditions. Due to its size (adults at ~5 mm being small enough to be reared in small volumes yet large enough to observe with the naked eye), ease of culture and short life span, *D. magna* is extensively used as a freshwater invertebrate model in toxicology studies (Tkaczyk et al., 2021). *D. magna* also has well documented phenotypic plasticity in response to predators and environmental variables. Kairomones (water-borne chemicals released by predators) induce the formation of morphological features such as 'helmets' (cranial extensions of the exoskeleton) and 'neckteeth' (small protrusions on the neck region accompanied by a strengthened carapace) in many developing *Daphnia* species, which are known to reduce predation rates (Tollrian et al., 2015). Chemical toxicants have also been shown to reduce growth and fertility via epigenetic mechanisms (Vandegehuchte et al., 2010). This phenotypic plasticity has led to proposals for the use of *D. magna* as a model for the study of environmental impacts on epigenetic developmental programmes (Harris et al., 2012).

Perhaps the most useful and unique trait of *D. magna* for use as a model organism to study environmental stressors such as underwater noise, is the ability to utilise its parthenogenic mode of reproduction in laboratory cultures, enabling the production and long-term maintenance of clonal lines to provide genetically identical test subjects for the stressor study of interest. This allows for the removal of genetics as a confounding factor; any differences in response variables between treatments can be attributed to either: 1) direct impact of the noise treatment on the test subjects and/or 2) resultant epigenetic factors of noise exposure (if early development or multiple generations are included within the observation period of the study). The ability of

individual females reared in isolation to reproduce parthenogenetically also allows for reproductive timing, fecundity, and death schedules to be measured accurately and precisely. Birth and death rates can then be calculated in the absence of density dependence to model how stressor impacts may manifest themselves at the population level in the field.

The two most widely used ecotoxicology tests on *D. magna* are the Organisation for Economic Co-operation and Development's (OECD) 'Test No. 202: *Daphnia* sp., Acute Immobilisation Test' and 'Test No. 211: *Daphnia magna* Reproduction Test' (OECD, 2004, 2008). The standard OECD Reproduction Test is a 21-day life cycle test, to investigate the effects of chemicals on the developmental timing and reproductive output over a single life cycle (< 24 hour female neonate to 21-day old female adult) (OECD, 2008). Offspring produced during this single-generation test can be collected and reintroduced to the same experimental conditions as the parent test subjects, which enables an investigation of whether, and how, chemical impacts change over multiple generations. This has allowed researchers to demonstrate how stressors that are seemingly 'low-harm' when inflicted on a single generation can increase the severity of their impacts over subsequent generations (Alonzo et al., 2008; Brennan et al., 2006), with some impacts retained in downstream generations even when the stressor is removed (Heyland et al., 2020). Based on the successful utilisation of *D. magna* as a model for multi-generational chemical impacts on a freshwater planktonic crustacean, it can be argued that it is also a strong candidate model organism for experiments assessing: (1) chronic impacts of anthropogenic noise on multiple generations of planktonic invertebrates; (2) carry-over effects of anthropogenic noise exposure (e.g. mother transferring noise-induced impacts to offspring). Due to their wide distribution across the Northern Hemisphere, there will undoubtedly be populations of *D. magna*

that are frequently exposed to anthropogenic noise e.g. narrowboats in canals or recreation/fishing vessels in large lakes.

This study assesses the impacts of anthropogenic noise playbacks on the survival, time-to-maturity, reproductive output and offspring survival of *D. magna*, extending the observational time to encompass four generations.

7.2 - Methods

7.2.1 - Daphnia magna and algal cultures

A starter culture of *D. magna* was purchased on 31/08/2020 and housed in the 'Aqualab' at Edinburgh Napier University. The Aqualab is an insulated temperature and light-controlled room, with soundproofing to isolate the room from the noise from other laboratories. The *D. magna* culture vessels were separated from the below shelving surface by anti-vibrational matting to minimise the transfer of vibration from any surrounding activities.

To ensure that all experimental animals were genetically identical across multiple generations, it was necessary to establish a clonal culture from the starter culture. This was achieved by isolating a single adult female from the starter culture on 15/09/2021 which then contributed to the new clonal culture via cyclical parthogenesis. After contributing offspring to the clonal culture, the original female was viewed under a microscope to ensure the species was *D. magna*, as opposed to similar species such as *D. pulex.* The presence of a deeply embayed dorsal margin of the postabdomen, ridges on either side of the top head shield mid-line and a lateral rib almost extending to posterior of carapace were used as defining characteristics (Benzie, 2005).

D. magna cultures were reared in synthetic freshwater at a temperature of 20 ^{o}C (± 0.1 ^{o}C) with a 16:8 h light cycle (1100 Lux at water surface during light periods). The synthetic freshwater was made up following the United States Environmental

Protection Agency protocol for 'Hard Synthetic Freshwater' (United States Enviromental Protection Agency, 2002), as recommended in OECD (2008) (Table 7.1; Table 7.2), as synthetic water mixes with hardness over 140 mg $CaCO_3$ equivalent have been shown to produce good reproductive performance. Synthetic water was aerated for a minimum of 24 hours in the temperature control room before use, to ensure chemical additives had fully dissolved and to allow temperature to reach 20 ^{o}C.

D. magna cultures were fed three times a week (Mon, Weds, Fri) with a suspension of *Chlorella vulgaris* (Beijerinck 1890) at a rate of 0.15 mg C/Daphnia/day (C = Organic carbon). *C. vulgaris* was cultured in Jaworski's medium (Appendix B) at 20 ^{o}C (± 0.1 ^{o}C) with an 18:6 h light cycle. Prior to each feeding session, the carbon content of the cultured *C. vulgaris* was determined by measuring absorbance at 685 nm using a Hach D2800 bench spectrophotometer and was then diluted to the feeding concentration of 0.15 mg C/Daphnia/day.

Table 7.1 Preparation of synthetic hard freshwater by adding reagent grade chemicals to deionised water.

Compound	Reagent Added (mg/L)[1]
$NaHCO_3$	192.0
$CaSO_4 \cdot 2H_2O$	120.0
$MgSO_4$	120.0
KCl	8.0

[1]Reagent grade chemicals added to deionized water

Table 7.2 Final water quality of prepared synthetic hard freshwater.

Water Quality Parameters[1]	
pH	8.2
Hardness	170[2]
Alkalinity	115[2]

[1]Measured after 24 h of aeration
[2]Expressed as mg $CaCO_3$ equivalent

7.2.2 - Sound Recordings and Playback

Two experimental sound exposure groups were compared: ferry noise and control. The audio used for these sound playback groups was compiled from sound recordings taken from Lake Windemere, UK (54° 22′ N, 2° 56′ W; altitude 39 m) in November 2014 by Bolgan et al. (2016). Acoustic recordings of the arrivals and departures of a passenger ferry were collected from the Bowness Pier (54°21.746' N, 002°55.415' W). Recordings of ambient sound levels in the same location with no ferries nearby were also taken. 10 minute recordings were taken as .wav files using a calibrated omni-directional hydrophone Aquarian H2a (sensitivity -180 dB re 1 V/Pa; frequency response 10 Hz-100 KHz) connected to a ZoomH1 recorder (sampling rate 44.1 kHz, 16-bit). The hydrophone was attached to a 1.5 m metal pole, which was anchored to the lake bottom with a concrete base. The hydrophone was tied to the approximate midpoint of the metal pole, giving an elevation from the lake bottom of 0.70 m. For further details of the field recording methods used, see Bolgan et al. (2016).

To produce the 'ferry noise' experimental sound track, audio of a single ferry arrival and departure from the Bowness Pier was replicated to match the schedule of said ferry (Bolgan et al., 2016), with the sections between this period filled with ambient sound taken from the Bowness Pier recording. For the first and last ferry of the day the track was cut so that it only contained the ferry leaving, or returning, respectively. This gave an audio track, with 4 ferry passages per hour from the hours of 06:00 to 20:00. The time between each vessel passage consisted of looped ambient sound from the same location. For the remaining hours of the 24 hour cycle where no vessel passages occur, a 5 minute clip of ambient sound from Bowness Harbour was looped. To produce the 'control' audio track, this same 5 minute clip of ambient sound from Bowness Harbour was looped continuously.

Using Ableton Live Suite software and an M-Audio M-Track Quad, the two experimental audio channels (ferry noise and control) were played from a single Lenovo Thinkpad laptop (Playback sample rate: 96 kHz). Each audio track output from the M-Track Quad was routed through a Pioneer A10-K Stereo Amplifier (2 x 50 W, frequency response: 20-20,000 Hz) amplifier to a DNH Sub Aqua 30 (30 W at 8 Ohms, Frequency response: 100 Hz - 20 kHz). (https://www.dnh.no/uploads/filer/365-AQUA30.pdf).

Experimental *Daphnia* were spatially positioned in sound exposure tanks as described in section 7.2.3. The received sound pressure levels at the position of each *Daphnia* were measured (HiTech HTI-94-SSQ with inbuilt preamplifier, Roland R-26 24-bit recorder, calibrated according to (Merchant et al., 2015) and subsequently altered (through changes in amplification) to give desired exposure pressures. Particle acceleration was measured using a custom-built, calibrated sensor developed by the Aquatic Noise Research Group at Edinburgh Napier University (Wale, 2017). This accelerometer was chosen due to it's small size, allowing recordings to be taken in small tanks and rearing vessels. The accelerometer output was recorded with a Roland R-26 24-bit recorder. Particle acceleration was recorded separately for all three axes and combined for a single analysis of total acceleration (scalar).

Power spectral density graphs were produced in Matlab R201 for sound pressure using PAMGuide (Merchant et al., 2015) and for particle acceleration using paPAM. The Hann window type was used, with window length of 1 second, 50% window overlap and Discrete Fourier Transformation length of 48 kHz (Figure 7.2). Root mean square (RMS) sound pressure levels were used for comparisons of overall sound levels received at each rearing vessel location and this information was used to select a grouping of 14 rearing vessels with similar sound exposures to be used for experiments (Figure 7.3).

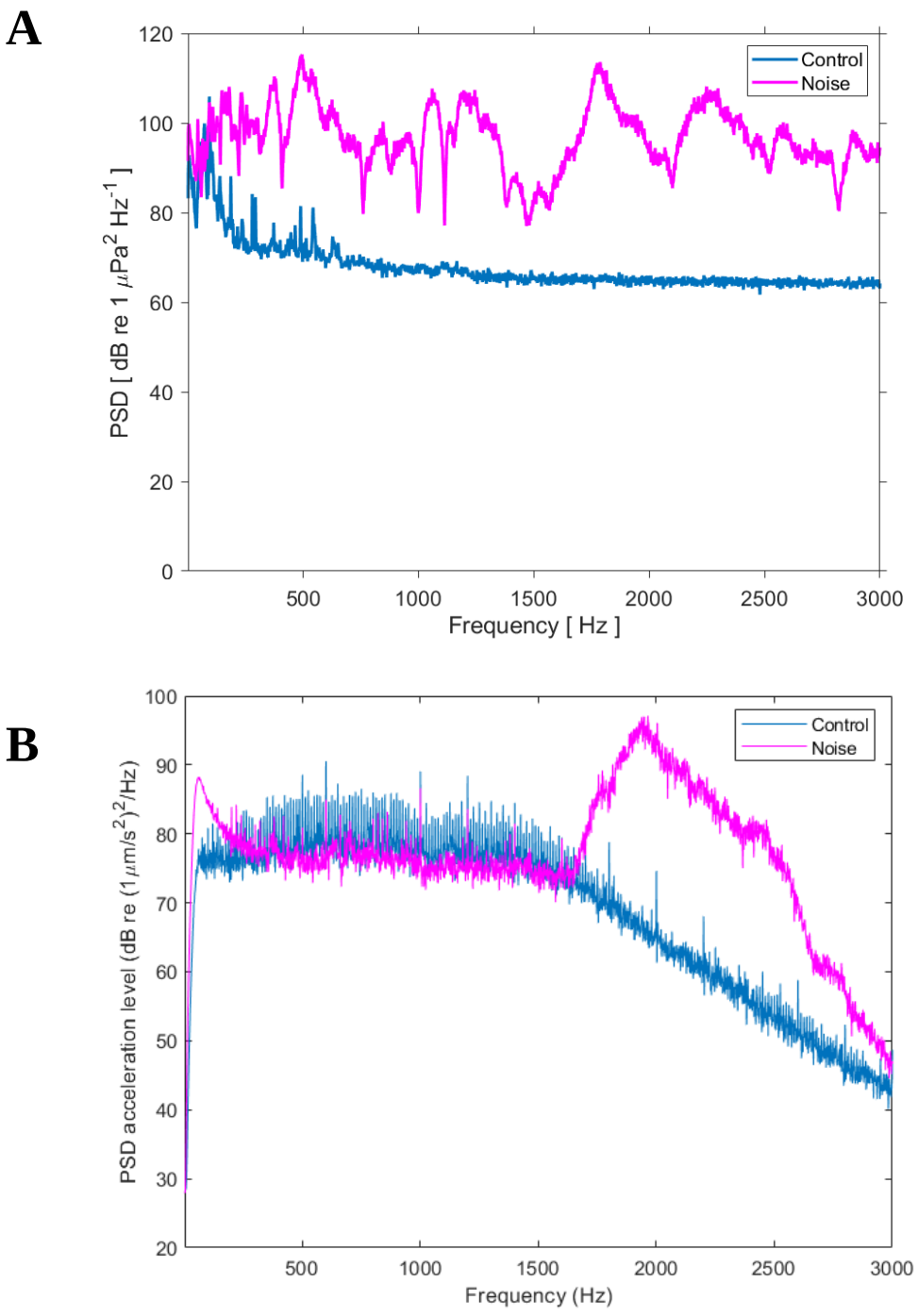


Figure 7.2 Representative power spectral density plots showing RMS levels of **A)** pressure and **B)** particle acceleration received by *Daphnia magna* (L.). Recordings were taken inside rearing cups C4 (see figure) in the centre of the array in control (blue line) and noise (pink line) tanks. DFT length: 48 kHz, recording lengths 30 s, Hann window length: 1 s.

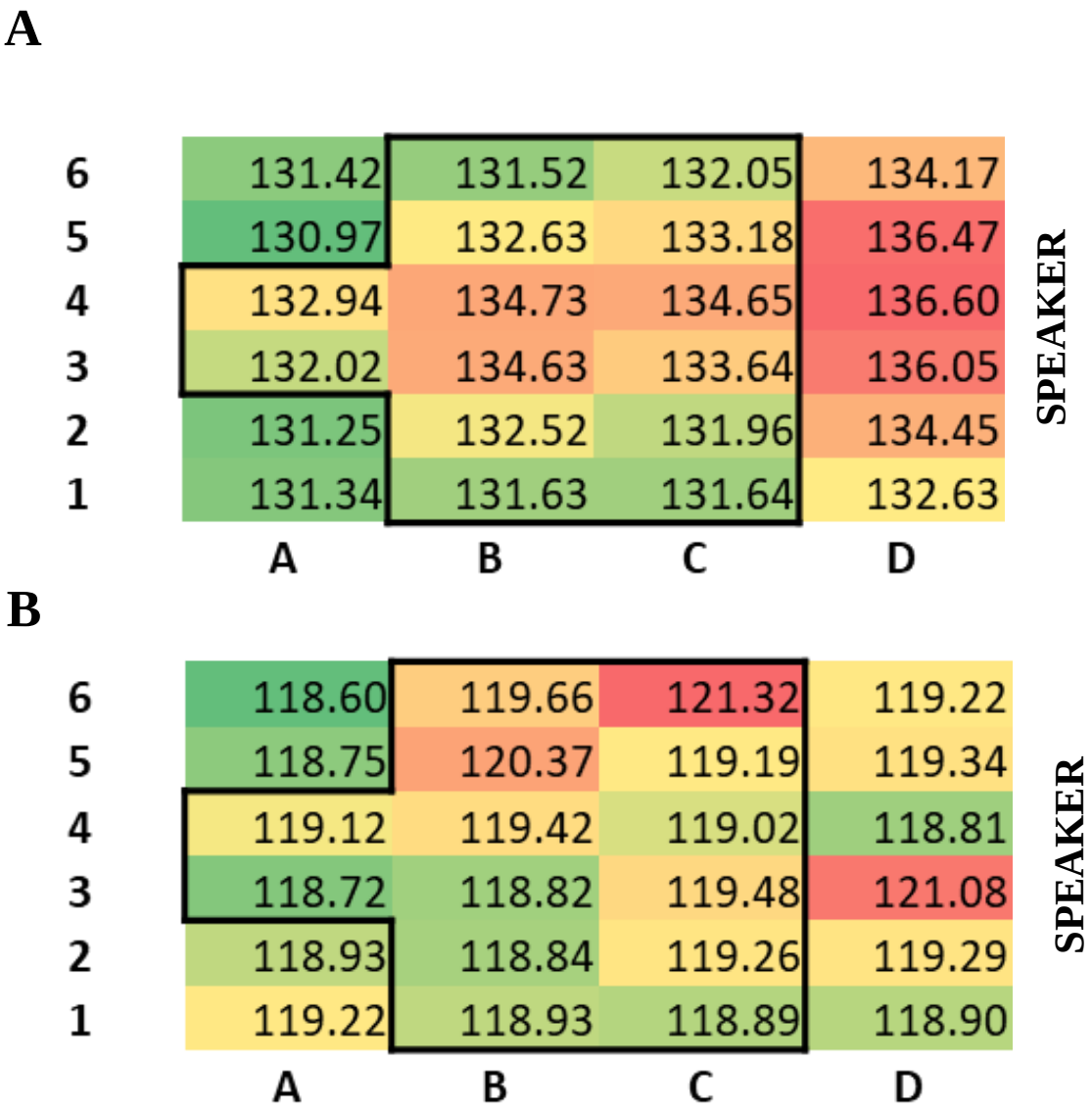


Figure 7.3 Sound exposure map showing RMS pressure levels (dB re: 1 µPa) received by *Daphnia magna* (L.) in **A)** noise tank and **B)** control tanks, averaged across the entire frequency domain to facilitate comparisons. Recordings were taken inside rearing vessels. Recording lengths 30s. Based on the observed sound cone across the noise tank rearing vessels (with higher received levels closer to the speaker and in the centre of the tank), A3, A4, B1-B6 and C1- C6 were used for experiments (black-bordered area) to minimise any differences in received levels and *D. magna* were repositioned randomly within this area at each water change to further reduce any differences in received levels. The same experimental area and random repositioning of *D. magna* was carried out in the control tank.

