Effects of Anthropogenic Sound and Environmental Chemistry on Early Life Stage Aquatic Invertebrates in a Multiple Driver Context

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

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: 06/06/2023

Abstract

Aquatic environments and the species therein are facing unprecedented pressures and challenges as a result of anthropogenic activities. These challenges often are considered and discussed individually, e.g. sound/noise, chemical pollution, nutrient availability, salinity etc., but in reality many pressures (drivers) are linked, with complex interplays and interactions often occurring across multiple levels of biological organisation. Given that aquatic sound levels from anthropogenic sources have increased substantially over the past century, and many marine ecosystems continue to be influenced by a wide range of physicochemical drivers, the aim of this thesis was to establish what, if any, interaction occurs between anthropogenic sound (as laboratory-based sound playbacks) and selected environmental drivers. Biological impacts of exposure to anthropogenic sound playbacks (pile driving and/or passenger ferry) were investigated across different chemical drivers characteristic of marine, brackish, and freshwater environments. Three respective model species were chosen (Norway lobster *Nephrops norvegicus*, Pacific oyster *Magallana gigas,* great pond snail *Lymnaea stagnalis*), each enabling assessment of a different early developmental stage. Anthropogenic sound exposure had significant impacts on two of the three species examined. In *N. norvegicus*, piling playbacks led to concentration-dependent interactions with waterborne cadmium, demonstrating both antagonistic and synergistic interactions with respect to larval mortality. In *L. stagnalis*, ferry playbacks interacted with waterborne calcium availability, with detrimental impacts on embryonic developmental success, and postulated fecundity-related energetic partitioning. In *M. gigas*, no significant differences in fertilisation rates, or immediate larval development, were observed following acute exposure of gametes to either

ferry- or piling playbacks at different salinities. This thesis evidences the need for wider, more integrated consideration of multiple drivers to environmental risk-assessment. It also reflects upon the inherent challenges in undertaking and interpreting such complex studies, helping to inform experimental design of future multiple driver studies.

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Table of Contents

List of Figures

List of Tables

Abbreviations

0-pk	zero to peak
ANOVA	analysis of variance
ASTM	American Society for Testing and Materials
ATP	adenosine triphosphate
c	sound speed
CAT	catalase
Cd	cadmium
dB	decibel
DNA	deoxyribonucleic acid
ES	exposure scenario
f	sound frequency
FDR	false detection rate
FFT	fast Fourier transform
λ	sound wavelength
GLMM	generalised linear mixed model
GpX	glutathione peroxidase
GSH	glutathione
Hz	hertz

I	intensity
ICP-MS	inductively coupled plasma mass spectrometry
IUCN	International Union of Conservation of Nature
kHz	kilohertz
LPO	lipid peroxidase
MT	metallothionein
NC3Rs	National Centre for the Replacement, Refinement & Reduction of Animals in Research
OECD	Organisation for Economic Co-operation and Development
p	sound pressure
Pa	Pascal
PCA	principal component analysis
PERMANOVA	permutation analysis of variance
pk-pk	peak to peak
PM	particle motion
PSD	power spectral density
PTS	permanent threshold shift
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
REML	residual maximum likelihood
ρ	density

RMS	root mean square
RO	reverse osmosis
ROS	reactive oxygen species
SD	standard deviation
SE	standard error
SEL	sound exposure level
SMR	standard metabolic rate
SOD	superoxide dismutase
SPL	sound pressure level
TBARS	thiobarbituric acid reactive substances
TTS	temporary threshold shift
µm	micrometre
µPa	micropascal
V	volt

Chapter 1
Overarching Introduction

Aquatic environments are a highly complex combination of biological, chemical, and physical factors, all of which have an overall bearing on the health and ecological success of the organisms therein. Such factors, when deviating from established normative ranges to an extent observed to provoke biological affects, are often referred to as pressures and/or drivers. Whilst these two terms have subtly different connotations, particularly in the context of socio-economic origins of anthropogenic factors (EEA, 1999), the term 'drivers' has more recently been proposed a catch-all descriptor of specific factors, either natural and anthropogenic, capable of affecting biological or ecological change (Boyd, Collins, et al., 2018) – simplifying and consolidating the terminology to improve reporting terminology. Yet, even knowing that organisms in aquatic environments are subject to numerous co-occurring drivers, scientific research tends towards a singular focus – investigating drivers of interest in isolation, rather than in a wider multiple driver context.

This thesis builds upon current understanding of how anthropogenic sound affects early life stage aquatic invertebrates, exploring how concurrent exposure to ecologically relevant environmental chemical drivers may impact upon effects of sound exposures, and vice versa, through a series of controlled laboratory studies. The novelty and scope of research addressed within this thesis makes a focused systematic literature review impractical. Rather, each experimental chapter outlines its specific justification, and is framed within background-specific literature, whilst the remainder of this overarching introductory chapter highlights key principles and more general

literature on both effects of sound, and multiple driver research. Where possible, the included literature aligns with the thesis' focus on invertebrates and early life stages. However, where invertebrate-specific examples of key concepts are lacking, examples are provided from other taxa to provide the wider context and fundamental knowledge base upon which to interpret the remainder of the thesis.

1.1 Importance of early life stage models

In the past decade or so, there has been an increasing movement towards the use of early life stage organisms for risk identification, particularly in specialist fields like ecotoxicology. An initial motivator for this was one of practicality, with embryo/larval assays being less resource intensive, with reduced lead-times compared to traditional organism testing models. This enables quicker and more efficient turnaround of studies, which is imperative in light of directives such as the European Union's Registration, Evaluation, Authorisation and restriction of CHemicals (REACH) which require a comprehensive understanding of hazards posed by all newly marketed chemicals (Capela et al., 2020). Use of embryo/larval assays also better achieves animal welfare imperatives including the likes of those spearheaded by the UK's National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) (Braunbeck et al., 2014; OECD, 2013).

Aside of the practical and logistic benefits of early life stage models, they also offer unique opportunities and specific benefits. For one, early life stage organisms are often more sensitive to environmental factors than adult stages (Braunbeck et al., 2014; Pineda et al., 2012), commonly providing improved limits of detection and resolution compared to their adult counterparts (Lange et al., 1995; K. S. Wang et al., 2011). Additionally, embryo/larval assays offer the ability to assess mutagenic, teratogenic and endocrine disruptive effects, all of which are of particular concern during

organogenesis (Bigsby et al., 1999; Bommarito et al., 2017; Mouche et al., 2011). Understanding such anthropogenic risks is particularly true of early life stages given they represent potential population bottlenecks (J. Pineda et al., 2010). Being able to develop early life stage models, particularly in species used as mature life stage model species, also opens the possibility to develop full life-cycle models, and hence to potentially assess multi-generational effects.

1.2 Underwater sound

1.2.1 Fundamentals of acoustics

Sound is a mechanical compression wave resultant of a vibratory or oscillatory source. Sound waves are comprised of, and can be characterised by, two components: a pressure wave, and a particle motion. Upon stimulation from a vibratory source, particles within a medium (solid, liquid, or gas) gain energy in the form of longitudinal oscillatory motion, which is in turn transferred to adjacent particles via contact, propagating the energy. During this process, the displacement of particles in the medium results in regional compression and rarefaction, leading areas of higher and lower pressure relative to the steady state of the medium, which propagate in the form of a pressure wave.

1.2.1.1 Basic terminology

Sound is commonly considered in terms of its pressure component, with descriptive metrics derived from the key characteristics of the sound wave (Figure 1.1). For a given periodic sound, the wavelength (λ) is the physical length of one full cycle of the sound wave – the distance between two sequential regions of either compression or rarefaction. The frequency of the sound wave (*f*) is a measure of the number of sound

waves cycles occurring in a one second duration, expressed in Hertz (Hz). The amplitude of the sound wave is the pressure differential from the steady-state pressure to the peak pressure.