7.2.3 - Experimental set-up

28 newly released 3rd brood, clonal *D. magna* neonates (<24 h) were transferred into polypropylene containers filled with 100 ml of synthetic freshwater (see table 7.1 for recipe) to initiate the experiment at generation F1. These containers were distributed at random between two water bath tanks (noise and control, n = 14 in each tank). Each tank contained a rigid plastic platform upon which the daphnia rearing containers were placed. The platforms were built to a height at which the surface water in the cups was exposed to the air, allowing gas exchange whilst preventing water mixing between the containers and the water bath tank. The containers were placed at random positions on the platform within a pre-assigned grid (Figure 7.3). At each water change, rearing vessels were removed and replaced, once again, at random positions in the grid, so that the position of each larvae within the grid changed 3 times per week.

The platform support legs rested on anti-vibrational rubber matting inside the tank and the tank itself was further isolated from surrounding vibration with a neoprene mat between the base of the tank and the shelving. The DNH Sub Aqua 30 speaker was suspended from above with transparent fishing line, with the speaker cone facing horizontally across the tank. The speaker support beam was surrounded with foam pipe insulation and rested across the top of the tank (Figure 7.4). The rearing vessel platform and the sound exposure speaker were situated at opposite ends of the tank (Figure 7.4).

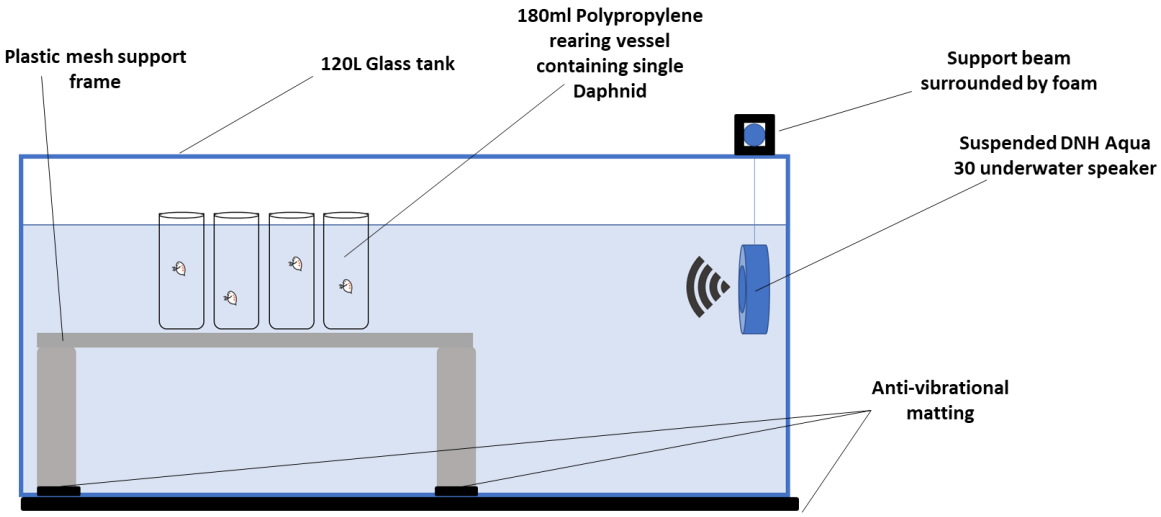


Figure 7.4 Aquarium set-up for the multigenerational *Daphnia magna* (L.) noise exposure experiment**.** Tank inner dimensions: 788 x 528 x 306 mm. Centre of speaker cone placed 80 mm from the right tank wall and 150 mm from the tank floor. *D. magna* reared individually within 180ml clear polypropylene vessels, held 150mm from the floor of the tank by a plastic mesh frame.

.

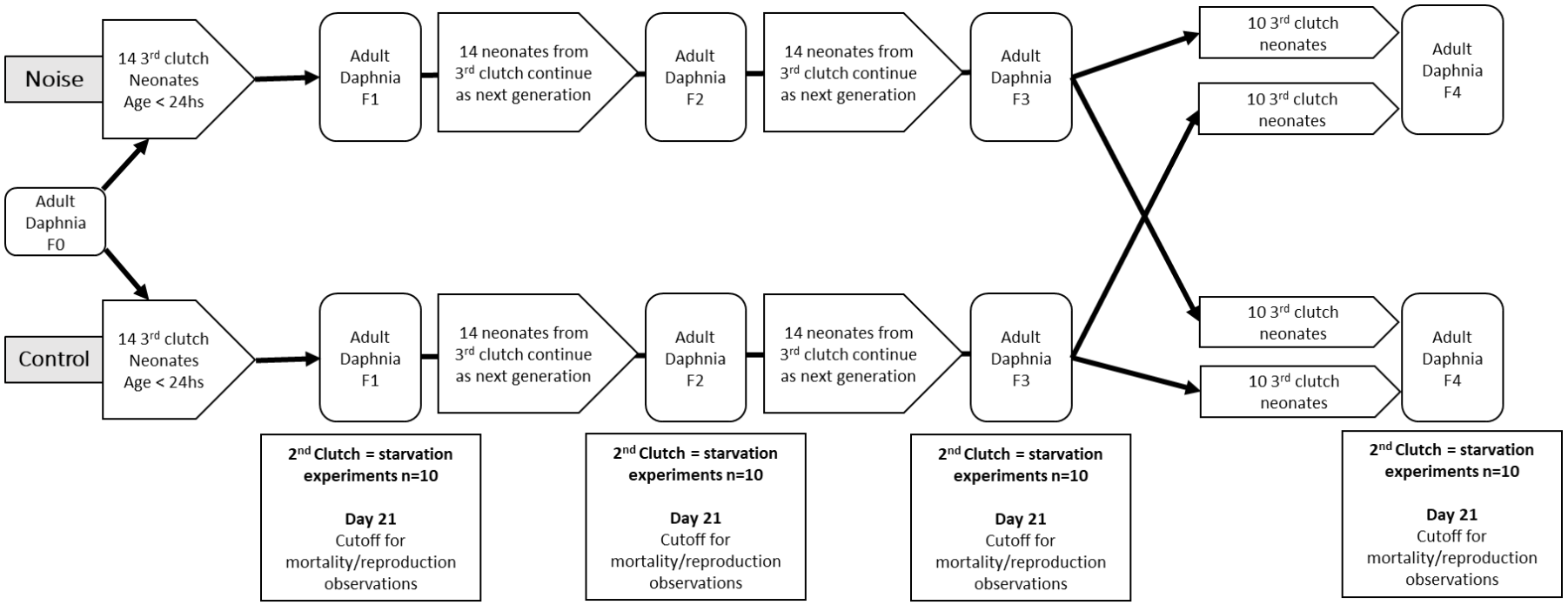


Figure 7.5 Experimental design. The effects of chronic boat noise playback exposure were assessed over a four-generation period (F1, F2, F3, F4). The neonates used to initiate each experimental generation were always taken from the 3rd brood of the parental generation to control for brood order effects. At generation F4, a crossover experiment occurs, where neonates from both noise and control lineages are each exposed to both noise and control conditions, to assess for the potential for control and treatment groups to reconverge within a single generation. 10 2nd brood neonates are used for starvation experiments at each generation.

7.2.4 - Transgenerational experimental design

28 newly released, 3rd brood, clonal *D. magna* neonates (<24 h) were used to initiate the first experimental generation F1 (noise n=14, control n=14). These daphniids were reared for a 21-day period, with daily observations for survival and reproduction (see sections 7.2.5.1 and 7.2.5.2), after which the experiment for that generation ended and the daphniids were removed from the experimental setup. *D. magna* neonates from the 3rd brood of F1 were collected and used to begin the next experimental generation F2. Noise exposed F1 females contributed 14 neonates which were then also reared in the noise exposure tank, whereas control F1 females contributed 14 neonates to F2 in the control tank. This process continued unchanged until generation F4, at which point a crossover was performed, whereby both the noise and control tanks were populated with: 1) 10 F4 neonates from control F3 females and 2) 10 F4 neonates from noise exposed F3 females. This allowed observation of the extent to which any noise-induced effects observed in generation F3 were reduced in F4 individuals reared in control conditions (Figure 7.5). At each generation, 10 *D. magna* neonates were collected at random from the 2nd broods of experimental females and were reared with no food to determine time-till-starvation. These individuals were reared in identical rearing vessels and water, but outside of the sound exposure tanks due to space constraints.

7.2.5 - Data collection

Each rearing cup was visually assessed daily for survival and reproduction.

7.2.5.1 - Survival

Animals that were unable to swim within 15 seconds, after gentle agitation of the test vessel, were deemed to be deceased. A mortality was recorded even when there was some slight movement of the antennae. In cases of a recorded mortality, this was verified post-hoc on the subsequent observation day. This was possible as the individual

turns an ‘off-white’ opaque colour in the 24 hours after mortality. If this didn’t happen and the animal was seen to be moving, the initial mortality recording would be revised, this was not required for this experiment however, as all animal mortalities were verified on the subsequent day.

7.2.5.2 - Reproduction

Rearing vessels were visually checked at 09:00 every day for the presence of neonates. Any released neonates were gently removed with a pipette. This allowed time till first reproduction (days) and subsequent daily reproductive output to be recorded over the 21-day testing period for each generation.

7.2.5.3 - Starvation

For each generation, 10 2nd brood neonates (< 24 h old) were selected at random and reared outside of the sound exposure tanks in 180 ml containers. No food was provided to the animals and time till starvation was recorded. Animals were checked daily and mortality was defined as in Section 2.5.1.

7.2.6 - Statistical analysis

All statistical analysis was conducted using R version 3.2.4 (R Core Team, 2017). For generations F1, F2 and F3, statistical comparisons were made between noise and control treatment groups. At generation F4, due to the crossover of noise-exposed and control lineages (See section 2.4 and Figure 7.5) 2-way comparisons were conducted between two factor groups: 1) Sound exposure (Noise vs control) and parental exposure (Pre-exposed vs naïve).

7.2.6.1 - Survival

The time-dependent mortality rates of different experimental groups were compared between treatment groups using a logrank test, testing against the null

hypothesis that survival curves are identical in the overall populations. For generation 4, the logrank test compared all groups as a single factor with four levels. Survival curves for each experimental group were fitted and compared using a Kaplan-Meier survival analysis with the *survival* (Therneau, 2021) package in R version 3.2.4.

7.2.6.2 - Reproduction

Time to first reproduction (days) and total reproductive output (defined as the number of offspring produced over the 21-day test period) were compared between treatment groups using Mann-Whitney U tests for each generation. At generation F4, a two-way ANOVA was used to test for effects of both noise exposure and parental noise exposure on reproductive response variables. Tukey's multiple comparisons tests were then carried out to test for differences between each factor level combination.

7.2.6.3 - Starvation

Time till starvation (days) of 2nd brood neonates was compared between treatment groups using Mann-Whitney U tests for each generation. At generation F4, a two-way ANOVA was used to test for effects of both noise exposure and parental noise exposure on reproductive response variables. Tukey's multiple comparisons tests were then carried out to test for differences between each factor level combination. Monitoring growth and survival under starvation conditions is a standard method to assess the ability of aquatic invertebrate larvae to respond to nutritional stress under different conditions (Pechenik and Rice, 2001; Qian and Pechenik, 1998; Wehrtmann, 1991; Yan et al., 2009).

7.2.6.4 - Population projections

As generation F4 was the final experimental generation in this study, the population growth rate was modelled from F4, forwards in time, with the assumption that the age-dependent survival and reproduction rates observed in F4 are maintained

into future generations. This assumption is based on some further assumptions: 1) Noise exposure continues to affect the age-dependent survival and reproduction of future generations in the same way as in F4, 2) No predation occurs, 3) No density-dependent effects on survival and reproduction occur. Population modelling was conducted for: 1) noise-exposed F4 individuals with a parental history of noise exposure and 2) control F4 individuals with a parental history of control conditions. In this way, the models were emulating *D. magna* populations residing in noisy and quiet environments.

For each group (noise and control), individuals were classified by age into classes with a width of 1 day, up to a maximum of 21 days (the experimental observation period) and the age-dependent survival and reproduction rates were calculated, resulting in the production of a Leslie matrix *A* with the following structure:

$$A = \begin{pmatrix} F_1 & F_2 & \cdots & F_{21} \\ P_1 & 0 & 0 & 0 \\ 0 & P_2 & 0 & 0 \\ 0 & 0 & \ddots & 0 \\ 0 & 0 & 0 & P_{21} \end{pmatrix}$$

Where F_t is the fertility rate at day t and P_t is the survival probability at day t.

The population size $\boldsymbol{n}$ at time $\boldsymbol{t}$ is given as:

$$n(t+1) = A_t\, n(t)$$

The stable age distribution and the dominant eigenvalue (which is equivalent to the finite growth rate (λ)) of each population model was calculated. Models were projected forwards in time for 100 days, starting with an initial population of 100 individuals. The starting individuals were divided between age classes according to the calculated stable age distribution for each population. All analysis was carried out in R version 3.2.4.and stable age distribution and the dominant eigenvalue were calculated using the *popbio* package (Stubben and Milligan, 2007).

7.3 - Results

7.3.1 - Survival

Survival of *D. magna* over the 21 day testing period was generally greater than 90% in all sound treatments, with the exception of noise exposed F2 group (85%). There were no differences in survival between experimental groups at any generation tested (F1: $X^2_{(1)} = 1$, $p = 0.3$; F2: $X^2_{(1)} = 0$, $p = 1$; F3: $X^2_{(1)} = 0.4$, $p = 0.5$; F4: $X^2_{(3)} = 1$, $p = 0.8$) (Table 7.3).

Table 7.3 Survival (%) of *Daphnia magna* (L.) over the 21-day testing period for each treatment group at each generation.

Generation	Sound Exposure	Parental Exposure	Survival (%)	n
1	Noise	N/A	93	14
1	Control	N/A	100	14
2	Noise	Noise	93	14
2	Control	Control	93	14
3	Noise	Noise	86	14
3	Control	Control	93	14
4	Noise	Noise	90	10
4	Control	Control	100	10
4	Noise	Control	100	10
4	Control	Noise	100	10

7.3.2 - Reproduction

7.3.2.1 - Time to first reproduction

No effect of boat noise playbacks on time to first reproduction was observed at generation F1 (the first generation of *D. magna* exposed to noise) ($U = 82$, $p = 0.48$). This apparent lack of noise exposure effect was not maintained over subsequent generations, with F2, F3 and F4 generations all exhibiting significantly delayed time to first reproduction when exposed to boat noise playbacks (F2: $U = 38$, $p = 0.0047$; F3: $U = 54.5$, $p = 0.043$; F4: $U = 16$, $p = 0.0076$) (Figure 7.6).

At generation F4, the factor of parental history of noise exposure (naïve vs pre-exposed) had a significant effect on time to reach first reproduction (Two-way ANOVA $F_{(1,36)} = 15.93$, $p < 0.01$), whereas the sound exposure received by F4 daphniids (noise vs control) did not (Two-way ANOVA $F_{(1,36)} = 0.85$, $p = 0.36$). The interaction between the factors of sound exposure received by F4 daphniids and parental sound exposure history was marginally insignificant (Two-way ANOVA $F_{(1,36)} = 3.39$, $p = 0.074$) . Tukey's post-hoc pairwise revealed that when exposed to noise, *D. magna* with a parental history of noise exposure took 18% longer to reach first reproduction than control group individuals with no parental history of noise exposure ($p = 0.007$) and 22% longer than control group individuals *with* a parental history of noise exposure ($p = 0.001$) (Figure 7.7).

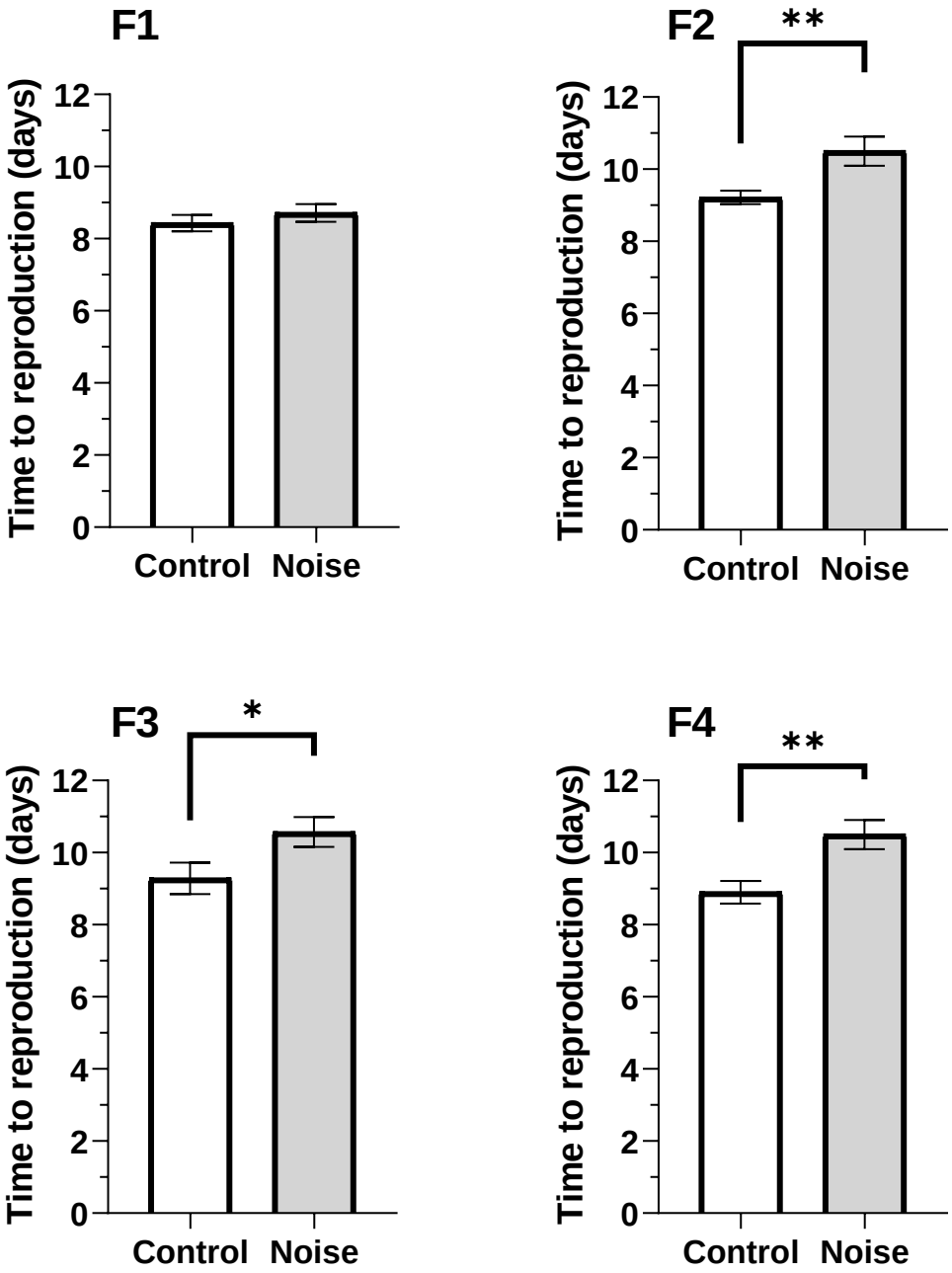


Figure 7.6 Mean (± SE) time to first reproduction (days) of *Daphnia magna* (L.) in response to different sound playback treatments (boat noise and control) over multiple generations of exposure (F1 – F4). * indicates statistically different groups (*: $p < 0.05$, **: $p < 0.01$) calculated using a Mann-Whitney U test. F*n* indicates experimental generation. N = 14 for generations F1-F3 and N=10 for generation F4.

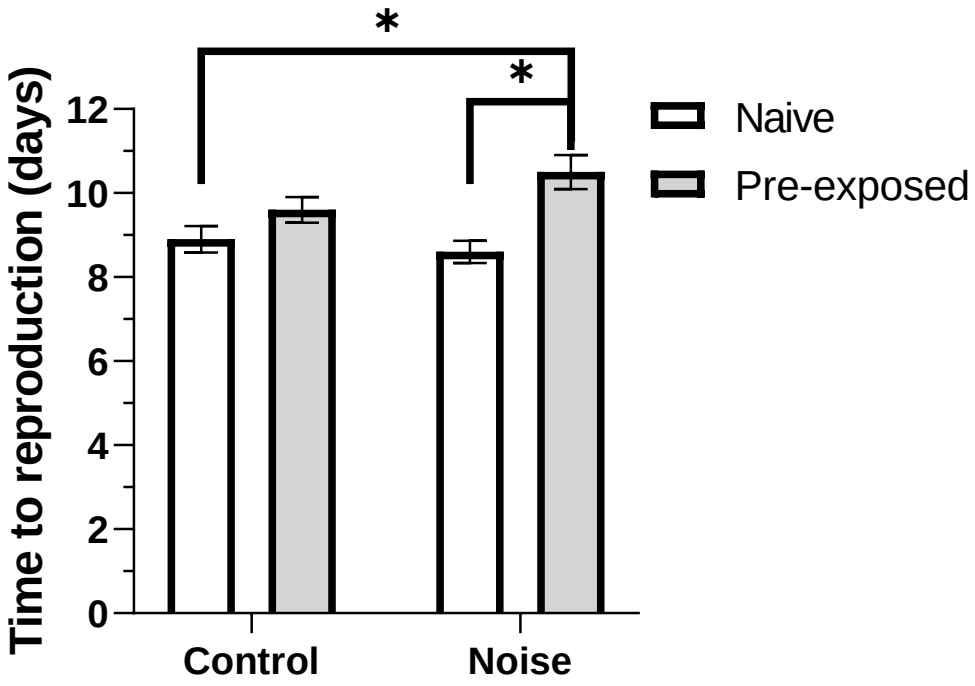


Figure 7.7 Mean (± SE) time to first reproduction (days) of generation F4 *Daphnia magna* (L.) with (pre-exposed) and without (naïve) parental history of noise exposure in response to different sound playback treatments (boat noise and control). * indicates statistically different groups ($p < 0.05$) calculated using a two-way ANOVA followed by Tukey HSD pairwise testing. N = 10 for each group.

7.3.2.2 - Reproductive output

Reproductive output (total neonates released over the 21-day test period) showed similar generational trends to the results observed for time to first reproduction (Section 7.3.2.1). No effect of boat noise playbacks on time to first reproduction was observed at generation F1 ($U = 74$, $p = 0.28$). However, boat noise playbacks significantly reduced reproductive outputs in subsequent generations (F2: $U = 49.5$, $p = 0.025$; F3: $U = 46$, $p = 0.015$; F4: $U = 21.5$, $p = 0.030$) (Figure 7.8).

At generation F4, the factor of parental history of noise exposure (naïve vs pre-exposed) had a significant effect on reproductive output (Two-way ANOVA $F_{(1,36)} = 9.49$, $p = 0.004$), whereas the sound exposure received by F4 daphniids (noise vs control) did not (Two-way ANOVA $F_{(1,36)} = 0.12$, $p = 0.73$). There was no interaction between the factors of sound exposure received by F4 daphniids and parental sound exposure history (Two-way ANOVA $F_{(1,36)} = 1.19$, $p = 0.28$) . Tukey's post-hoc pairwise revealed that under control conditions, *D. magna* with a parental history of noise exposure had 19% fewer offspring than control group individuals with no parental history of noise exposure ($p = 0.027$) (Figure 7.9).

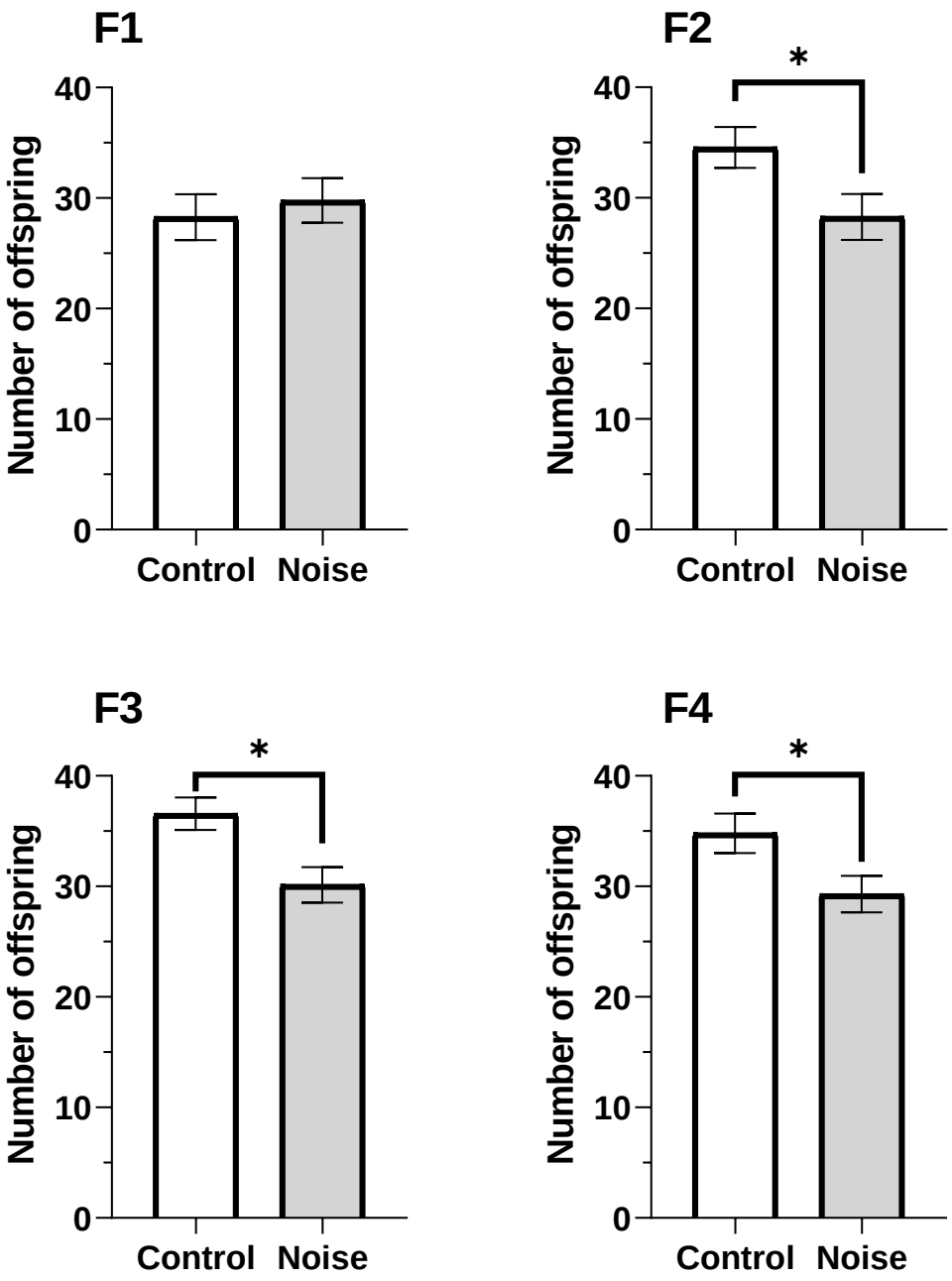


Figure 7.8 Mean (± SE) offspring produced over a 21-day period by *Daphnia magna* (L.) in response to different sound playback treatments (boat noise and control) over multiple generations of exposure (F1 – F4). * indicates statistically different groups ($p < 0.05$) calculated using a Mann-Whitney U test. F*n* indicates experimental generation. N = 14 for generations F1-F3 and N=10 for generation F4.

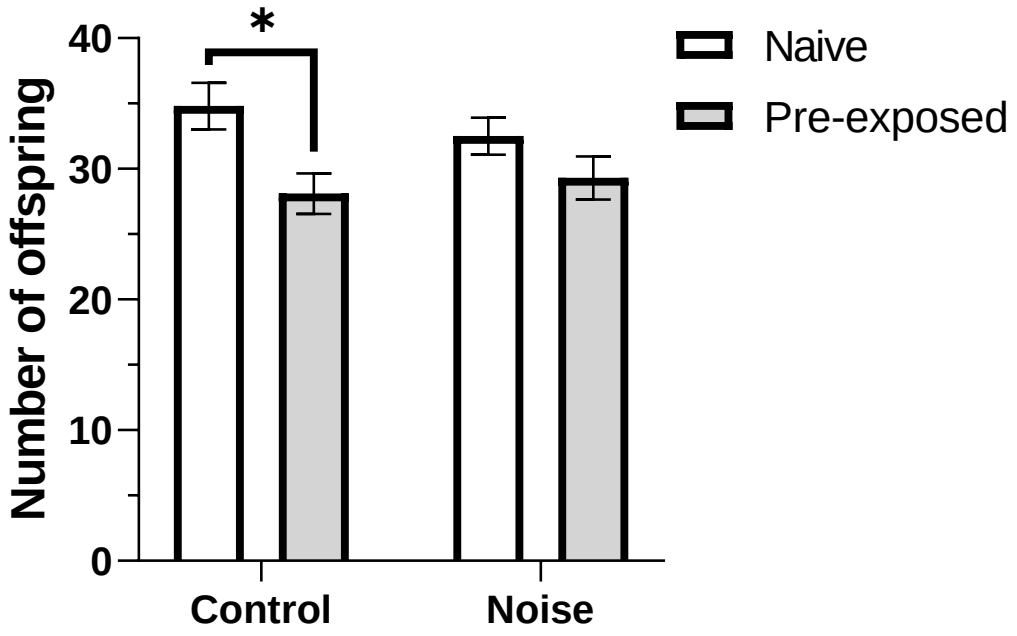


Figure 7.9 Mean (± SE) offspring produced over a 21-day period by generation F4 *Daphnia magna* (L.) with (pre-exposed) and without (naïve) parental history of noise exposure in response to different sound playback treatments (boat noise and control). * indicates statistically different groups ($p < 0.05$) calculated using a two-way ANOVA followed by Tukey HSD pairwise testing.

7.3.3.3 - Starvation

No effect of boat noise playbacks were observed on time-to-starvation of 2nd brood neonates at any generation (F1: U = 43, p = 0.62; F2: U = 41, p = 0.51; F3: U = 46, p = 0.79; F4: U = 45.5, p = 0.75) (Figure 7.10).