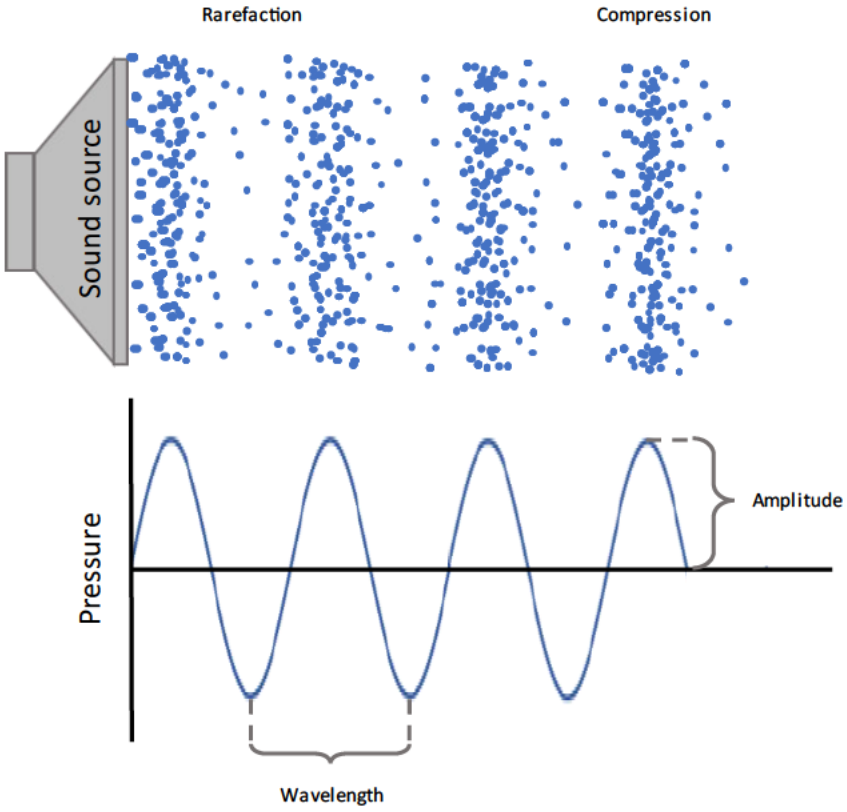

Figure 1.1: Visual schematic of sound. *Top: Representation showing regions of compression and rarefaction of particles within the media. Bottom: Representation of corresponding sound pressure waveform.*

Particle motion, being the physical motion element of sound, can be measured as either displacement, speed, or acceleration of the particle. Unlike sound pressure, which is a scalar measurement, particle motion has both magnitudinal and directional elements, and can therefore be expressed as a vector.

1.2.1.2 Sound speed and propagation

For any given sound speed (c), the frequency (f) and wavelength (λ) of sound are relate accordingly: $c = \lambda f$

The speed of sound (c) in liquids is determined by the density (ρ) and the bulk modulus (K_s) of the fluid according to the relationship:

$$c = \sqrt{\rho / K_s}$$

Correspondingly, although the precise speed of sound will vary according to specific properties of the medium, sound travels far quicker in water (~1500 ms^{-1}) compared with air (~330 ms^{-1}). This is because of the reduced distance/time oscillating particles must travel before colliding with a neighbouring particle and propagating their additional energy/motion. During propagation, energy is inevitably lost to the environment (attenuated) consequent of thermodynamic laws, with these losses being proportional to the rate at which the pressure within the medium is being distorted. This leads to frequency dependent rates of sound attenuation, with high frequency sounds dissipating more rapidly that low frequency sounds. Likewise, interactions of sound waves with environmental features (waves, sea floor, sea surface, etc.) can lead to refraction, reflection, scattering and absorption of sound, all of which will alter how the profile of a sound changes from source to receiver.

1.2.1.3 Measuring sound

The decibel

Sound is commonly discussed in the unit of decibels (dB), though the decibel is not *strictly* a unit in and of itself. Rather, a decibel is a *relative* logarithmic unit measured against a stated reference unit, calculated as:

$$dB\ re\ i_{ref} = 10 \log_{10} \left(i / i_{ref} \right)$$

i = measured value, and i_{ref} = reference value.

As such, the term decibel is meaningless without the context of the reference unit. The use of decibel units enables values varying over large ranges to be reduced to more manageable numbers.

Energy, power, and intensity

As stated above, in its simplest form sound is simply a form of energy. At its source, energy equates to the square of the sound pressure, with the power (measured in Watts) being the rate at which sound energy is being produced. At any distance from the source, sound will be subject to propagation losses and dispersion of sound energy across the sound wavefront; this is measured as intensity (*I*), which is the sound energy per unit area, where:

$$I = \frac{p^2}{\rho c} \qquad p = \text{pressure}, \rho = \text{density}, c = \text{speed of sound}$$

Substituting intensity into the decibel equation and simplifying gives the following:

$$\text{dB re } p_{ref} = 10 \log_{10} \frac{p^2}{{p_{ref}}^2} \qquad p = \text{pressure}, \ p_{ref} = \text{reference pressure}$$

For acoustics in water, p_{ref} is given as 1 µPa. For sound pressure level (SPL) calculations, this further simplifies the equation to:

$$\text{SPL (dB re 1 µPa)} = 20 \log_{10} p \qquad p = \text{pressure}$$

Waveform metrics

Broadly speaking, sound can be classified as being either non-impulsive (e.g. ships/boats) or impulsive (e.g. impact pile driving, seismic air guns, sonar), with impulsive sound signals displaying traits such as rapid onset, short rise-times, and high degrees of kurtosis (Hastie et al., 2019; Martin et al., 2020; Southall et al., 2019). However, there remains no defined threshold and/or mathematical function delineating the boundary between non-impulsive and impulsive sounds (International Organization for Standardization, 2016).

Although wavelength and amplitude are sufficient for characterising idealised periodic waveforms (e.g. sine waves), they are less meaningful for characterising real-world sounds which often vary by amplitude, contain multiple sound frequencies, and have varying degrees of impulsivity. Resultantly, additional metrics exist which have more applicability for comparing scalar sound waveforms, both of pressure and/or particle motion.

- **Zero to peak (0-pk)** – the magnitudinal differential between the steady-state and the maximum signal amplitude (positive or negative)
- **Peak to peak (pk-pk)** – the magnitudinal differential between the maximum positive signal amplitude and the maximum negative signal amplitude
- **Root mean square (RMS)** – mean magnitudinal signal amplitude
- **Sound exposure level (SEL)** – a metric for measuring the sum intensity of a sound standardised to stated equivalent duration (normally 1 second).

  For sound pressure in water, a reference value of 1 μPa^2, and an SEL standardised to 1 second, this equates to:

$\text{SEL (dB re 1 µPa}^2\text{.s)} = 10\ \log_{10}(\frac{1}{t}\sum_{i=1}^{n} {p_i}^2)$ t = time (s), p = pressure (µPa)

- **Single strike SEL (SELss)** – As SEL, but applied to single instance of impulsive signal, and calculated over the portion of the signal corresponding to the 90% energy window.
- **Cumulative SEL (SELcum) –** As SEL, but accounting for the entirety of sound exposure during a given time over a prolonged exposure (assuming data for SEL measures does not encompass the entire exposure duration).