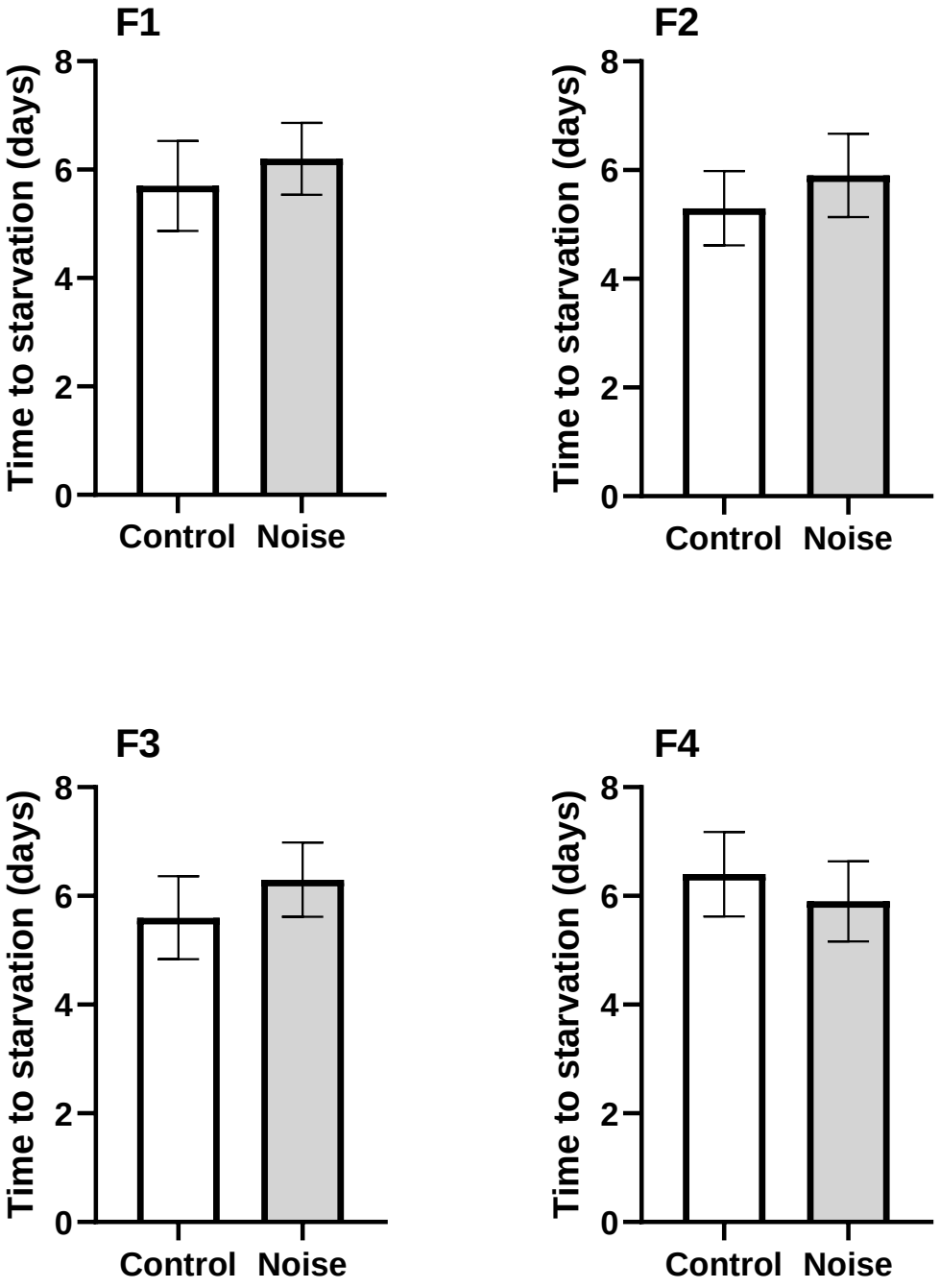


Figure 7.10 Mean (± SE) time to starvation (days) of *Daphnia magna* (L.) neonates (2nd brood) produced by females reared in different sound playback treatments (boat noise and control) over multiple generations of exposure (F1 – F4). * indicates statistically different groups ($p < 0.05$) calculated using a Mann-Whitney U test. F*n* indicates experimental generation. N = 10 for all groups.

7.3.3.4 - Population projections

D. magna population growth was projected using Leslie matrices computed for generation F4 individuals. Noise exposed F4 populations with noise exposed parental lineages were compared to control F4 populations with control parental lineages – thereby modelling the likely trajectory of population growth if both populations remained in noisy or control conditions (assuming no predation or density dependent reductions in population growth).

The projected noise-exposed and control populations had dominant eigenvalues (representing finite population growth rates (λ)) of 1.27 and 1.33 respectively. Noise exposure reduces growth rates; Starting from 100 individuals at stable age distribution, the noise exposed group reaches a population size of 13.72 million after 50 days, whereas the control group reaches a population size of 120.67 million.

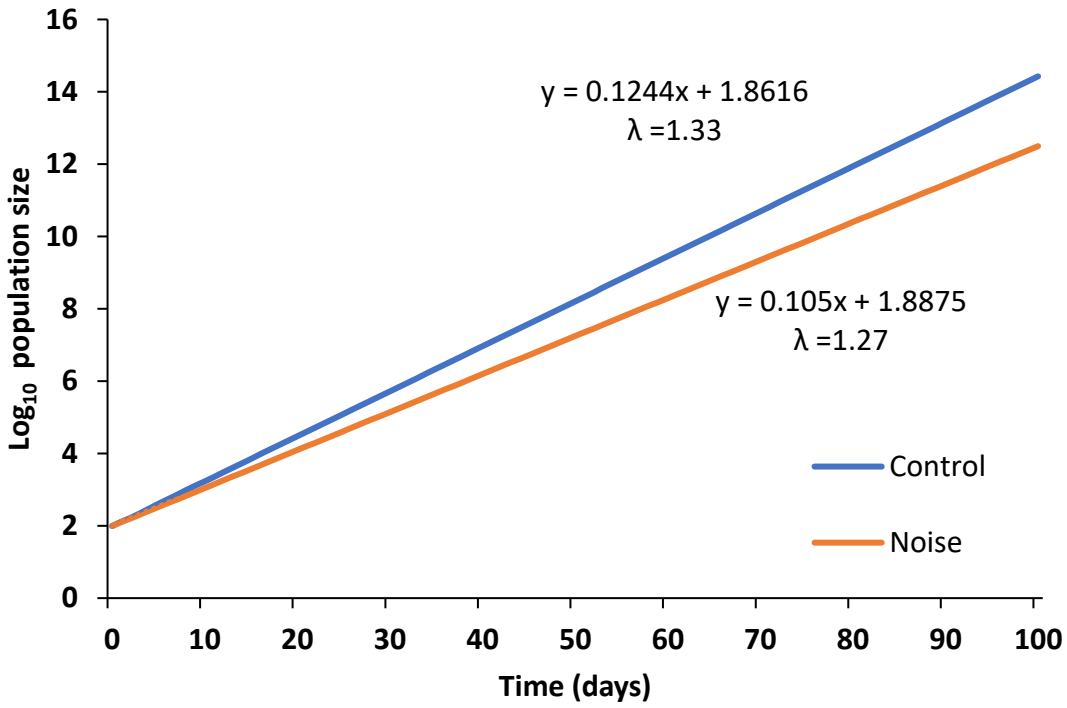


Figure 7.11 Population projections for generation F4 *Daphnia magna* (L.) reared in noise-exposed and control environments, with an initial population size of 100 individuals at stable age distribution. Population projections applied on Leslie matrices.

7.4 - Discussion

To knowledge, this is the first study to assess the chronic impacts of underwater noise across multiple generations on any animal. Underwater anthropogenic noise is increasing globally and its potential impacts on aquatic ecosystems is a growing concern (de Soto, 2016; Popper and Hastings, 2009; Williams et al., 2015). Acute exposures to noise can be useful in determining the threshold noise levels at which harms occur and how they manifest (e.g. behavioural change or physiological stress) (e.g. (de Soto et al., 2013; Kastelein et al., 2017; Wale et al., 2019), but studies utilising these types of exposure often do not provide information as to whether an organism is able to acclimate or habituate to the stressor over time. Whether an organism's response to noise changes over time, and whether the long-term effects of noise change over multiple generations is crucial information when attempting to understand how noisy environments may be impacting wild populations over the long term, as has been similarly argued for a range of other stressors/pollutants (Li et al., 2017; Stark et al., 2004; Wennergren and Stark, 2000).

7.4.1 - Acute noise exposure results in no major changes to observed life history parameters of naïve Daphnia magna *over a single generation*

No significant effects of boat noise playbacks were observed on the life history traits (survival, time to maturity and reproductive output) of *D. magna* at generation F1. If taken in isolation, these results would suggest that noise of the character and intensity used in this experiment is insufficient to impact the 'fitness' of *D. magna* as it pertains to a laboratory setting with no food limitation or predation. The fact that noise impacts do occur in later generations, as discussed below, highlights the vital importance of long-term studies of this type to fully understand the impacts of noise in the field.

4.2. Noise exposure impacts the life history parameters of Daphnia magna *over multiple generations*

Whilst no effects of noise occurred over a single generation, time to maturity was delayed and reproductive output reduced over subsequent generations of noise exposed *D. magna*. The magnitude of these impacts remained relatively consistent over generations F2, F3 and F4, suggesting that any impacts of noisy environments on *D. magna* in the wild are likely to be stable over time. The fact that no changes were observed in the initial generation F1 suggests that the noise exposure impact mechanism either occurs: 1) directly upon developing embryos in the mother's brood pouch; 2) via a cue passed from noise-exposed mother to developing embryo or 3) by altering the mother's energetic investment into developing embryos. If the 3rd hypothesis was true, the time to starvation of the offspring of noise exposed mothers would be expected to differ from control mother offspring. *D. magna* neonates with high maternal lipid content can survive starvation up to twice as long as neonates with low lipid content (Tessier et al., 1983). This is not what was observed however, with no differences in time till starvation of the offspring of any group, at any generation. Taken with the fact that generation F4 neonates with noise-exposed mothers showed delayed maturity times and reduced reproduction even in control conditions, compared to control neonates with control parental lineages, this gives weight to the 1st and 2nd hypotheses. Either noise exposure negatively impacts *D. magna* embryonic development directly, which has been observed for mollusc embryos (de Soto et al., 2013; Nedelec et al., 2015), or via a cue passed on from the mother, a noise impact route that has never been demonstrated to date. Exposure of *D. magna* to silver nanoparticles has been shown to produce reproductive output reductions and increases in anti-oxidant gene expression that persist over two further unexposed generations (Ellis et al., 2020), and 5-azacytidine, a chemical known to affect DNA methylation in animals (a key epigenetic mechanism

(Harris et al., 2012)), reduces DNA cytosine methylation of both directly exposed *D. magna* and two further unexposed generations (Vandegehuchte et al., 2010). These studies demonstrate the possibility for transgenerational inheritance of environment-induced epigenetic changes in nonexposed subsequent generations.

Regardless of the impact mechanism, a key finding of this study is that long term anthropogenic noise exposure can impact key life history parameters of *D. magna*. Seemingly small changes to survival and reproductive rates can lead to large changes in population growth dynamics, particularly in organisms that have relatively short lifespans and generational turnover (Holgate and Caswell, 1990). This is exemplified by the fact that control group *D.* magna are modelled to have a population size an order of magnitude larger than noise exposed *D. magna* after 50 days (120.67 million as opposed to 13.72 million). In the wild, unrestricted population growth of the type used in this model would not occur due to predation and density-dependent life history parameters, but the model nevertheless demonstrates the potential for noise to impact population growth. *Daphnia* spp. are dominant grazers of phytoplankton and drive the seasonal 'clear-water' phase (caused by removal of phytoplankton) observed in many freshwater systems (Lampert et al., 1986). As a preferred prey species, they form a key trophic linkage between planktonic producers and predatory fish and invertebrates. Reduction of *Daphnia* population growth in a lake may therefore result in a greener lake (with associated water quality reductions), a delayed clear-water phase, and reduced prey availability for freshwater fish. Thus, subtle sublethal noise effects on Daphnia may drive significant impacts on entire lake ecosystems. The population modelling displayed here should be interpreted with caution however, as of course in real-world systems the complexity of noise impacts, interacting with other stressors, and potentially impacting numerous species in varying ways must be considered. For example, additional environmental sound reduces the feeding rate of damselfly larvae on daphnia

(Villalobos et al., 2017). In this way, reduced availability of *D. magna* as a prey source due to noise induced reductions in growth and reproductive output reported here may be coupled with impaired abilities of predators to successfully consume *D. magna*, further exacerbating the reduction in trophic linkage between phytoplankton and secondary consumers.

7.4.3 - Future directions

Given the negative noise impacts on *D. magna* demonstrated in this study, and the species' high suitability as an epigenetic model organism (Harris et al., 2012), further work is warranted to investigate any epigenetic changes that may be occurring due to noise exposure, and the mechanisms by which they are retained by future generations. Multi-generational noise impacts are a novel area of research and this study may provide the foundation for the emergence of *D. magna* as a model organism for such research.

This study observed the potential for recovery of noise exposed *D. magna* lineages over a single generation (F4). A single generation of control conditions was not sufficient for response variables to return to levels comparable to the control group. Further work ought to be done, extending the number of unexposed generations to observe how long noise-induced effects persist in populations after exposure is ceased.

Finally, combined with the results presented in this study, such follow-up laboratory studies would provide good grounds to refine the scope and identify the response parameters of interest for field experiments to test whether the observed laboratory results transfer to real-world populations of *D. magna* living in noisy conditions in the field.

7.5 - References

Alonzo, F., Gilbin, R., Zeman, F. A. and Garnier-Laplace, J. (2008). Increased effects of internal alpha irradiation in *Daphnia magna* after chronic exposure over three successive generations. *Aquatic Toxicology*, *87*(3), 146–156. https://doi.org/10.1016/j.aquatox.2008.01.015

Benzie, J. A. H. (2005). *Cladocera: The Genus Daphnia (including Daphniopsis) (Anomopoda: Daphniidae)*. Backhuys Publishers.

Bolgan, M., Chorazyczewska, E., Winfield, I. J., Codarin, A., O'Brien, J. and Gammell, M. (2016). First observations of anthropogenic underwater noise in a large multi-use lake. *Journal of Limnology*, *75*(3), 644–651. https://doi.org/10.4081/jlimnol.2016.1405

Brennan, S. J., Brougham, C. A., Roche, J. J. and Fogarty, A. M. (2006). Multi-generational effects of four selected environmental oestrogens on *Daphnia magna*. *Chemosphere*, *64*(1), 49–55. https://doi.org/10.1016/j.chemosphere.2005.11.046

Cousyn, C., De Meester, L., Colbourne, J. K., Brendonck, L., Verschuren, D. and Volckaert, F. (2001). Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(11), 6256–6260. https://doi.org/10.1073/pnas.111606798

de Soto, N. A. (2016). Peer-Reviewed Studies on the Effects of Anthropogenic Noise on Marine Invertebrates: From Scallop Larvae to Giant Squid. In A. N. Popper & A. Hawkins (Eds.), *The Effects of Noise on Aquatic Life II* (pp. 17–26). Springer New York. https://doi.org/10.1007/978-1-4939-2981-8_3

de Soto, N. A., Delorme, N., Atkins, J., Howard, S., Williams, J. and Johnson, M.

(2013). Anthropogenic noise causes body malformations and delays development in marine larvae. *Scientific Reports*, *3*(1), 2831. https://doi.org/10.1038/srep02831

Drenner, R. W., Strickler, J. R. and O'Brien, W. J. (1978). Capture Probability: The Role of Zooplankter Escape in the Selective Feeding of Planktivorous Fish. *Journal of the Fisheries Research Board of Canada*, *35*(10), 1370–1373. https://doi.org/10.1139/f78-215

Ellis, L. A., Kissane, S., Hoffman, E., Brown, J. B., Valsami-Jones, E., Colbourne, J. and Lynch, I. (2020). Multigenerational Exposures of *Daphnia magna* to Pristine and Aged Silver Nanoparticles: Epigenetic Changes and Phenotypical Ageing Related Effects. *Small*, *16*(21), 2000301. https://doi.org/10.1002/smll.202000301

Elser, J. J., Fagan, W. F., Denno, R. F., Dobberfuhl, D. R., Folarin, A., Huberty, A., Interlandi, S., Kilham, S. S., McCauley, E., Schulz, K. L., Siemann, E. H. and Sterner, R. W. (2000). Nutritional constraints in terrestrial and freshwater food webs. *Nature*, *408*(6812), 578–580. https://doi.org/10.1038/35046058

Hairston, N. G., Kearns, C. M., Demma, L. P. and Effler, S. W. (2005). Species-Specific *Daphnia* Phenotypes : A History of Industrial Pollution and Pelagic Ecosystem Response. *Ecology*, *86*(7), 1669–1678. https://www.jstor.org/stable/3450609

Hambright, K. D. and Hall, R. O. (1992). Differential zooplankton feeding behaviors, selectivities, and community impacts of two planktivorous fishes. *Environmental Biology of Fishes*, *35*(4), 401–411. https://doi.org/10.1007/BF00004992

Harris, K. D. M., Bartlett, N. J. and Lloyd, V. K. (2012). Daphnia as an Emerging Epigenetic Model Organism. *Genetics Research International*, *2012*, 1–8. https://doi.org/10.1155/2012/147892

Heyland, A., Bastien, T. and Halliwushka, K. (2020). Transgenerational reproductive effects of two serotonin reuptake inhibitors after acute exposure in *Daphnia magna* embryos. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *238*(August), 108875. https://doi.org/10.1016/j.cbpc.2020.108875

Holgate, P. and Caswell, H. (1990). Matrix Population Models. *Biometrics*, *46*(4), 1238. https://doi.org/10.2307/2532473

Ives, A. R., Carpenter, S. R. and Dennis, B. (1999). Community Interaction Webs And Zzooplankton Responses To Planktivory Manipulations. *Ecology*, *80*(4), 1405–1421. https://doi.org/https://doi.org/10.1890/0012-9658(1997)080[1405:CIWAZR]2.0.CO;2

Kastelein, R. A., Jennings, N., Kommeren, A., Helder-Hoek, L. and Schop, J. (2017). Acoustic dose-behavioral response relationship in sea bass (*Dicentrarchus labrax*) exposed to playbacks of pile driving sounds. *Marine Environmental Research*, *130*, 315–324. https://doi.org/10.1016/j.marenvres.2017.08.010

Kleiven, O. T., Larsson, P. and Hobek, A. (1992). *Sexual Reproduction in* Daphnia magna *Requires Three Stimuli*. *65*(2), 197–206.

Lampert, W., Fleckner, W., Rai, H. and Taylor, B. E. (1986). Phytoplankton control by grazing zooplankton: A study on the spring clear-water phase. *Limnology and Oceanography*, *31*(3), 478–490. https://doi.org/10.4319/lo.1986.31.3.0478

Li, Y., Wang, W. X. and Wang, M. (2017). Alleviation of mercury toxicity to a marine copepod under multigenerational exposure by ocean acidification. *Scientific Reports*, *7*(1), 1–9. https://doi.org/10.1038/s41598-017-00423-1

Merchant, N. D., Fristrup, K. M., Johnson, M. P., Tyack, P. L., Witt, M. J., Blondel, P. and Parks, S. E. (2015). Measuring acoustic habitats. *Methods in Ecology and*

Evolution, *6*(3), 257–265. https://doi.org/10.1111/2041-210X.12330

Nedelec, S. L., Radford, A. N., Simpson, S. D., Nedelec, B., Lecchini, D. and Mills, S. C. (2015). Anthropogenic noise playback impairs embryonic development and increases mortality in a marine invertebrate. *Scientific Reports*, *4*, 5891. https://doi.org/10.1038/srep05891

OECD. (2004). Test No. 202: *Daphnia* sp. Acute Immobilisation Test. In *OECD Guideline for the Testing of Chemicals* (Issue April). OECD. https://doi.org/10.1787/9789264069947-en

OECD. (2008). Test No. 211: *Daphnia magna* Reproduction Test. In *OECD Guideline for the Testing of Chemicals* (Issue October). OECD Publishing. https://doi.org/10.1787/9789264070127-en

Popper, A. N. and Hastings, M. C. (2009). The effects of anthropogenic sources of sound on fishes. *Journal of Fish Biology*, *75*(3), 455–489. https://doi.org/10.1111/j.1095-8649.2009.02319.x

Pechenik, J. A. and Rice, M. E. (2001). Influence of delayed metamorphosis on postsettlement survival and growth in the sipunculan Apionsoma misakianum. *Invertebrate Biology*, *120*(1), 50–57. https://doi.org/10.1111/j.1744-7410.2001.tb00025.x

Qian, P. Y. and Pechenik, J. A. (1998). Effects of larval starvation and delayed metamorphosis on juvenile survival and growth of the tube-dwelling polychaete Hydroides elegans (Haswell). *Journal of Experimental Marine Biology and Ecology*, *227*(2), 169–185. https://doi.org/10.1016/S0022-0981(97)00267-0

R Core Team. (2017). *R: A Language and Environment for Statistical Computing*. https://www.r-project.org/

Sarnelle, O. (1993). Herbivore Effects on Phytoplankton Succession in a Eutrophic Lake. *Ecological Monographs*, *63*(2), 129–149.

Sarnelle, O. (2005). Daphnia as keystone predators: Effects on phytoplankton diversity and grazing resistance. *Journal of Plankton Research*, *27*(12), 1229–1238. https://doi.org/10.1093/plankt/fbi086

Stark, J. D., Banks, J. E. and Vargas, R. (2004). How risky is risk assessment: The role that life history strategies play in susceptibility of species to stress. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(3), 732–736. https://doi.org/10.1073/pnas.0304903101

Stubben, C. and Milligan, B. (2007). Estimating and analyzing demographic models using the popbio package in R. *Journal of Statistical Software* 22(11), 1–23

Tessier, A. J., Henry, L. L., Goulden, C. E. and Durand, M. W. (1983). Starvation in *Daphnia*: Energy reserves and reproductive allocation. *Limnology and Oceanography*, *28*(4), 667–676. https://doi.org/10.4319/lo.1983.28.4.0667

Therneau T (2021) A Package for Survival Analysis in R. R package version 3.2-10

Tkaczyk, A., Bownik, A., Dudka, J., Kowal, K. and Ślaska, B. (2021). *Daphnia magna* model in the toxicity assessment of pharmaceuticals: A review. *Science of The Total Environment*, *763*, 143038. https://doi.org/10.1016/j.scitotenv.2020.143038

Tollrian, R., Duggen, S., Weiss, L. C., Laforsch, C. and Kopp, M. (2015). Density-dependent adjustment of inducible defenses. *Scientific Reports*, *5*, 1–9. https://doi.org/10.1038/srep12736

United States Enviromental Protection Agency. (2002). Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms Fifth Edition October 2002. *Environmental Protection*, *October*, 266.

Vandegehuchte, M. B., Lemière, F., Vanhaecke, L., Vanden Berghe, W. and Janssen, C. R. (2010). Direct and transgenerational impact on *Daphnia magna* of chemicals with a known effect on DNA methylation. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *151*(3), 278–285. https://doi.org/10.1016/j.cbpc.2009.11.007

Villalobos, G., Alison, J. and Christopher, M. D. (2017). Environmental noise reduces predation rate in an aquatic invertebrate. *Journal of Insect Conservation*, *21*(5), 839–847. https://doi.org/10.1007/s10841-017-0023-y

Wale, M. A. (2017). *The Effects of Anthropogenic Noise Playbacks on Marine Invertebrates. PhD Thesis*. Edinburgh Napier University.

Wale, M. A., Briers, R. A., Hartl, M. G. J., Bryson, D. and Diele, K. (2019). From DNA to ecological performance: Effects of anthropogenic noise on a reef-building mussel. *Science of The Total Environment*, *689*, 126–132. https://doi.org/10.1016/j.scitotenv.2019.06.380

Wehrtmann, I. S. (1991). How important are starvation periods in early larval development for survival of *Crangon septemspinosa* larvae? *Marine Ecology Progress Series*, *73*(2/3), 183–190. http://www.jstor.org/stable/24825407

Wennergren, U. and Stark, J. (2000). Modeling long-term effects of pesticides on populations: Beyond just counting dead animals. *Ecological Applications*, *10*(1), 295–302. https://doi.org/10.1890/1051-0761(2000)010[0295:MLTEOP]2.0.CO;2

Williams, R., Wright, A. J., Ashe, E., Blight, L. K., Bruintjes, R., Canessa, R., Clark, C. W., Cullis-Suzuki, S., Dakin, D. T., Erbe, C., Hammond, P. S., Merchant, N. D., O'Hara, P. D., Purser, J., Radford, A. N., Simpson, S. D., Thomas, L. and Wale, M. A. (2015). Impacts of anthropogenic noise on marine life: Publication patterns, new discoveries, and future directions in research and management. *Ocean and*

Coastal Management, *115*, 17–24. https://doi.org/10.1016/j.ocecoaman.2015.05.021

Yan, X., Zhang, Y., Huo, Z., Yang, F. and Zhang, G. (2009). Effects of starvation on larval growth, survival, and metamorphosis of Manila clam *Ruditapes philippinarum*. *Acta Ecologica Sinica*, *29*(6), 327–334. https://doi.org/https://doi.org/10.1016/j.chnaes.2009.09.012

Chapter 8

Synthesis

E. L. Bolger

Chapter 8

Synthesis

In this thesis, the current trends in invertebrate stressor research have been reviewed and existing literature concerning the responses of early life invertebrates to underwater sound analysed. Based on these analyses, knowledge gaps have been identified, namely: 1) Whether, and how, the early life history stages of most ecologically and commercially important taxonomic groups respond to noise exposure; 2) To what extent noise impacts can be extrapolated across different taxa and 3) Whether, and how, noise-induced impacts carry over from one life stage to the next, or from one generation to the next. Here, these knowledge gaps have been addressed in a series of experimental laboratory trials. Firstly, focusing on 5 model species, this thesis has shown that 4 of the 5 were significantly and adversely affected by anthropogenic noise playbacks to varying degrees, evidencing that anthropogenic noise can impact the biology of varied taxonomic groups of invertebrates even before adult life stages begin. When exposed to the same noise playback regimes, the larvae of the taxonomically relatively close relatives *Nephrops norvegicus* (L.) and *Homarus gammarus* (L.) (*Nephrops* and *Homarus* being the only two genera in the family Nephropidae (true lobsters)) had markedly different responses, highlighting the need for caution if attempting to generalise noise induced impacts across seemingly similar groups. *Loligo forbesii* (Steenstrup, 1857) embryos increase their oxygen consumption rate in response to acute noise and *Ostrea edulis* (L.) larvae have reduced survival and lower active swimming behaviour in the presence of noise and absence of preferred settlement substrate. Using *Daphnia magna* (Straus, 1820) as a model species, for the first time the multi-generational impacts of anthropogenic noise on an aquatic invertebrate have been described. The data produced in this thesis has broadened understanding of how

anthropogenic noise impacts commercially and ecologically invertebrates at sensitive early life stages and *D. magna* has emerged as a promising candidate for a model species for multi-generational noise impact research i.e. the epigenetic effects of noise and this manifests across generations.

8.1 – Main findings

The analysis of trends in stressor research focussing on early life aquatic invertebrates over the previous 20 years (see chapter 2) has shown that the impacts of noise remains an understudied field (only 3% of all stressor studies over the previous decade), with greater attention paid to stressors such as temperature and chemical stress (34% and 29% of studies over the previous decade respectively). Further analysis of the aquatic invertebrate sound impact literature has revealed that only 37% of studies have focused on the early life stages. The majority of these early life studies have focused on the effects of natural sounds on larval settlement (43%), orientation (21%) and behaviour (21%). The following knowledge gaps were identified:. Firstly, the effects of anthropogenic noise exposure on early life aquatic invertebrates in the medium to long term remains largely unknown, particularly with regard to temperate species. Secondly, no studies have yet considered the potential for anthropogenic noise impacts to carry over across life history stages in aquatic invertebrates. There is a need to address these knowledge gaps in the context of increasing anthropogenic noise levels in many aquatic systems and protective legislation such as the MSFD that needs an evidence base to inform its implementation.

The effects of anthropogenic noise on early life stage aquatic invertebrates were investigated for a total of five model organisms comprising both marine and freshwater species, *N. norvegicus* (Family: Nephropidae), *H. gammarus* (Family: Nephropidae), *L. forbesii* (Family: Loliginidae), *O. edulis* (Family: Ostreidae) and *D. magna* (Family:

Daphniidae). These species represented a wide range of invertebrate taxonomies, phenotypes, and life history strategies, both in their larval and adult lives.

N. norvegicus and *H. gammarus* (see chapters 3 and 4 respectively) were chosen to represent the early life stages of mobile benthic Nephropidae crustaceans colloquially known as 'true lobsters', with both species developing via three planktonic larval stages before metamorphosing into benthic juveniles. Larval *N. norvegicus* exhibited delayed development when reared in ship noise playback treatments. Developmental delays occurred in both Busy (12 hours of ship noise playbacks per 24 hours) and Occasional (3 hours of ship noise playbacks per 24 hours), with the delays occurring later in development in the Occasional group compared to the Busy (increased zoeal stage III duration, compared to increased zoeal stage II duration, respectively). Naïve larvae (having never experienced experimental ship noise playbacks) increased oxygen consumption during acute noise exposure, evidencing a stress response. In contrast, pre-exposed larvae *reduced* their oxygen consumption when subjected to an additional noise exposure, indicating that larvae can alter their behavioural responses over repeated exposures, rather than habituating. Noise-induced impacts carried over from larval to juvenile stages, with juvenile *N. norvegicus* reared in noisy conditions showing greatly reduced ability to perform repeated tail-flicking escape-responses compared to the control group. This could be due to a reduction in energy reserves in the tail muscles as a consequence of changes in energy investment due to noise stress, or, alternatively the noise impacted juveniles could have developed impaired or desensitised particle motion sensory systems that did not respond to the hydrodynamic changes caused by an approaching anterior object. No effects of anthropogenic noise were observed on the presence of oxidative stress biomarkers in juvenile *N. norvegicus*, indicating that noise impacts were not severe enough to significantly alter metabolism and release ROS as a result.

Unlike *N. norvegicus*, *H. gammarus* larvae exhibited no changes to developmental rate in response to anthropogenic noise. Due to low survival in all treatments, follow-on experiments on juveniles were not possible.

Eggs/embryos of the squid *L. forbesii* (see chapter 5) were chosen as an early life stage representative of highly mobile, predatory, neritic cephalopod species. Of all invertebrate taxa studied, adult cephalopods so far have the greatest body of evidence supporting their ability to detect, respond to, and be negatively impacted by underwater sound. In contrast to its adult mode of life, early life *L. forbesii* develops in sessile egg strands for 1-2 months and is therefore unable to move away from unwanted noise for this period. Like *N. norvegicus,* naïve *L. forbesii* embryos significantly increased oxygen consumption in response to noise, however this was stage-dependent, with no effect of noise observed on embryos that were almost ready to hatch (possibly due to the effect being masked by the pre-hatching increase in oxygen consumption across all treatment groups).

Larvae of the oyster *O. edulis* were chosen as early life stage representative models for sessile bivalves. The settlement rate and success of settlement-competent *O. edulis* pediveliger larvae were unaffected by ship noise noise playbacks, regardless of whether they were reared in proximity to preferred settlement substrate or not. However, the survival and active swimming behaviour of *O. edulis* larvae were significantly reduced by ship noise when suitable settlement substrate was absent.

The water flea *D. magna* was chosen as a model for a freshwater species as: 1) it is omnipresent in many lake systems and plays key ecological roles in these systems and 2) it has desirable characteristics for a multi-generational noise impact model species. Anthropogenic noise playbacks both significantly delayed the development of newly hatched *D. magna* and reduced reproductive output compared to ambient sound controls, but only after *two* generations of repeated exposure. Fourth generation

individuals reared in control conditions after 3 generations of boat noise exposure retained a significantly reduced reproductive output, demonstrating that noise impacts can be transmitted from parent to offspring and persist for at least one generation.