  For non-impulsive signals:

  $\text{SELcum} = \text{SEL} + 10\log_{10} t$ t = total duration of sound exposure

  For impulsive signals:

  $\text{SELcum} = \text{SELss} + 10\log_{10} n$ n = number of impulsive instances

Fast Fourier Transform

In addition to basic waveform analyses, sound signals can be transformed to ascertain component frequencies in a process known as Discrete Fourier Transform (DFT). For digital (or digitised) signals, DFT is more commonly estimated using a Fast Fourier Transform (FFT) algorithm, which is computationally more efficient and therefore better suited to large data sets. This involves windowing and overlapping discrete length-segments of signals, with the resolution of both the time and frequency components influenced by the width of these windows, with higher temporal resolution coming at the expense of frequency resolution, and vice versa.

In application, FFT can be used to determine not only the component frequencies of sound, but distribution of sound power across these frequencies. This data can be maintained in a time-domain, allowing visualisation and calculation of how power

distributions vary across the duration of a sound using spectrograms. Alternatively, the time dimension of the spectrographic data can be collapsed, allowing better estimation of sum power distribution within a sound; this is known as power spectral density (PSD).

1.2.2 Sound in an ecological context

Far from being the "silent world" made famous by Jacque Cousteau (Cousteau & Dumas, 1953), aquatic environments are in reality teeming with sound. These sounds can be broadly categorised into three groups based upon their origins – geophony, biophony, and anthrophony. Geophonic sounds have natural, non-biological origins, such as those produced by tectonic activity, weather, or waves. Biophony refers to sounds with biological origins, either those produced actively (e.g. vocalisations and other communicative sounds) or passively (e.g. sounds arising from movement, feeding etc.). Meanwhile anthrophonic sounds are those with purely anthropogenic origins, for example sounds resultant of shipping, construction, seismic surveying etc.

Given sound's ability to efficiently propagate through water, especially compared to light which rapidly attenuates, many aquatic organisms have evolved to utilise sound. Although use of sound in aquatic environments is mostly associated with marine mammals, sound is also used by many other aquatic taxa for various purposes (Duarte et al., 2021). Examples of sound utilisation by non-mammals include communication in fish (Ladich, 2019; A. N. Radford et al., 2014), orientation in crustacean and cnidarian larvae (Jeffs et al., 2003; Montgomery et al., 2006; Vermeij et al., 2010), and predation by the likes of pistol shrimp (Koukouvinis et al., 2017). Additionally, molluscs, cnidarians, ascidians, and echinoderms have all shown a sensitivity to sound in one form or another (Charifi et al., 2017; McDonald et al., 2014; Mooney et al., 2010; Vazzana et al., 2020).

1.2.3 Invertebrate sound perception

Current literature above notwithstanding, significant knowledge gaps remain in our understanding of the relationships between invertebrates and sound (Hawkins et al., 2015). Though there has been increased interest in addressing such knowledge gaps in the past couple of decades, huge disparities in the relative quantities of literature pertaining to invertebrates remain (Wale et al., 2021).

All known auditory systems function by vibratory displacement of sensory hair cells (Nedelec et al., 2016). In mammals, as a result of past evolutionary adaptations to non-aquatic lifestyles (Duarte et al., 2021), sound pressure must be converted into fluid-based particle motion within the inner ear (Ekdale, 2016). Meanwhile aquatic invertebrates, lacking any evolution favouring air-based sound sensitivity, being directly coupled to water through their body forms, and lacking air-filled cavities, are better evolved to detect particle motion directly (Nedelec et al., 2016), with many invertebrates having mechanosensory structures physiologically analogous to the mammalian inner ear (Hawkins & Popper, 2017).

Notably, given the sensitivity of aquatic invertebrates to particle motion, benthic invertebrates are almost certainly sensitive to substrate vibration (Hawkins et al., 2021; Roberts & Elliott, 2017). How aquatic invertebrates delineate between waterborne particle motion and substrate vibration (if at all) is uncertain. From the perspective of evolution, it nonetheless seems likely that marine invertebrates can distinguish between the two; possibly through specific differences in the particle motion behaviours between media (Hazelwood & Macey, 2021; Hill, 2009).

Based upon attempts to ascertain the sound frequency sensitivities of invertebrates, and the attenuation of sound particle motion, the prevailing understanding is that the auditory capabilities of aquatic invertebrates are generally sensitive to particle motion

at frequencies below 1000 Hz (Hawkins et al., 2015), though sensitivities up to 3000 Hz (Lovell et al., 2005) and 5000 Hz (Pye & Watson, 2004) have been independently ascertained for decapod crustacean species. Moreover, a recent study showed that decapod crustacea may have increased sensitivity to a combination of particle motion and sound pressure stimulus than to particle motion alone (C. A. Radford et al., 2022), challenging the current paradigm that only particle motion is relevant to sound detection in invertebrates.

1.2.4 Impacts of anthropogenic sound

Anthrophonic sounds are a growing concern to aquatic (particularly marine) environments given their increasing pervasiveness resultant of activities including shipping, offshore construction, and seismic surveying (Hildebrand, 2004; Simmonds et al., 2014). Despite growing awareness and evidence of negative impacts of anthropogenic sound on marine organisms, sound-producing activities face little in the way of regulation and mitigation. Despite acceptance of anthropogenic sound as a pollutant, it does not currently fall within the specific remit of any internationally agreed regulatory framework (Y. C. Chang & Zhang, 2021). Indeed, Germany is currently the only nation to impose legal restrictions on anthropogenic sound levels, placing limits on peak-to-peak sound pressure levels and sound exposure levels generated by offshore pile driving (Müller et al., 2019). In a similar vein, though noise abatement techniques and technologies exist, questions remain over their efficacy given contextual differences in efficiency, and uncertainties in their employment (Burnham et al., 2021; Tsouvalas & Metrikine, 2016). Moreover, where these restrictions and abatement techniques are applied, it is with the principal intention of preventing permanent harm to marine mammals, and bears little regard to impacts on non-mammals, and more widespread sub-acute effects of sound (Popper et al., 2020).

Examples of such ecological and physiological impacts of anthropogenic sound are given below.

1.2.4.1 Acoustic masking

One concern of anthropogenic sounds is their ability to disrupt or interfere with communicative and orientative applications of sound. To avoid masking, the term for a given sound being obstructed from detection by a louder competing sound of similar frequency (Oxenham, 2013), many species have evolved to utilise sound at frequencies and/or in temporal windows where geophonic and biophonic competition is minimal or non-existent (Hart et al., 2021; Krause & Farina, 2016). Indeed, in passive acoustic monitoring (PAM), frequency is a key component for ascertaining presence of organisms, and even identifying species (Coquereau et al., 2016; Lin et al., 2013; Mouy et al., 2018; L. Zhang et al., 2012). Likewise, sound is a known orientation cue, with evidence that some planktonic larvae are attracted to, and encouraged to settle towards the sounds of biogenic reefs (Lillis et al., 2013, 2015). Unfortunately, many anthropogenic sounds comprise sound frequencies which can mask communicative sounds, reducing their effective range (Clark et al., 2009). Similarly, anthropogenic sounds can interfere with auditory settlement cues to the detriment larval recruitment (Lecchini et al., 2018). The potential negative impacts notwithstanding, there is at least some evidence that organisms can adapt to the challenges of masking through behavioural plasticity (Parks et al., 2007; Simpson et al., 2010). Concerns of masking are not solely restricted to non-impulsive anthropogenic sounds, as the impulsive traits of sound diminish during propagation, leading to increased signal durations (Hastie et al., 2019). Resultantly, repetitive impulsive sources such as pile driving or seismic airguns can effectively manifest as continuous, non-impulsive sounds at distance (Clark et al., 2009).