In summary, the complementary set of laboratory experiments conducted here has revealed a range of adverse anthropogenic noise-induced effects impacting the early life stages of a diverse group of invertebrate model species. Notably, it was demonstrated that impacts can be carried over from larvae to juvenile, as in the case of *N. norvegicus* or from mother to offspring as in the case of *D. magna*. The experimental findings, summarised in terms of the hypotheses generated in section 2.3.3 are as follows:

H_1: Underwater noise will increase the oxygen consumption of the exposed early life stages of the study species (chapters 3 and 5)

Finding: Hypothesis is accepted for N. norvegicus *zoeae and* L. forbesii *embryos*

H_2: Underwater noise will delay the development of the exposed early life stages of the study species (chapters 3, 4 and 7)

Finding: Hypothesis is accepted for N. norvegicus *zoeae and* D. magna*, rejected* for H. gammarus *zoeae*

H_3: Underwater noise will reduce the survival of the exposed early life stages of the study species (chapters 3 – 7)

Finding: Hypothesis is accepted for O. edulis *pediveligers in the absence of preferred settlement substrate. Rejected for all other species.*

H_4: Underwater noise will increase oxidative stress biomarker levels of the exposed early life stages of the study species (chapter 3)

Finding: Hypothesis is rejected for N. norvegicus *juveniles*

H_5: Underwater noise will change the behaviour of the exposed early life stages of the study species (chapters 3 and 6)

Finding: Hypothesis is accepted for N. norvegicus *juveniles (reduced escape responses) and* O. edulis *pediveligers (reduced swimming activity)*

H_6: Underwater noise will reduce the larval settlement rate of the exposed early life stages of the study species (chapter 6)

Finding: Hypothesis is rejected for O. edulis *pediveligers (in the absence of natural reef sounds)*

H_7: Underwater noise will reduce reproductive output/fecundity of the exposed early life stages of the study species (chapter 7)

Finding: Hypothesis is accepted for D. magna, *over multiple generations*

8.2 - Implications

Having addressed the knowledge gaps identified (chapter 2) with a series of controlled laboratory experiments (chapters 3-7, findings summarised in section 8.1), the resultant implications, both in terms of future research needs and regarding policy, are summarized below for all study species and respective experimental outcomes. The relative cause for concern of each experimental finding is summarised in figure 8.1 and the implications of the findings for each model species is summarised in figure 8.2.

The findings of noise impacts on the development, behaviour, and physiology of *N. norvegicus* larvae (chapter 3) highlight the urgent need to consider invertebrate larvae as receptors of noise pollution. By tracking the chosen larval response parameters over the entirety of their planktonic development (zoeal stages I, II and III), it was possible to determine the effects of repeated noise exposure on the survival and development of a sensitive life planktonic stage which has been described as a bottleneck allows a clearer picture of the likely biological significance of noise exposure to be developed than would be possible if acute exposure experiments were used in isolation. By combining acute effects of noise (increased oxygen consumption of noise exposed zoea), with chronic effects (delayed larval development and reduced escape response stamina in juveniles), this thesis has produced a holistic understanding of an organism that is stressed by loud environments in its early life stages and these impacts not only impair development but carry over into reduced anti-predator behaviour beyond the larval stages. If replicated in the field this would possibly lead to critical changes in recruitment success to an already pressured fishery species.

The lack of noise impacts on the development of the *H. gammarus* (like *N. norvegicus* a member of the Nephropidae family) larvae (chapter 4) highlights the difficulty in extrapolating noise impact findings between invertebrate species. Hearing

sensitivities and behavioural responses are likely to be highly adapted according to the particular life history strategy that a species adopts, so divergent responses to noise.

The increase in oxygen consumption of developing *L. forbesii* embryos (chapter 5) indicates that their statocysts are already competent to sense noise and cause changes in physiology even prior to hatching. These results complement the impacts of noise on behaviour and statocyst damage that have been observed in adult squid, building a picture of an animal that can be impacted by anthropogenic noise throughout its entire life cycle. Managers may wish to consider this information if noisy short-term construction projects are planned to be conducted during time periods when *L. forbesii* eggs strands are developing locally.

The lack of observed negative effects of ship noise playbacks on the settlement rate of *O. edulis* pediveligers (chapter 6) means that there is currently no evidence to suggest that *O. edulis* restoration projects across the North-East Atlantic (see Pogoda *et al.* (2019)) should expect an impaired settlement rate of *O. edulis* pediveligers as a result of localised boating traffic noise. This is of course provided that they can secure a reliable supply of larvae and ensure that other important settlement prerequisites such as suitable substrate (e.g. dead oyster shells (cultch)) and biofilms are present. However, the reduced swimming activity and survival of noise-exposed *O. edulis* larvae in the absence of suitable substrate, if present in the field also, would impact their transport over areas of e.g. the North Sea with poor settlement substrate and busy shipping lanes. The main recommendation based on the experimental findings, is that restoration managers should aim to promote retention of released larvae in their restored beds by increasing the 3-D complexity and settlement surface area of the sea floor with the addition of cultch and manage expectations for long distance larval transport (and the genetic-flow benefits this may bring) until a substantial network of close proximity restored reefs is achieved.

The fact that noise impacted *D. magna* (see chapter 7) showed no observable impacts of anthropogenic noise until the second generation of exposure highlights the importance of long-term studies of this type, as an acute noise study observing a single generation would likely have concluded that boat noise impacts do not meaningfully impact *D. magna*. 4th generation individuals reared in control conditions after 3 generations of boat noise exposure retained a reduced reproductive output, demonstrating that noise impacts can be transmitted from parent to offspring and persist for at least one generation. These findings are the first to suggest that anthropogenic noise can elicit multigenerational impacts on aquatic animals. This has implications for the ecological dynamics of freshwater systems, as the population growth of this key microalgae grazer and prey species over the spring and summer may be delayed in noisy systems. The effects this may have on predator species is further complicated by the fact that they themselves could be impacted by noise (Villalobos et al., 2017). The multi-generational impacts of noise observed on *D. magna* has implications for the wider noise impact research field. Even taxa where the impacts of noise are thought to be well-understood, such as marine mammals, have not to my knowledge been investigated for multi-generational noise impacts, quite understandably given the long life-spans and difficulty in quantifying the extent to which highly mobile parents and offspring have been exposed to noise in the field. *D. magna* has therefore emerged as a convenient and potentially fruitful model organism to begin explorations in this area.

Taken together, the effects of underwater noise playbacks on aquatic invertebrates reported in this thesis, ranging from reduced survival to no effect, can be summarised in terms of cause for concern as in figure 8.1. The increase in O_2 consumption by *N. norvegicus* and *L. forbesii* in response to acute noise playbacks, whilst a concern (as this is indicative of a stress response), is ranked lower than other impacts as it is difficult to extrapolate from an acute increase in metabolism to long term negative effects.

Following the same reasoning, the developmental delays observed in *D. magna* and *N. norvegicus* are ranked as higher concern due to their chronic nature and the increased likelihood of predation occurring before an individual can reproduce. The reduction in survival of *O. edulis* pediveligers in response to noise playbacks (and in the absence of preferred settlement substrate) is ranked higher still, due to reduced survival being deemed of more concern to wild populations than developmental delays. Finally, the greatest causes of concern are the reduced reproductive output of *D. magna* and the reduced escape response stamine of *N. norvigus*. In the case of *D. magna* if the reduced reproductive output occurs in the wild, then as a result of *D. magna*'s key role as both prey species and microalgae grazer, the entire freshwater ecosystem may be affected, with reductions in preferred prey species for predatory invertebrates and fish and increases in microalgae in the system, possibly altering water quality. In the case of *N. norvegicus* the stark reductions in the escape responses juveniles reared in noisy conditions is of great concern, as the impact on individual fitness (increased predation) is clear and the fact that individuals retain negative impacts beyond the larval stages is also of critical importance (figure 8.1).

The impacts observed for all model species in this thesis can be used to inform legislative decision makers and managers of the effects of anthropogenic noise on early life invertebrates. In turn, decision makers could combine the results reported here with knowledge of the spatial distribution of anthropogenic noise in the field, to estimate the extent to which impacts may be occurring and generate strategies and further research to uphold their commitments to limiting the impacts of noise on the marine environment.

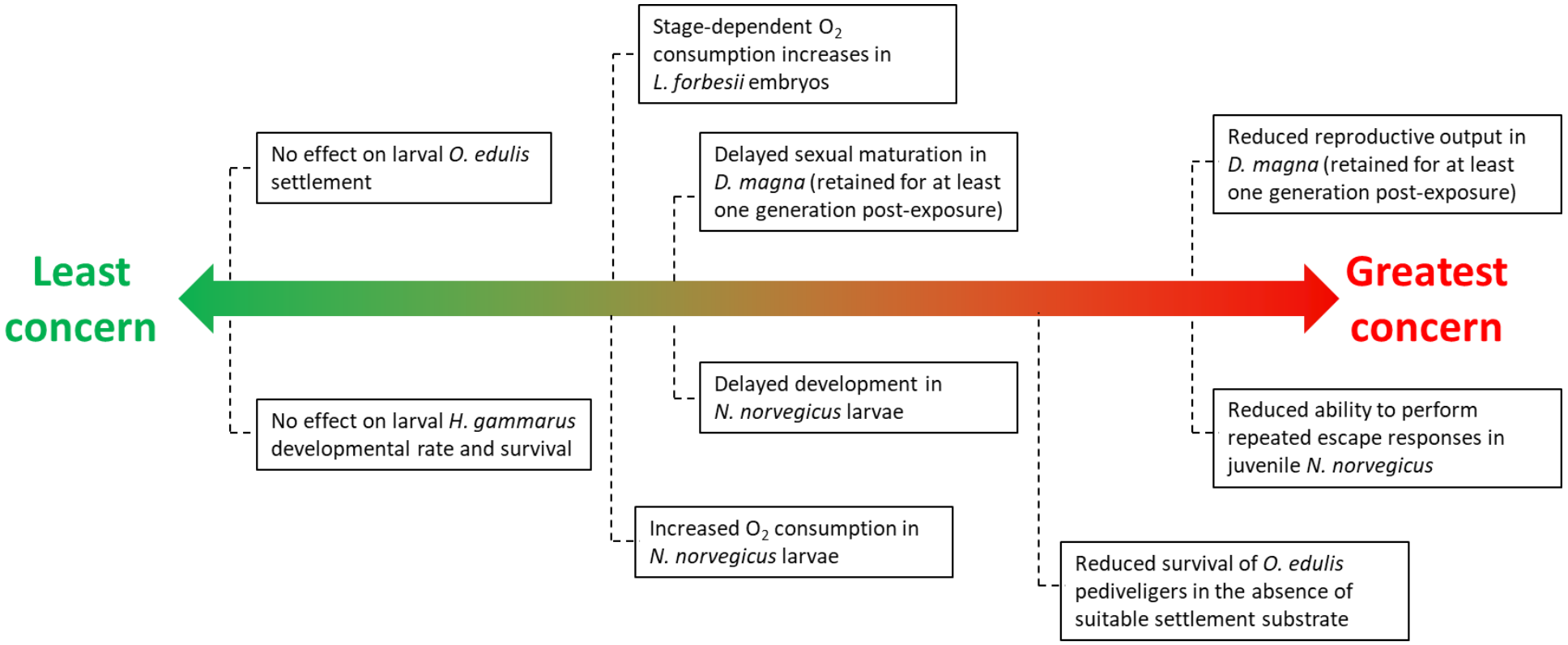


Figure 8.1 The findings of the studies reported in this thesis, ranked according to the relative grounds for concern regarding the likely impacts on the survival, reproduction and recruitment to, wild populations of the species of concern if reproduced in the field.

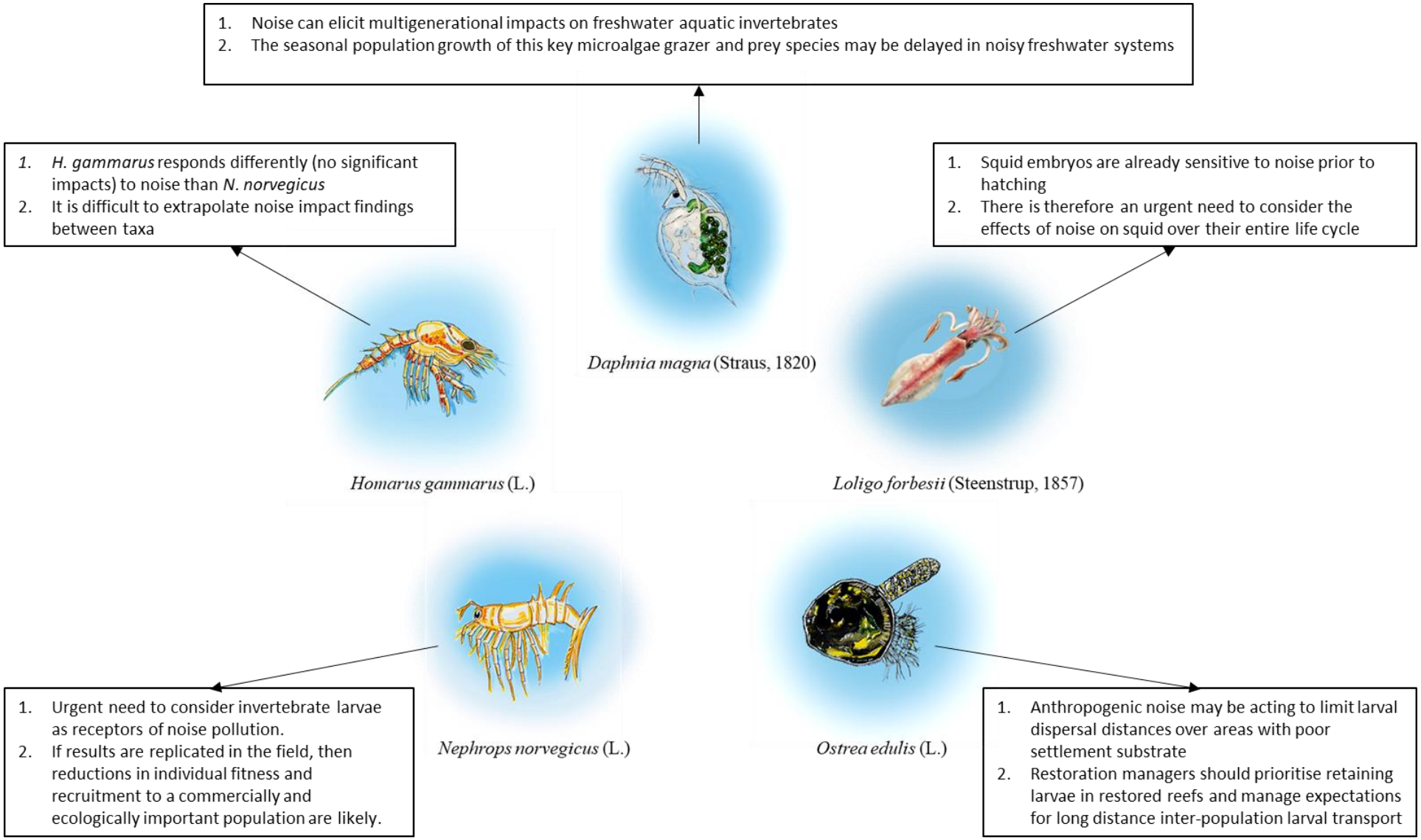


Figure 8.2 The key implications of the noise impact findings presented in this thesis for each model species studied. For picture credits, see appendix C.

8.3 – Future directions

The work produced in this thesis provides a foundation to continue research in several directions. Firstly, the experimental setups utilising individual rearing of larvae whilst simultaneously exposing them to the same sound playback source has proven to be an effective method for investigating noise impacts on early life invertebrates. Researchers who wish to further explore the responses of early life aquatic invertebrates to underwater sound can adapt and replicate these methods for use with a wide range of invertebrate larvae, as there are many ecologically important species yet to be investigated in this way. Experimental set-ups using communal rearing of larvae have tended to produce very high mortality rates (personal observation). One limitation of the methods used here however was the extra effort needed for changing the water and adding food to each individual vial by hand, which of course limits maximum sample sizes and time available for testing of additional response parameters. Given that noise effects can be subtle, future work should aim to replicate sample sizes utilised here (e.g. chapter 3). Development of reliable automated feeding and water change systems, whilst costly, would allow for this work to be carried out more efficiently.

The work conducted on *N. norvegicus, L. forbesii, O. edulis* and *D. magna* has shown biologically significant noise impacts affecting mortality, reproduction, developmental rate, behaviour, and physiology. Future work could attempt to unravel the mechanistic pathways via which these observed impacts occur, either through further exploration of biochemical stress marker levels or changes in gene expression.

Researchers could also transfer some of the analyses used in this thesis to trials in the field, where noise impacts can be observed in a natural environment. Whilst this poses numerous challenges of its own, the results the experimental laboratory work conducted here can help refine the scope of field experiments, identify the noise sources and levels of interest, and highlight appropriate response parameters.

Whilst anthropogenic noise has been the focus of this thesis, there are a range of other potential stressors in the aquatic environment that could not only cause impacts in addition to noise stress, but may also interact with noise in potentially unexpected ways. Stressors can combine in ways that are additive, synergistic (more than the sum of their parts), antagonistic (less than the sum of their parts) or potentiometric (where a non-stressor facilitates the action of another stressor) (Côté et al., 2016). Further work that takes into account the interaction between multiple stressors, including noise, is urgently needed to elucidate the likely overall impacts of a complex and stressful aquatic environment.

Given the multigenerational impacts of anthropogenic noise on *D. magna* and its high suitability as a model organism (e.g. quick generational turnover, clonal mode of reproduction, ease of rearing), there is great potential for future work to begin utilising *D. magna* as a model organism for both the multi-generational impacts of noise and the epigenetic impacts of noise.

8.4 – References

Côté, I. M., Darling, E. S. and Brown, C. J. (2016). Interactions among ecosystem stressors and their importance in conservation. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1824), 20152592. https://doi.org/10.1098/rspb.2015.2592

Pogoda, B., Brown, J., Hancock, B., Preston, J., Pouvreau, S., Kamermans, P., Sanderson, W. and Nordheim, H. Von. (2019). *Living Resources The Native Oyster Restoration Alliance (NORA) and the Berlin Oyster Recommendation : bringing back a key ecosystem engineer by developing and supporting best practice in Europe*.

Villalobos, G., Alison, J. and Christopher, M. D. (2017). Environmental noise reduces predation rate in an aquatic invertebrate. *Journal of Insect Conservation*, *21*(5), 839–847. https://doi.org/10.1007/s10841-017-0023-y

Appendices

Appendix A – Standard Operating Procedures

Appendix A.1 – Glutathione (GSH)

Reduced glutathione (GSH) in gill tissue homogenates is determined according to Smith et al. (2007)[11], adapted from Owens & Belcher (1965)[40]. Briefly, to each well of a 96-well microtiterplate are added (in triplicate) 20 µl gill tissue homogenate, 20 µl 10 mM nitrobenzoic acid (DNTB), 260 µl assay buffer (100 mMol K_2HPO_4, 5 mMol EDTA, pH 7.5), 20 µl 2 U ml^{-1} glutathione reductase (GR). The reaction is then initiated by the addition of 20 µl NADPH and absorbance measured at 412 nm after 6 minutes against a 5-20 µMol GSH standard and total GSH expressed as µMol mg $protein^{-1}$.

References

Smith, C.J., Shaw, B.J., Handy, R.D., 2007. Toxicity of single walled carbon nanotubes to rainbow trout (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects. Aquatic Toxicology 82, 94-109.

Owens, C.W.I., Belcher, R.V., 1965. A colorimetric micro-method for the determination of glutathione. Biochemical Journal 94, 705-711.

GR from Sigma G3664 (168 U/mg protein – 2.77 mg protein in 0.22 ml) = 1:1000 dilution (assay buffer) for 2 U ml^{-1}.

Appendix A.2 – Thiobarbituric acid reacting substances (*TBARS)*

Background

Measurement of thiobarbituric acid reacting substances (TBARS) is one of the most widely used procedures for evaluation of free radical-mediated oxidation of biological systems containing polyunsaturated fatty acids (PUFA). Lipid peroxidation can be viewed as a series of reactions in which first dienes are formed, followed by lipid hydroperoxides (LOOHs), finally terminating with the fragmentation of the PUFA chains to carbonyl compounds that are TBARS (Camejo *et al.*, 1998).

Reagents

1 mol/L butylated hydroxytoluene (2,6-Di-O-tert-butyl-4-methylphenol) (BHT in ethanol)

PBS 1 mmol/L EDTA (pH 7.4)

50% (w/v) trichloroacetic acid (TCA) in distilled water

1.3% (w/v) thiobarbituric acid (TBA) in 0.3% (w/v) NaOH

0.5–25 nmol 1,1,3,3-tetraethoxypropane (TEP in ethanol) (1 mol=220.31 g L^{-1})

Method

Standard preparation

Stock TEP: 1 mol=220.31 g L^{-1}; 97%

919 g L density

919*0.97/220.3 = 4.046 M/l

1)	10ul of TEP Stock in 10ml =		4.046	mMol	
	c1	v1		c2	v2
					final volume
2)	4.046uM	10ul of 1		101.15uM	400ul
3)	100uM	100ul of 2		25.29uM	400ul
4)	25.29uM	200ul of 3	=	15.19uM	333ul
5)	15.19uM	150ul of 4		5.06uM	450ul
6)	5.06uM	200ul of 5		2.53uM	400ul
7)	2.53uM	100ul of 6		0.51uM	500ul

To each well in a 96-well microtiterplate add (in triplicate):

Add 40 µl 0 - 25 µmol TEP
10 µl BHT
140 µl PBS
50 µl TCA
75 µl TBA

Homogenize tissue by hand (using a glass homogenizer) on ice in 5 volumes PBS

To each well in a 96-well microtiterplate add (in triplicate):
40 µl of homogenate
10 µl BHT
140 µl PBS
50 µl TCA
75 µl TBA

Incubate at 60°C (60 min) and cool on ice

Record absorbance first at 530 nm and second at 630 nm.

$$Abs = A_{530nm} - A_{630nm}$$

Determine total protein concentration in homogenate (Bradford, 1976).

Prepare a BSA standard stock solution (1 mg l^{-1})
Prepare a dilution series (1, 0.8, 0.6, 0.4, 0.2, 0 mg l^{-1})
Pipette 10 µl of each standard dilution to five separate wells.
Pipette 290 µl of Bradford reagent.
Incubate for 5 mins, read absorption at 595 nm.

Pipette 10 µl of each homogenate into 5 replicate wells
Homogenates will probably need diluting 1:10
Pipette 290 µl of Bradford reagent.
Incubate for 5 mins, read absorption at 595 nm.

$$TBARS = \frac{Abs}{Protein}\ \mu mol\ mg\ protein^{-1}$$

If diluted, multiply the homogenate protein calculations by the dilution factor!

References

Bouskill, N. J., Handy, R. D., Ford, T. E. & Galloway, T. S. (2006). Differentiating copper and arsenic toxicity using biochemical biomarkers in Asellus aquaticus and Dreissena polymorpha. *Ecotoxicol. Environ. Safety* 65: 342-349.

Bradford, M. M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* 72: 248-254.

Camejo, G., Wallin, B. & Enojärvi, M. (1998). Analysis of oxidation and antioxidants using microtiter plates. In *Free Radical and Antioxidant Protocols. Methods in Molecular Biology*. D. L. Armstrong ed(s), vol. 108: 377-386. Humana Press Inc., Totawa, NJ,.

Smith, C. J., Shaw, B. J. & Handy, R. D. (2007). Toxicity of single walled carbon nanotubes to rainbow trout (Oncorhynchus mykiss): Respiratory toxicity, organ pathologies, and other physiological effects. *Aquat. Toxicol.* 82: 94-109.

PRE-ASSAY PREPARATION

Reagent Preparation

NOTE: Methanol is no longer supplied in this kit. It can be purchased separately under Item No. 707016 or you can supply your own.

1. **Catalase Assay Buffer (10X) – (Item No. 707010)**

   Each vial contains 5 ml of Assay Buffer. Dilute 2 ml of Catalase Assay Buffer concentrate with 18 ml of HPLC-grade water. This final Assay Buffer (100 mM potassium phosphate, pH 7.0) should be used in the assay. When stored at 4°C, this diluted Assay Buffer is stable for at least two months. Prepare the additional vial as needed.

2. **Catalase Sample Buffer (10X) – (Item No. 707012)**

   Each vial contains 10 ml of Sample Buffer. Dilute 5 ml of Catalase Sample Buffer concentrate with 45 ml of HPLC-grade water. This final Sample Buffer (25 mM potassium phosphate, pH 7.5, containing 1 mM EDTA and 0.1% BSA) should be used to dilute the formaldehyde standards, Catalase (Control), and CAT samples prior to assaying. When stored at 4°C, this diluted Sample Buffer is stable for at least two months. Prepare the additional vial as needed.

3. **Catalase Formaldehyde Standard - (Item No. 707014)**

   The vial contains 4.25 M formaldehyde. The reagent is ready to use as supplied.

4. **Catalase (Control) – (Item No. 707013)**

   Each vial contains a lyophilized powder of bovine liver CAT and is used as a positive control. Reconstitute the Catalase (Control) by adding 2 ml of diluted Sample Buffer to the vial and vortex well. Take 100 µl of the reconstituted enzyme and dilute with 1.9 ml of diluted Sample Buffer. A 20 µl aliquot of this diluted enzyme per well causes an absorbance of approximately 0.29 after subtracting the background absorbance. The diluted enzyme is stable for 30 minutes. The reconstituted Catalase (Control) is stable for one month at -20°C.

5. **Catalase Potassium Hydroxide – (Item No. 707015)**

   Each vial contains 4 ml of 10 M potassium hydroxide (KOH). The reagent is ready to use as supplied.

6. **Catalase Hydrogen Peroxide – (Item No. 707011)**

   The vial contains an 8.82 M solution of H_2O_2. Dilute 40 µl of Catalase Hydrogen Peroxide with 9.96 ml of HPLC-grade water. The diluted Hydrogen Peroxide solution is stable for two hours.

7. **Catalase Purpald (Chromogen) – (Item No. 707017)**

   Each vial contains 4 ml of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) in 0.5 M hydrochloric acid. The reagent is ready to use as supplied.

8. **Catalase Potassium Periodate – (Item No. 707018)**

   Each vial contains 1.5 ml of potassium periodate in 0.5 M potassium hydroxide. The reagent is ready to use as supplied.

Sample Preparation

Overheating can inactivate catalase. The enzyme should be kept cold during sample preparation and assaying. In general, catalase is very unstable at high dilution. It is recommended to store samples concentrated and assay within 30 minutes after dilution.

Tissue Homogenate

1. Prior to dissection, either perfuse tissue or rinse tissue with a phosphate buffered saline (PBS) solution, pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue on ice in 5-10 ml of cold buffer (*i.e.*, 50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) per gram tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell Lysate

1. Collect cells by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Homogenize or sonicate the cell pellet on ice in 1-2 ml of cold buffer (*i.e.*, 50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Plasma and Erythrocyte Lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month.
3. Remove the white buffy layer (leukocytes) and discard.
4. Lyse the erythrocytes (red blood cells) in four times its volume of ice-cold HPLC-grade water.
5. Centrifuge at 10,000 x g for 15 minutes at 4°C.
6. Collect the supernatant (erythrocyte lysate) for assaying and store on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.

Serum

1. Collect blood without using an anticoagulant. Allow blood to clot for 30 minutes at 25°C.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.

Tissue Homogenization using the Precellys 24 Homogenizer

- Prior to dissection, either perfuse or rinse tissue with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots.
- Freeze organs immediately upon collection and then store at -80°C. Snap-freezing of tissues in liquid nitrogen is preferred.
- Add cold 50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA.
- Homogenize the tissue sample using the Precellys 24 according to appropriate settings.
- Spin the tissue homogenates at 10,000 x g for 15 minutes at 4°C.
- Collect supernatant and assay samples according to the kit booklet protocol. Samples may need to be diluted appropriately for assay and should be normalized using a protein assay.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. We suggest that there be at least two wells designated as positive controls. A typical layout of formaldehyde standards and samples to be measured in duplicate is shown in Figure 1. We suggest you record the contents of each well on the template sheet provided on page 22.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	B	B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	C	C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	D	D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	E	E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	F	F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	G	G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	+	+	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

A-G = Standards
+ = Positive controls
S1-S40 = Sample wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 240 µl in all the wells.
- All reagents except samples must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- If the expected CAT activity of the sample is not known or if it is expected to be beyond the range of the standard curve, it is prudent to assay the sample at several dilutions.
- It is recommended that the samples and formaldehyde standards be assayed at least in duplicate.
- Use the diluted Assay Buffer in the assay.
- Monitor the absorbance at 540 nm using a plate reader.

Standard Preparation

Preparation of the Formaldehyde Standards - Dilute 10 µl of Catalase Formaldehyde Standard (Item No. 707014) with 9.99 ml of diluted Sample Buffer to obtain a 4.25 mM formaldehyde stock solution. Take seven clean glass test tubes and mark them A-G. Add the amount of formaldehyde stock and diluted Sample Buffer to each tube as described in Table 1 (below).

Tube	Formaldehyde (µl)	Sample Buffer (µl)	Final Concentration (µM formaldehyde)*
A	0	1,000	0
B	10	990	5
C	30	970	15
D	60	940	30
E	90	910	45
F	120	880	60
G	150	850	75

Table 1
*Final formaldehyde concentration in the 170 µl reaction.

Performing the Assay

1. Formaldehyde Standard Wells - Add 100 µl of diluted Assay Buffer, 30 µl of methanol, and 20 µl of standard (tubes A-G) per well in the designated wells on the plate (see Sample plate format, Figure 1, page 13).
2. Positive Control Wells (bovine liver CAT) - Add 100 µl of diluted Assay Buffer, 30 µl of methanol, and 20 µl of diluted Catalase (Control) to two wells.
3. Sample Wells - Add 100 µl of diluted Assay Buffer, 30 µl of methanol, and 20 µl of sample to two wells. To obtain reproducible results, the amount of CAT added to the well should result in an activity between 2-35 nmol/min/ml. When necessary, samples should be diluted with diluted Sample Buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 100,000 to bring the enzymatic activity to this level.
4. Initiate the reactions by adding 20 µl of diluted Hydrogen Peroxide to all the wells being used. Make sure to note the precise time the reaction is initiated and add the diluted Hydrogen Peroxide as quickly as possible.
5. Cover the plate with the plate cover and incubate on a shaker for 20 minutes at room temperature.
6. Add 30 µl of Potassium Hydroxide to each well to terminate the reaction and then add 30 µl of Catalase Purpald (Chromogen) (Item No. 707017) to each well.
7. Cover the plate with the plate cover and incubate for 10 minutes at room temperature on the shaker.
8. Add 10 µl of Catalase Potassium Periodate (Item No. 707018) to each well. Cover with plate cover and incubate five minutes at room temperature on a shaker.
9. Read the absorbance at 540 nm using a plate reader.

ANALYSIS

Calculations

Determination of the Reaction Rate

1. Calculate the average absorbances of each standard and sample.
2. Subtract the average absorbance of standard A from itself and all other standards and samples.
3. Plot the corrected absorbance of standards (from step 2 above) as a function of final formaldehyde concentration (µM) from Table 1. See Figure 2 for a typical standard curve.

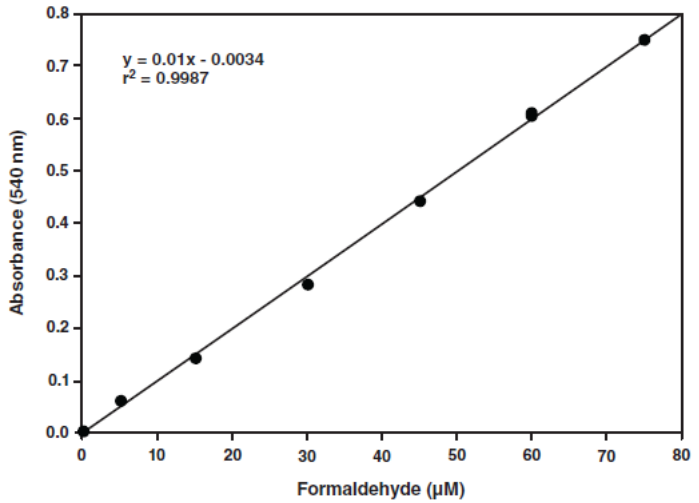


Figure 2. Formaldehyde standard curve

4. Calculate the formaldehyde concentration of the samples using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample.

$$\text{Formaldehyde } (\mu M) = \left[\frac{\text{Sample absorbance - (y-intercept)}}{\text{Slope}}\right] \times \frac{0.17 \text{ ml}}{0.02 \text{ ml}}$$

5. Calculate the CAT activity of the sample using the following equation. One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25°C.

$$\text{CAT Activity} = \frac{\mu M \text{ of Sample}}{20 \text{ min.}} \times \text{Sample dilution} = \text{nmol/min/ml}$$

Performance Characteristics

Sensitivity:

The dynamic range of the assay is limited only by the accuracy of the absorbance measurement. Most plate readers are linear to an absorbance of 1.2. Samples containing CAT activity between 2-35 nmol/min/ml can be assayed without further dilution or concentration.