1.2.4.2 Sensory impairment

Anthropogenic sound exposure has the potential to impair the sound reception capabilities of a species, either temporarily or permanently. Hearing impairment is arguably a more persistent concern given onset of its impacts extend beyond the duration of sound exposure, as opposed to masking where onset of impacts are restricted to the exposure period. Again, research in hearing impairment is heavily weighted towards mammals, and particularly the induction of temporary threshold shifts (TTS) or permanent threshold shifts (PTS) in auditory sensitivity (Ryan et al., 2016a). The pathologies of both TTS and PTS are numerous and complex, but in short, recoverable TTS is caused by fatigue of the auditory systems, whereas non-recoverable PTS results from irreparable physical damage (Ryan et al., 2016b). Threshold shifts are dependent upon the sound exposure, with onset and recovery period from TTS being a function of both the amplitude and duration of sound exposures (Hirsh & Bilger, 1955). Impulsive sounds are also more likely to result in hearing impairment than non-impulsive sounds, as reflected in the current commonly accepted TTS and PTS onset threshold estimates for marine mammals (Southall et al., 2019).

Auditory impairment has also been observed in non-mammals, with TTS evidenced in fish and shown to correspond to the same linear, cumulative trends as in mammals (Amoser & Ladich, 2003; M. E. Smith et al., 2004). Despite some defined auditory thresholds of aquatic invertebrates including cephalopods (Hu et al., 2009; Mooney et al., 2010) and crustacea (Lovell et al., 2005; C. A. Radford et al., 2016), literature on TTS and PTS in invertebrates is notably absent from current literature; possibly reflecting the difficulties and uncertainties in ascertaining reliable auditory thresholds in such creatures (Hawkins et al., 2015). Nonetheless, histological observations have revealed anthropogenic sound-induced structural damage to hearing apparatus in crustaceans

(Day et al., 2019), cniderians (Solé et al., 2016), and molluscs (André et al., 2011; Day et al., 2017; Solé et al., 2013). Long-term implications of damage to hearing systems in invertebrates are seemingly uncertain, as is the potential and propensity for recovery; though Day et al (2020) observed rock lobsters (*Jasus edwardsii*) apparent ability to cope with and/or adapt to such damage. Sensory impairment resultant of sound exposure may not solely be limited to changes in acoustic/vibratory sensitivity, as evidence of cross-modal impairment to olfactory cues has also been postulated (Roberts & Laidre, 2019).

1.2.4.3 Behavioural changes

Anthropogenic sound exposures can have behavioural and physical impacts on invertebrates extending beyond merely how sound signals are perceived. Boat sounds have been shown to provoke positive phonotaxis and induced settlement in the ascidian *Ciona intestinalis* (McDonald et al., 2014), and negative phonotaxis in the crab *Neohelice granulata* (Sal Moyano et al., 2021); both with potential to hamper larval recruitment. However negative phototaxis and/or avoidance behaviours are a classic example of a behavioural stress responses, with such avoidance behaviours leading to the exclusion of species from habitats during offshore construction (Russell et al., 2016). Stress-induced behavioural changes are particularly evident in shoaling fish, with pile driving playbacks resulting in seabass *(Dicentrarchus labrax)* shoaling behaviour becoming less cohesive, with individuals displaying lower correlation in swimming speed and directionality (Herbert-Read et al., 2017). Field-based air-gun exposures similarly led to fish increasing swimming depth and forming tighter and exhibiting faster shoaling behaviour (Fewtrell & McCauley, 2012). Stress-related behaviours have also been observed in bivalve molluscs, with anthropogenic sound

exposures resulting in decreased valve activity, ventilation, and algal clearance rates (Charifi et al., 2017; Shi et al., 2019).

Another means by which anthropogenic sound can illicit behavioural change is via distraction. Anthropogenic sound has been observed to impact factors including foraging success, with three-spined stickleback (*Gasterosteus aculeatus*) showing reduced prey consumption resultant of decreased predation success rate during sound exposures (Voellmy et al., 2014), with similar reductions in feeding rates demonstrated by damselfly larvae (*Ischnura elegans*) consequent of increased food handling time (Villalobos-Jiménez et al., 2017). There is also evidence that anthropogenic sound exposures can hamper cognitive behaviours, with fish exposed to boat playbacks showing reduced capacity in learning to recognise, and adapt to, novel predators to the detriment of survival under future predation (Ferrari et al., 2018).

1.2.4.4 Survival and development

In addition to general knowledge gaps pertaining to effects of sound on invertebrates, a specific need to understand responses of different life stages, particularly early life stages, has previously been stated (Carroll et al., 2017; Hawkins et al., 2015).

Seismic pulse playbacks increased developmental duration and rates of developmental abnormality in New Zealand scallops (de Soto et al., 2013), with brachyuran crab development being slower in response to turbine playbacks (Pine et al., 2012). Boat sound playbacks promoted mortality of larval and early life stage sea hares (*Stylocheilus striatus*) (Nedelec et al., 2014), and reduced the size of newly hatched cod (*Gadus morhua*) (Nedelec et al., 2015).

1.2.4.5 Biochemical responses

Less obvious are biochemical and biomolecular responses to anthropogenic sound. Such responses are often difficult to contextualise but can act as biomarkers of exposure and provide mechanistic insight. Increased concentrations of cortisol and catecholamines in cetacean blood samples has evidenced adrenal stress resultant of anthropogenic sound in cetaceans (Romano et al., 2004; Yang et al., 2021). Increased cortisol levels have also been observed in sound-exposed cod (*Gadus morhua*) (Sierra-Flores et al., 2015), whilst scallops (*Pecten fumatus*) displayed reduced homeostatic capability and potential immunodeficiency arising from altered haemolymph biochemistry (Day et al., 2017). Other bivalve species demonstrate responses to anthropogenic sound including decreased respiration rate and ATP content, and neurotoxic effects (Shi et al., 2019), and elevated incidence of DNA damage and elevated levels of biomarkers consistent with lipid peroxidation (Wale et al., 2019). Oxidative stress biomarkers have also shown tissue-specific impacts of sound in the crab, *Neohelice granulata* (Morena et al., 2022).

1.3 Environmental chemistry

1.3.1 Disambiguation, definition, and scope

As a term, ‘environmental chemistry’ is used somewhat loosely in scientific literature, being used in subtly different but equally valid contexts between different scientific disciplines, such ecotoxicology, environmental fate, and bio/geochemistry (Cooper et al., 2001). This undoubtedly reflects the different focus of each discipline, but also the scope and scales (physical or temporal) across which they are being considered. At a fundamental level, any stable amalgamation of matter can be considered either a

chemical or chemical species (Amouroux et al., 2012). Consequently, if all matter is a chemical, the question of what constitutes an environment, as ever, is arguably a matter of judgement and scale. Likewise, the changes in the compositional and chemical balance of environments, such as chemical influxes/effluxes etc., and baselines against which these are judged, is a matter of temporal scale.

For the context of this thesis, environmental chemistry is taken to mean physicochemical composition of the water in which an organism exists. From an experimental perspective, this encompasses both the intended and consequential physicochemical composition resultant of modifications made to the ionic balance of the environment. Three different aspects of environmental chemistry were chosen for study as part of this thesis, each addressing a different environmental consideration; 1) an anthropogenic chemical pollutant and known detrimental toxicant (i.e. cadmium – Chapter 3) ; 2) a naturally occurring chemical of known nutritional importance (i.e. calcium – Chapter 4); 3) a naturally occurring environmental chemical gradient of no intrinsic biological value or detriment, but nonetheless provides a homeostatic challenge (i.e. salinity – Chapter 5).

1.3.2 Physicochemical considerations of chemical solubility in water

What we consider water almost inevitably comprises more than merely H_20, with it likely also containing dissolved ionic chemical species consequent of its solvent nature (Chaplin, 2001). However, the solubility of chemicals in water is not necessarily constant, and can be affected by both physical and chemical factors, some of which are described in brief below.

1.3.2.1 Temperature and pressure

Temperature is a fundamental factor in water's solvent capacity – its maximum capacity to form a solution with one or more chemicals. According to Le Chatelier's principle, which describes shifts in dynamic equilibria to maintain and re-establish balance – where the dissolution of a chemical is an endothermic process, warmer water will generally promote solubility, and conversely reduce solubility where dissolution is exothermic (Reger et al., 2009). Consequently, for solids and liquids most of which (though not all) tend to undergo endothermic dissolution, increasing temperature increases solubility. Conversely, for gases, which undergo exothermic dissolution, increasing temperature reduces solubility (Averill & Eldredge, 2007). However, even where temperature meaningfully alters solvent capacity, this relationship is not necessarily linear as the specifics of the chemical's nature (e.g. bond strength, molecular size etc.) also factor into the equation (Stoker, 2015).

Pressure does not greatly affect the solubility of solid or liquid solutes in water due to the general incompressibility of these states of matter, however increasing pressure makes compressible gases more soluble in accordance with Henry's law (Averill & Eldredge, 2007).

1.3.2.2 Chemical speciation

Dissolution of ionic and covalent compounds occurs through the process of dissociation, whereby the compound separates into anionic and cationic species which are then maintained in solution. For any given chemical compound, this dissociation exists according to a dynamic solubility equilibrium existing according to Le Chatelier's principle. However, where multiple compounds are dissolved in water, those sharing commonality in dissociated species will contribute the solubility equilibria of each

other, in turn potentially altering both the specific speciation present, and resultantly the overall solubility of each chemical (Scholz & Kahlert, 2019). Moreover, the introduction of multiple anionic and cationic species introduces the potential for cross-reactivity, where dissociated ions can form new combinations, with the potential to affect chemical species proportions/concentrations, and even form chemicals with solubility below the current solvent capacity of the water, leading to precipitation. Such concerns are a large contributory factor in solutes often demonstrating lower solubility in practice than would be expected from theoretical solubility characteristics (Scholz & Kahlert, 2019).

1.3.2.3 pH, acidity, basicity, and alkalinity

Rather than being some intrinsic characteristic, pH and alkalinity are specific examples of the concepts of dissolution and speciation mentioned above. pH is simply a measure of the relative proportion of H^+ and OH^- species present within an aqueous solution – acidic and alkaline solutions containing proportionally more H^+ and OH^- ions respectively. Indeed, the difference between weak and strong acids/bases is their ionisation constant, describing their propensity to dissociate into ions. Similarly, whilst there is a common appreciation that concentrated acids and bases are both corrosive, it is lesser known that this is because excessively high proportions of H^+ or OH^- ions can cause large-scale ionisation of chemical compounds, increasing their propensity to further dissociate and react with other available ions.

In contrast to acidity or basicity which describe the pH of a solution, alkalinity is a measure of how well aqueous solutions resists acidification. Rather than being determined by a specific chemical species, alkalinity is determined by a host of conservative ions – those ions with speciation generally unaffected by temperature, pressure, or pH. Although many chemical species are capable of contributing to

alkalinity, in natural aquatic environments it principally equates to carbonate alkalinity, describing the specific stoichiometric equilibria between the related dissociations:

$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \quad \text{and} \quad HCO_3^- \rightleftharpoons 2H^+ + 2CO_3^{2-}$$

Because both carbonate (HCO_3^-) and bicarbonate (CO_3^{2-}) ions can accept H^+, the presence of such ions provides great pH buffering qualities of a solution – but once again merely demonstrates an example of how the presence of multiple chemical species can mediate the proportionality/concentration of one another.

1.3.2.4 Photooxidation

Exposure to light can cause and accelerate the oxidation and/or degradation of some chemicals. This typically occurs via a catalytic chain reaction facilitated by increased presence of free-radicals resultant of exposure of certain chemical species to ultraviolet radiation (Frankel, 2012). Thus, the lighting conditions, particularly the wavelength and intensity, can also influence speciation of some chemicals in aqueous solutions.

1.3.2.5 Consequences for experimental design and interpretation

The physicochemical concepts mentioned above strongly influence how ecotoxicological studies are conducted and interpreted, given variations in temperature and pH in particular can fundamentally affect the bioavailability of chemicals, as well as potentially modifying the biological responses of model species. Consequently, standardised aquatic test methods typically provide environmental parameters to which experiments should conform – chosen not only to ensure compatibility with the model species, but also the consistency of the environmental chemistry – facilitating better data quality, reproducibility, and comparability between

studies. Likewise, all environmental conditions, including water quality, should be monitored and reported wherever possible.

1.3.3 Chemistries featured in this thesis

Discussing the biological impacts of environmental chemistry is complicated given the vast scope of considerations this could entail. Similarly, as discussed above, chemical environments are dynamic, and shift with every interaction. Matters are further complicated given that biological responses are fundamentally biochemically driven, and thus capable of reacting to environmental chemistry in very direct, but variable ways. In the interests of focus, the environmental chemistry impacts outlined below are restricted to those forming the focus of the remainder of the thesis.

1.3.3.1 Cadmium

Cadmium is heavy metal, naturally existing as various minerals within the Earth's crust. As such, cadmium can naturally be found in soils and sediments resultant of weathering and erosion. Cadmium is also found naturally within the food chain (albeit at very sparing concentrations) as a consequence of its uptake from plants, and bioaccumulation and biomagnification through the trophic levels. Despite its presence within the food chain, with the exception of some marine phytoplankton (Lane & Morel, 2000; Park et al., 2008; Y. Xu et al., 2007), cadmium provides no nutritional value, and is instead widely considered to be exclusively toxic to life, even at low levels.

Cadmium is also an historic environmental pollutant, having had long and broad industrial applications including use in batteries, pigments, and as an alloying material (Hasanuzzaman & Fujita, 2013), and being formed as a by-product of processes including zinc refinement (Shiel et al., 2010). These anthropogenic sources, though

responsible for globally lower emissions than natural sources across comparable time scales, are often highly concentrated, leading to substantially elevated cadmium concentrations in localised regions (Kubier et al., 2019). For example, following construction of the Morowali district's mining port in Indonesia, concentrations of cadmium in the local seawater spiked at over 100 µg L^{-1} (Delly et al., 2021). In comparison, mean open-ocean surface concentrations of cadmium range between 0.001-0.1 µg L^{-1} (Neff, 2002), whilst those in terrestrial groundwaters are typically around 5 µg L^{-1} but vary notably dependent on the underlying geology and hydrology (Kubier et al., 2019). The discrepancy in the typical cadmium concentration between fresh and marine waters is consequent of cadmium's tendency to complex with ionic species more abundant in seawater, reducing the fraction existing in solution (Kubier et al., 2019; Neff, 2002).

Cadmium is known to cause toxicity and biochemical disruption in invertebrates through several modes of action. At a molecular level, cadmium promotes the creation of reactive oxygen species (ROS) promoting oxidative stress, and damaging and inhibiting the repair of DNA (Zhang & Reynolds, 2019). In early life stage invertebrates specifically, cadmium-induced oxidative stress has been shown to drive cellular apoptosis in larvae of the fruit fly, *Drosophila melanogaster* (Yang et al., 2022) and induced damage and structural changes in sperm of the of freshwater crab, *Sinopotamon henanense* (Ma et al., 2013). These cytotoxic effects contribute greatly to the carcinogenic and teratogenic nature of cadmium, with freshwater biota generally being more sensitive than marine biota due to differing physicochemical properties between the two environments (Eisler, 1993).