Precision:

When a series of 45 CAT measurements were performed on the same day, the intra-assay coefficient of variation was 3.8%. When a series of 45 CAT measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 9.9%.

Linearity of the Assay

The dose-response relationship for purified CAT from bovine liver was linear from 5-80 ng of protein (see Figure 3, below). Tissue homogenates, cell lysates, plasma, serum, and erythrocyte lysates also exhibited a linear relationship between the amount of sample and CAT activity over a wide range.

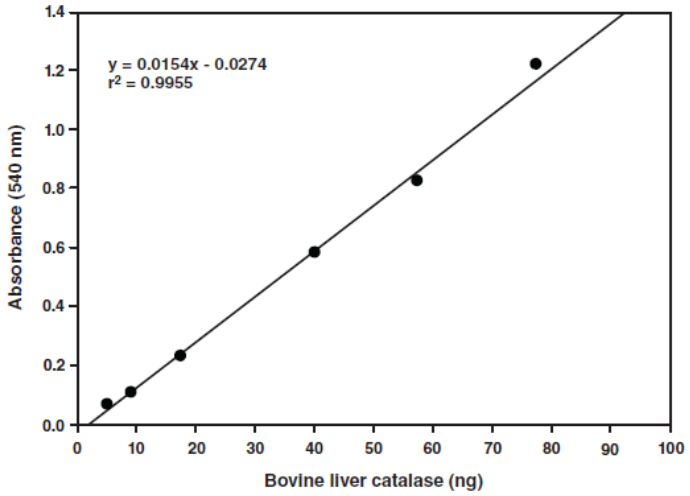


Figure 3. **Absorbance *versus* bovine liver catalase** (ng)

INTRODUCTION

Background

Glutathione peroxidase (GPX) catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and functions to protect the cell from oxidative damage. With the exception of phospholipid-hydroperoxide GPX, a monomer, all of the GPX enzymes are tetramers of four identical subunits.[1,2] Each subunit contains a selenocysteine in the active site which participates directly in the two-electron reduction of the peroxide substrate.[1,2] The enzyme uses glutathione as the ultimate electron donor to regenerate the reduced form of the selenocysteine.[1,2]

About This Assay

Cayman's GPX Assay measures GPX activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPX, is recycled to its reduced state by GR and NADPH:

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GPX}} \text{R-O-H} + \text{GSSG} + \text{H}_2\text{O}$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+$$

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPX activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPX activity in the sample.[3] The Cayman GPX Assay Kit can be used to measure all of the glutathione-dependent peroxidases in plasma, erythrocyte lysates, tissue homogenates, and cell lysates.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **GPX Assay Buffer (10X) - (Item No. 703110)**

   This vial contains 5 ml of a 10X Assay Buffer solution. Once thawed, mix 5 ml GPX Assay Buffer (10X) with 45 ml of HPLC-grade water. This final Assay Buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA) should be used in the assay. When stored at 4°C, this diluted Assay Buffer is stable for at least six months. Prepare the additional vial as needed.

2. **GPX Sample Buffer (10X) - (Item No. 703112)**

   This vial contains 3 ml of a 10X Sample Buffer solution. Once thawed, mix 2 ml GPX Sample Buffer (10X) with 18 ml of HPLC-grade water. This final Sample Buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA and 1 mg/ml BSA) is used to dilute the GPX control (Item No. 703114) and the samples prior to assaying. When stored at 4°C, this diluted Sample Buffer is stable for at least one month.

3. **Glutathione Peroxidase (Control) - (Item No. 703114)**

   This vial contains 50 µl of bovine erythrocyte GPX. To avoid repeated freeze/thaw, the GPX should be aliquoted into several small vials and stored at -20°C. Prior to use, transfer 10 µl of the supplied enzyme to another vial on ice, and add 490 µl of diluted Sample Buffer. The diluted enzyme is stable for four hours on ice. *NOTE: A 20 µl aliquot of this diluted enzyme per well results in a decrease of approximately 0.051 absorbance unit/minute under the standard assay conditions described in* ***Performing the Assay*** *(see page 14).*

4. **GPX Co-Substrate Mixture - (Item No. 703111)**

   This vial contains lyophilized glutathione and glutathione reductase. Reconstitute the contents of the vial with 6 ml of diluted GPX Assay Buffer. One vial of GPX Co-Substrate is sufficient reagent to assay 96 wells. Reconstitute additional vials as needed. The reconstituted GPX Co-Substrate is stable at room temperature for two hours or for two days when stored at 4°C. *NOTE: Do not freeze the reconstituted reagent.*

5. **GPX NADPH - (Item No. 703119)**

   This vial contains lyophilized NADPH. Reconstitute the vial with 6 ml of diluted GPX Assay Buffer. One vial of GPX NADPH is sufficient reagent to assay 96 wells. Reconstitute additional vials as needed. The reconstituted NADPH is stable at room temperature for two hours or for two days when stored at 4°C.

6. **GPX Cumene Hydroperoxide - (Item No. 703118)**

   The 96-well kit contains one 2.5 ml vial of cumene hydroperoxide. The 480-well kit contains one 12 ml vial of cumene hydroperoxide. The reagent is ready to use as supplied.

Sample Preparation

Tissue Homogenate

1. Prior to dissection, perfuse or rinse tissue with a PBS (phosphate buffered saline) solution, pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (*i.e.*, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT) per gram tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell Lysate

1. Collect cells by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes, rather use a rubber policeman.
2. Homogenize the cell pellet in cold buffer (*i.e.*, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Plasma and Erythrocyte Lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month.
3. Dilute the plasma 1:2 with Sample Buffer before assaying.
4. Remove the white buffy layer (leukocytes) and discard.
5. Lyse the erythrocytes (red blood cells) in 4 volumes of ice-cold HPLC-grade water.
6. Centrifuge at 10,000 x g for 15 minutes at 4°C.
7. Collect the supernatant (erythrocyte lysate) for assaying and store on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.
8. Dilute the erythrocyte lysate 1:10-1:20 with Sample Buffer before assaying.

NOTE: It has been reported that heme peroxidase activity of hemoglobin can lead to falsely elevated GPX activity in erythrocyte lysates. There was no significant effect in the GPX activity when assayed with Cumene Hydroperoxide as the substrate. Therefore, it is not necessary to treat the sample with Drabkin's Reagent (potassium ferricyanide/potassium cyanide) to convert hemoglobin to cyanmethemoglobin before assaying.

Tissue Homogenization using the Precellys 24 Homogenizer

- Freeze organs immediately upon collection and then store at -80°C. Snap-freezing of tissues in liquid nitrogen is preferred.
- Add 1 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA and 1 mM DTT) per 100 milligrams of tissue.
- Homogenize the sample using the Precellys 24 according to appropriate settings:

Organ	Speed (rpm)	Cycle Length (seconds)	Cycle Break (seconds)	Number of Cycles	Beads
Heart (aorta)	5,000	30	30	3	CK28 Large Ceramic

- Spin the tissue homogenates at 10,000 x g for 15 minutes at 4°C.
- Collect supernatant and assay samples according to the kit booklet protocol. Samples may need to be diluted appropriately for assay and should be normalized using a protein assay.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as non-enzymatic or background wells. The absorbance rate of these wells must be subtracted from the absorbance rate measured in the GPX sample and control wells. We suggest that there are at least three wells designated as positive controls and that you record the contents of each well on the template sheet provided on page 22.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	B	7	7	7	15	15	15	23	23	23
B	PC	PC	PC	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

B - Background Wells
PC - Positive Control Wells
1-30 - Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells.
- Use different tips to pipette the diluted Assay Buffer, Co-Substrate, NADPH, enzymes, and Cumene Hydroperoxide.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 190 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- The assay is performed at room temperature.
- Monitor the decrease in absorbance at 340 nm.

Performing the Assay

1. **Background Wells** - add 70 µl of Assay Buffer, 50 µl of Co-Substrate Mixture, and 50 µl NADPH to three wells.
2. **Positive Control Wells** - add 50 µl of Assay Buffer, 50 µl of Co-Substrate Mixture, 50 µl NADPH, and 20 µl of diluted GPX (control) to three wells.
3. **Sample Wells** - add 50 µl of Assay Buffer, 50 µl of Co-Substrate Mixture, 50 µl NADPH, and 20 µl of sample to three wells. To obtain reproducible results, the amount of GPX added to the well should decrease the absorbance to between 0.02 and 0.135/min. When necessary, samples should be diluted with Sample Buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to this level. *NOTE: The amount of sample added to the well should always be 20 µl. To determine if an additional sample control should be performed see the Interferences section (page 18).*
4. Initiate the reactions by quickly adding 20 µl of Cumene Hydroperoxide to all the wells being used.
5. Carefully shake the plate for a few seconds to mix.
6. Read the absorbance once every minute at 340 nm using a plate reader to obtain at least 5 time points. *NOTE: The initial absorbance of the sample wells should not be above 1.2 or below 0.5.*

ANALYSIS

Calculations

1. Determine the change in absorbance (ΔA_{340}) per minute by:
   a. Plot the absorbance values as a function of time to obtain the slope (rate) of the linear portion of the curve (a graph is shown on page 16 using bovine erythrocyte GPX)

   OR

   b. Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

$$\Delta A_{340}/\text{min.} = \frac{*|\, A_{340}\,(\text{Time 2}) - A_{340}\,(\text{Time 1})\,|}{\text{Time 2 (min.)} - \text{Time 1 (min.)}}$$

*Use the absolute value.

2. Determine the rate of ΔA_{340}/min. for the background of non-enzymatic wells and subtract this rate from that of the sample wells.
3. Use the following formula to calculate the GPX activity. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of 0.00373 μM^{-1}*. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to $NADP^+$ per minute at 25°C.

$$\text{GPX activity} = \frac{\Delta A_{340}/\text{min.}}{0.00373\ \mu M^{-1}} \times \frac{0.19\ \text{ml}}{0.02\ \text{ml}} \times \text{Sample dilution} = \text{nmol/min/ml}$$

*The actual extinction coefficient for NADPH at 340 nm is 0.00622 $\mu M^{-1}cm^{-1}$. This value has been adjusted for the pathlength of the solution in the well (0.6 cm).

Appendix A.5 – Superoxide dismutase (SOD)

Sigma-Aldrich.

www.sigmaaldrich.com

19160 SOD Determination Kit

Application

Superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. In order to determine the SOD activity, several direct and indirect methods have been developed. Among these methods, an indirect method using nitroblue tetrazolium (NBT) is commonly used due to its convenience and ease of use. However, there are several disadvantages to the NBT method, such as poor water solubility of the formazan dye and the interaction with the reduced form of xanthine oxidase.

The SOD Determination Kit allows very convenient SOD assaying by utilizing the highly water-soluble tetrazolium salt, WST-1 [2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt] that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 are linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD, as shown in Figure 1. Therefore, the IC_{50} (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method.
Figure 2 shows the absorption spectrum of WST-1 formazan. Since the absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity can be quantified by measuring the decrease in the color development at 440 nm.

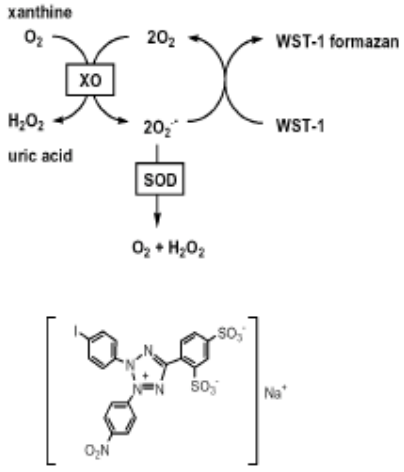


Figure 1: Principle of the SOD Assay Kit

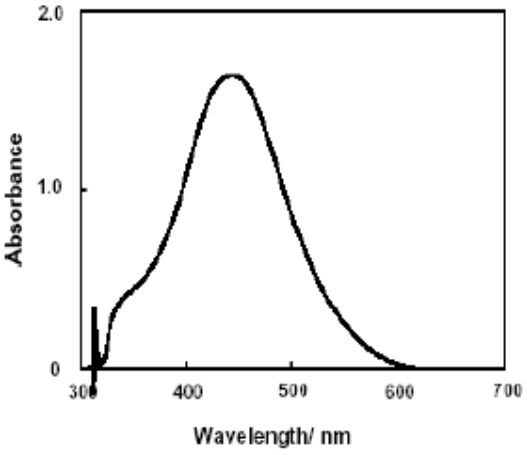


Figure 2. Absorption spectrum of WST-1 formazan.

Components

- WST Solution 5 ml
- Enzyme Solution 100 µl
- Buffer Solution 100 ml
- Dilution Buffer 50 ml

Notes

1) For an accurate measurement, the use of multiple wells per sample is recommended (see Figure 3).
2) Since superoxide will be released immediately after the addition of Enzyme Working Solution to a well, use a multi-channel pipette to avoid the reaction time lag of each well.
3) Inhibition activity can also be determined by a kinetic method. Determine an incubation time range that has a linearity of the slope before the assay. A good linearity should be observed up to 20 min. For the calculation, use the following equation:

SOD activity (inhibition rate %) = {[(S1 - S3) - (SS - S2)] / (S1 – S3)} x 100%
S1: slope of Blank 1
S2: slope of Blank 2
S3: slope of Blank 3
SS: slope of Sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	SOD 200 U/ml			Blank 1			Blank 2			Blank 3		
B	SOD 100 U/ml			SOD 0.05 U/ml			SOD 0.01 U/ml			SOD 0.001 U/ml		
C	SOD 50 U/ml			Sample 1			Sample 7			Sample 13		
D	SOD 20 U/ml			Sample 2			Sample 8			Sample 14		
E	SOD 10 U/ml			Sample 3			Sample 9			Sample 15		
F	SOD 5 U/ml			Sample 4			Sample 10			Sample 16		
G	SOD 1 U/ml			Sample 5			Sample 11			Sample 17		
H	SOD 0.1 U/ml			Sample 6			Sample 12			Sample 18		

Figure 3: Sample and blank arrangement on a 96-well plate including SOD standard solutions.

Interfering agents

Reducing agents, such as ascorbic acids and reduced forms of glutathiones, interfere with the SOD assay. The following are the concentrations of materials that cause 10% increase in the O.D. value. Please note that since the increase in the O.D. values can be subtracted as the O.D. of Blank 2, these materials do not interfere with the actual SOD assay.

Bovine Serum Albumin	5% w/v
Ascorbic acid	0.1 mM
Glutathione, reduced form	5 mM

Appendix B – Jarowski's Medium Recipe

culturecollection of algae and protozoa

media recipes

JM (Jaworski's Medium)

Freshwater algae

Stocks		**per 200 ml**
(1)	$Ca(NO_3)_2.4H_2O$	4.0 g
(2)	KH_2PO_4	2.48 g
(3)	$MgSO_4.7H_2O$	10.0 g
(4)	$NaHCO_3$	3.18 g
(5)	EDTAFeNa	0.45 g
	$EDTANa_2$	0.45 g
(6)	H_3BO_3	0.496 g
	$MnCl_2.4H_2O$	0.278 g
	$(NH_4)6Mo_7O_{24}.4H_2O$	0.20 g
(7)	Cyanocobalamin	0.008 g
	Thiamine HCl	0.008 g
	Biotin	0.008 g
(8)	$NaNO_3$	16.0 g
(9)	$Na_2HPO_4.12H_2O$	7.2 g

Medium	**per litre**
Stock solutions 1 - 9	1 ml each

Make up to 1 litre with deionized water. For agar, add 15.0 g per litre of Bacteriological Agar (Oxoid L11)*. Autoclave at 15 psi for 15 minutes.

Supply
* Unipath Ltd, Wade Road, Basingstoke, Hants, RG24 0PW, UK

CCAP (Culture Collection of Algae and Protozoa), Dunstaffnage Marine Laboratory,
Oban, Argyll, PA37 1QA, UK
Tel: +44 (0)1631 559000 Fax: +44 (0)1631 559001 Email: ccap@sams.ac.uk Web: www.ccap.ac.uk

Appendix C – Pictures used

All watercolour paintings and drawings used for the chapter title pages in this thesis have been produced by Sophie Lane specifically for this thesis, except for the veined squid image (chapter 5) which is modified from a public domain image originally produced by Jatta di Guiseppe (1896) in Cefalopodi viventi nel Golfo di Napoli (sistematica), Berlin: R. Friedländer & Sohn.