Cadmium can also have endocrine disruptive effects in early life stage organisms, affecting expression of regulatory hormones (Takiguchi & Yoshihara, 2006). Cadmium

can cause changes in reproductive organs to the detriment of reproductive fitness (Paschoalini et al., 2019; Takiguchi & Yoshihara, 2006). Early life stage growth and development can be retarded by cadmium exposure, with cadmium well-documented to alter expression of key growth hormones (Abidi et al., 2016; Jones et al., 2001; Rodríguez Moreno et al., 2003; Zou, 2005).

1.3.3.2 Calcium

Calcium is an alkaline earth metal, forming the fifth most abundant element in the Earth's crust, where is exist as various sulphate, silicate, and carbonate minerals. Weathering of calcium minerals accounts for significant proportion of calcium in aqueous environments, thus the underlying geology and hydrology strongly the concentrations and availability of calcium in solution. The riverine transportation of dissolved calcium over geological time has led to calcium being highly concentrated in marine environments in comparison to freshwater environments (Weyhenmeyer et al., 2019). Indeed, calcium availability is considered one of the foremost limiting factors to the distribution of freshwater fauna (Zehmer et al., 2002) given its practically universal importance as a macronutrient

Calcium is among the most important macronutrients, with a role in a host of biochemical processes. Among calcium's commonly recognised roles is as a structural material, with calcium carbonate forming the predominant mineral of mollusc shells (Bevelander, 1952; Clark et al., 2020; Weiss et al., 2002) and an ossifying component in some crustacean exoskeletons (Gbenebor et al., 2016; Luquet, 2012). The deposition of calcium into such structural elements is an active process linked with metabolic activity (Clark et al., 2020), whilst the acquisition of calcium from the environment can occur either passively or actively depending upon uptake route and the prevailing chemical gradient (Ebanks et al., 2010b; Greenaway, 1971).

Calcium ions (Ca^{2+}) play a fundamental role in the establishment and discharge of cellular electropotentials, as regulated by voltage-gated Ca^{2+} channels within cellular membranes (Senatore et al., 2016). This electropotential regulation forms the basis of nervous transmission with discharged calcium ions acting as a primary cell signal (He et al., 2018). However, calcium ions are extensively involved secondary signalling (Kudla et al., 2018), contributing to the government and modification of many intracellular functions including endo/exocytosis (Bagur & Hajnóczky, 2017), ATP production and respiration rates (Fink et al., 2017), gene expression (Puri, 2020), cellular proliferation (Munaron et al., 2004), and cellular apoptosis (Berridge et al., 1998).

More specific to early life stage biology, calcium signalling has established roles in modulating reproductive biology, with this particularly well documented in mammals. However, many calcium signalling roles are conserved across taxa, although the specific mechanistic pathways involved can vary between taxa (Stein et al., 2020). A critical role of calcium signalling is in egg activation, whereby it triggers the initiation of embryonic development following gamete fusion (Wakai et al., 2011). Notably, in aquatic invertebrates, the onset of germinal vesicle breakdown (GVBD) in bivalves has been attributed to calcium signalling (Whitaker, 2006), which would also account for large releases of intracellular calcium observed during fertilisation of the sea urchin, *Lytechinus pictus* (Steinhardt et al., 1977).

Despite its stated importance in cellular functionality, excessively high concentrations of calcium ions can nonetheless cause cellular disruption and toxicity (Bagur & Hajnóczky, 2017). Thus, whilst much is known about multiple specific cellular pathways involving calcium ions, disentangling the wider interactions and effects of calcium availability, especially at an organismal level, can be challenging.

1.3.3.3 Salinity

Salinity is a term referring to the concentration of salts dissolved in water. In natural environments, these salts predominantly originate from the weathering of rock. Salinity is used practically to differentiate water into different categories, thought the precise delineations often vary. Typically, freshwater is considered as having salinity below 0.5 parts per thousand (ppt or ‰), with levels above this being described as increasingly saline. In comparison, seawater averages a salinity of around 35 ‰, with estuarine environments having salinities intermediating these ranges, and are often described as being brackish (Olson et al., 2022; Velasco et al., 2019). Seawater is saline in comparison to freshwater due to the concentration of dissolved salts in ocean basins consequent of riverine transport and evaporation of water from ocean sinks over geological time (Stacey & Hodgkinson, 2013). Estuarine regions have a mixture of freshwater and seawater inputs, contributing to their intermediate, but also variable salinities. Similarly, partially and/or fully enclosed basins are more subject to influxes of freshwater, and thus variable or otherwise extreme salinities. Intertidal rockpools may become hyposaline during heavy precipitation, or hypersaline consequent of heavy evaporation (Legrand et al., 2018). Likewise, basins with limiting flushing and freshwater inputs, such as the Dead Sea, can reach salinities far exceeding those typical of standard seawater (Khlaifat et al., 2020).

Salinity as a measurement does not discern between contributary ionic species. However, seawater is broadly conservative in its constitution, with over 99 % of all present salts represented by just with six ionic species (Lewis & Schwartz, 2004). Whilst differences in the precise composition of seawater may result in different physicochemical properties (as discussed in Section1.3.2), in local environments where salinity is predominantly dictated by fluxes in freshwater, the relative proportion of

ionic species is mostly constant. Therefore, for species adapted to their respective local environmental chemistries, difficulties posed to aquatic invertebrates by changeable salinity are ultimately underpinned by challenges in maintaining osmotic homeostasis. These challenges place increasing pressure on cellular energy production associated with active ionic transport, in addition to changing demand for the expression of antioxidant proteins and enzymes (Hauton, 2016).

Differences in salinity affected the propensity for a/sexual reproduction of the hydrozoan *Eleutheria dichotoma*, and with hypersaline conditions reducing planulae survival (Dańko et al., 2020). Meanwhile, the brackish species *Gammarus lawrencianus* demonstrates reproductive plasticity, produced fewer but larger eggs in decreased salinities (Steele & Steele, 1991). Salinity can also affect the quality of gametes with a significant decrease in Pacific oyster, *Magallana. gigas*, sperm motility decreasing from salinities of 33 ‰ to 13 ‰ (Falkenberg et al., 2019). Germinal vesicle breakdown (GVBD) of oyster oocytes positively correlates with increasing salinity, with significantly reduced GVDB ratios evident at salinities below 24 ppt (Li et al., 2021; Y. Qin et al., 2018).

In a multiple driver context, salinity is amongst the most studied marine drivers, both in general, and more specifically in relation to early life stage invertebrates (Przeslawski et al., 2015; Velasco et al., 2019). Though no known studies have addressed salinity in the context of anthropogenic sound, a meta-analysis of various biological responses of multiple early life stage invertebrate phyla found changes in salinity typically results in synergistic driver interactions (Przeslawski et al., 2015).

1.4 Multiple driver studies

Most scientific studies have restricted study designs to a single experimental driver – an approach that garners results that are generally assessable using simplistic cause-effect relationships. However, natural environments are highly complex, comprising multiple co-occurring drivers. Thus, whilst single driver studies are generally robust and easy to interpret, they lack environmental realism. Although the answer to this is seemingly simple – merely add additional drivers to the experimental design – the realities are more complicated. Not only does inclusion of additional drivers increase the logistic complexity of experimental design (Boyd, Collins, et al., 2018), but it also increases the complexity of statistical analysis (see Chapter 2 and Chapter 6 for details). Moreover, co-occurring drivers can interact in various ways (discussed below) – thus their impacts of multiple drivers rarely reflect the sum of their parts.

1.4.1 Design approaches

Multiple driver study design requires a clear understanding of one's research questions and specific objectives. Whilst full-factorial designs – where individual factors (drivers), and each factor level, exists both in isolation and every conceivable combination – are the most compressive and robust design approaches (Ferreira et al., 2017), they can rapidly become logistically and/or resource limited with introduction of additional drivers (Boyd, Collins, et al., 2018). To counteract this, reduced or collapsed designs may be adoptable dependent upon one's specific research objectives, albeit each with consequences to understanding and inference (Gunst & Mason, 2009). Examples of different reduced and collapsed designs are show in Figure 1.2: Visual schematic exampling study design approaches for a three-driver study with six levels per driver.. Experimental designs can be reduced or collapsed in several ways, such as reducing the scope by limiting the number or drivers and/or driver levels being considered. If known

combinations of drivers are of particular interest, these can be prioritised in a deliberate fashion, and other driver combinations excluded from consideration. Alternatively, driver combinations for consideration can be selected for systematically and/or randomly to best enable coverage of your drivers.

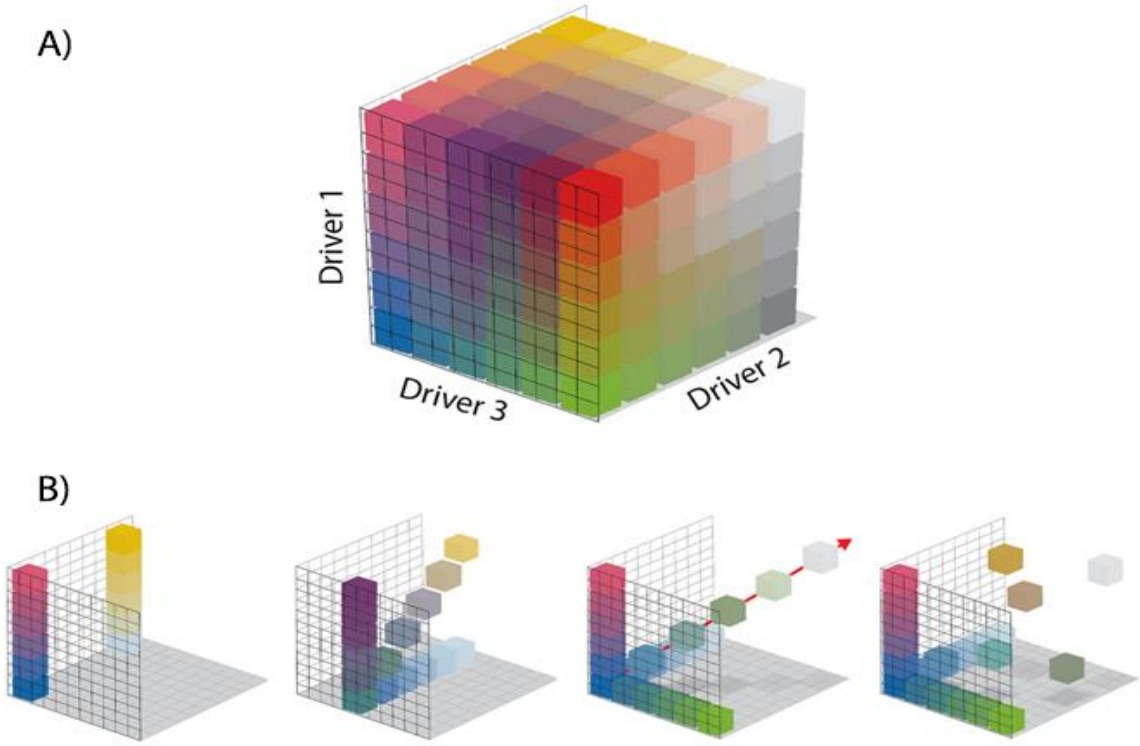

Figure 1.2: Visual schematic exampling study design approaches for a three-driver study with six levels per driver. *A) full-factorial design; B) reduced design examples. Modified from (Boyd et al., 2018).*

1.4.2 Driver interactions – definitions, causes, and propensity

There are three fundament driver interactions: additive, antagonism, and synergism (Folt et al., 1999; J. J. Piggott et al., 2015). These terms, by definition, describe how the drivers combine mechanistically, comparing effects resultant of driver combinations to theoretical effects based on additive null-model observations of each driver acting independently (Boyd & Brown, 2015; Folt et al., 1999), giving the following definitions:

Additive – a combined-driver effect equal to the sum of the individual driver effects.

Antagonism – a combined-driver effect lesser than the sum of the individual driver effects.

Synergism – a combined-driver effect greater than the sum of the individual driver effects.

A fourth interaction, potentiation, where an otherwise inert driver increases the effect of another driver has also been described (Syberg et al., 2017), however potentiation in this context is arguably a highly specific form of synergism.

Although these interaction terms are considered mechanistic definitions, they serve only to describe the difference between independent and interactive effects of drivers – not the underlying cause of these differences. In this way, the term 'mechanistic' pertaining to driver interactions can lead to confusion, given in wider literature mechanistic more commonly implies causation.

Driver interactions may arise from physicochemical or physiological origins (Boyd & Brown, 2015; Rider et al., 2014). Physicochemical interactions are abiotic, arising where the physical and/or chemical properties of individual drivers alter the properties of another driver, influencing factors such as bioavailability. For example, changes in temperature and pH can alter metal speciation altering biological uptake kinetics (Cornu et al., 2016; Spurgeon et al., 2006). Influences including photodegradation, chemical sorption, and potentiative effects can also alter the bioavailability of chemical compounds (Vulava et al., 2016; Y. Zhang et al., 2020).

Physiological interactions are biotic, occurring when drivers share common biological mechanistic pathways. As with physicochemical interactions, these interactions may

arise from changes uptake kinetics, for example a decrease in ion uptake resultant of competitive saturation of shared cellular uptake channels (Mei et al., 2014), or resource competition (Siedler et al., 2020). However physiological interactions can also occur at response level, where physiological effects of drivers coincide or otherwise mediate one another.

The likely form multiple driver interactions take depends upon the mechanistic pathways of the individual drivers (Singh et al., 2017). Additive responses generally occur where drivers have different mechanistic pathways, and thus act completely independently of one another. Antagonistic and synergistic interactions often occur via toxicokinetic interactions. Where both drivers share a toxicokinetic pathway, antagonism is likely. For example, many heavy metals cause toxicity via induction of reactive oxygen species (ROS) production, and thus antioxidant responses to one metal can mediate similar impacts of other metals in an antagonistic manner (Balali-Mood et al., 2021; Y. Wang et al., 2020). Likewise, cellular upregulation in the metal-sequestering protein metallothionein caused by zinc exposure also offers protection against other co-occurring metals such as cadmium (Peraza et al., 1998). Synergistic interactions occur where driver toxicokinetic pathways mediate the metabolism of each other, such as reducing clearance rate, inhibiting protective mechanisms, and/or overwhelming of tolerance capacity (Medda et al., 2020; Spivey, 2012). Correspondingly, as antagonism and synergism both relate to toxicokinetics, the propensity for each interaction may also be dose dependent.

It is noteworthy that responses to drivers can also have wider systemic ramifications. Coors and De Meester (1998) observed greater parasite infestation rates in daphnia increased resultant of an immunomodulatory effects of pesticide exposure –

highlighting that driver exposures are likely to affect multiple responses, which may themselves lead to wider interactions.

1.4.3 Interactions in aquatic environments

A meta-analysis of 286 responses from 88 multi-driver studies conducted by Jackson et al (2016) found antagonistic interactions predominated in freshwater studies, accounting for 56% of responses, followed in commonality by synergistic (28%) and additive (16%) interactions. A similar meta-analysis of marine-based studies by Crain et al. (2008) uncovered a broadly similar trend, though less defined (38% antagonistic, 36% synergistic, 26% additive), whereas others have found all three interactions to be equally present in aquatic environments (Fong et al., 2017).

Fong et al. (2017) postulated that additive effects are most prevalent in biodiversity and community structure responses – reflecting drivers affecting independent species differently within a community. Additive effects also occur in energy-dependent responses such as growth reduction where drivers add cumulative to energy burdens (Coors & De Meester, 1998). Conversely, synergism and antagonism are most common in specific physiological and molecular traits within individual organisms. Therefore, the apparent differences in interaction propensity between freshwater and marine environments more likely reflect the specific drivers and responses covered in each meta-analysis rather than an ecologically mediated difference. Further uncertainty may also arise from inconsistencies in reporting of potentiative effects. Microplastics were shown to potentiate triclosan toxicity in the marine copepod *Acartia tonsa* (Syberg et al., 2017). Phototoxic potentiation of sulfonamide antibiotics was evident in *Daphnia magna* (Jung et al., 2008), whilst predator chemical cues have been suggested to potentiate toxicity, albeit with inconsistent results (G. Qin et al., 2011). Styf et al. (2013) postulated potentiating effects of elevated temperature and pH on sensitivity

to hypercapnia, however decoupling related physicochemical drivers in this manner is problematic – highlighting the need for consensus on a scientific approach to conducting and reporting multi-driver studies.

1.4.4 Existing multiple driver studies featuring anthropogenic sound

To date, only three known studies have considered anthropogenic sound in a multiple driver context – all having been conducted under controlled laboratory conditions using sound playbacks.

Ginnaw et al. (2020) investigated the impacts of white noise and tonal sounds on the shoaling behaviour of freshwater three-spined stickleback *Gasterosteus aculeatus* under both illuminated and dark conditions, finding no evidence that sound exposures altered swimming dynamics, and only subtle suggestions of any driver interactions.

The remaining two studies both focused on the combination of anthropogenic sound and cadmium exposure on marine bivalve molluscs, with Shi et al. (2019) investigating effects of pile driving playbacks on the blood clam *Tegillarca granosa,* and Charifi et al. (2018) effects of cargo ship playbacks in the Pacific oyster *Magallana gigas*. Each study used heavily reduced study designs, with both studies lacking any assessment of sound in the absence of cadmium, and Charifi et al. (2018) further lacking a treatment absent of cadmium. Resultantly, despite collectively addressing a wide range of behavioural and physiological responses, and both studies evidencing that sound playbacks significantly influenced responses to cadmium exposure, neither study allows for a mechanistic understanding of how the interaction between the two drivers, nor any context on the effects of sound exposure in isolation.

1.5 Study species

Three different study species were used in this thesis: Norway lobster, *Nephrops norvigucus* (Chapter 3), great pond snail, *Lymnaea stagnalis* (Chapter 4), and Pacific oyster, *Magallana gigas* (Chapter 5). The rationale for using multiple species rather than a singular focal species was one of practicality and pragmatism, facilitating a robust assessment of the specific research objectives detailed in Section1.6. The objective of addressing key phases of early life stage development – fertilisation, embryonic development, larval development – was considered infeasible using a single species, or would have otherwise risk producing suboptimal data. Rather, species best facilitating assessment of each specific research objectives were selected, with ecologically and/or economically value species prioritised where multiple candidate species were identified. The use of multiple species also alleviated logistical concerns of seasonal availability of larvae, and long-term husbandry of broodstock which may have been a greater challenge with a single species. A brief justification for the selection of each species is given below.

Norway lobster, *Nephrops norvegicus*

The Norway lobster, *Nephrops norvegicus* (henceforth *Nephrops*), is a decapod crustacean common to the shallow-water regions of the North-East Atlantic and North Sea regions (Fisheries Global Information System (FAO-FIGIS), 2016).

Nephrops was chosen to assess larval developmental endpoints as the species undergoes a planktonic larval development comprising several well-defined zoeal larvae stages over a six-week (Powell & Eriksson, 2013), providing multiple developmental milestones against which to monitor developmental rate, and a larval duration of suitable length to provide temporal resolution to chronic exposure studies. Furthermore, from personal experience, larvae of *Nephrops* are comparatively robust

and easier to maintain compared to those of some other commercially valuable decapods such as European lobster (*Homarus gammarus*), brown crab (*Cancer pagarus*), both of which are notoriously difficult to culture in experimental systems. The well documented larval development of *Nephrops* (Powell & Eriksson, 2013; Spicer & Eriksson, 2003), good mechanistic understanding of related processes including moulting (Philp & Marteinsdottír, 2013; Sardà, 1991), and context for how environmental factors impact upon *Nephrops* developmental rate (Dickey-Collas et al., 2000; H. L. Wood et al., 2015) all provide a solid foundation upon which to build inferences. There are also studies addressing effects of metal pollutants on the species (Canli et al., 1997; Cenov et al., 2018), and an assessment of the audiological capability of the species (Goodall et al., 1990), providing specific context to the aims and objectives of the thesis.

Nephrops is socioeconomically valuable, representing the third largest target-species fishery in Scotland by mass (The Scottish Government, 2020), yet its habitat greatly overlaps regions of high density of existing and/or planned fixed structures, the construction of which usually requires some degree of pile driving. Thus, understanding the potential impacts of anthropogenic sound and accompanying drivers is of particular importance and interest.

Great pond snail, *Lymnaea stagnalis*

The great pond snail *Lymnaea stagnalis* is a freshwater pulmonate gastropod common to north-western Europe and North America (Kuroda and Abe, 2020). The species was chosen as model species for assessing fecundity due to its established role as an OECD guideline species for assessing chemical reproductive toxicity in molluscs (OECD, 2016). This guideline provides specific experimental environmental parameters developed and ring-trialled to ensure robustness and consistency of resultant experimental

outputs. Several methods for undertaking embryo assays with the species have also been proposed, taking advantage of an established high sensitivity to chemical pollutants and water quality (Gomot, 1998; Mazur et al., 2016; Munley et al., 2013). The fecundity of *L. stagnalis* is well-studied with existing literature providing context on contributary factors including mating behaviour (Koene et al., 2007; van Duivenboden et al., 1985), mediating neurological and biochemical pathways (Janse et al., 1996), and diet (Reátegui-Zirena et al., 2016). Likewise, a similar mechanistic understanding is present for factors effecting embryonic development, including energetic and metabolic factors (Byrne et al., 2009; Zotin and Kleimenov, 2006), neurological development (Croll et al., 1999; Nagy & Elekes, 2000; Voronezhskaya et al., 1999), and environmental drivers (Vaughn, 1953). Specifically, the specific roles of the macronutrient calcium as pertaining to reproduction, growth, and development are well documented for *L. stagnalis* (Dalesman & Lukowiak, 2010; Ebanks et al., 2010a, 2010b; Grosell & Brix, 2009; Piggott & Dussart, 1995), aiding mechanistic understanding.

L. stagnalis was also deliberately chosen to contribute to the currently sparsity of studies concerning impacts of anthropogenic sounds on freshwater invertebrates (Morley et al., 2014; Villalobos-Jiménez et al., 2017).

Pacific oyster, *Magallana gigas*

The Pacific oyster, *Magallana gigas* is a species with wide environmental tolerance, which has seen it gain both commercial and food-security value (Willer & Aldridge, 2020), but also increasing notoriety as an invasive species (Faust et al., 2017; King et al., 2021). *M. gigas* were selected as a study species to assess gamete fusion due to its use as a recognised International Council for the Exploration of the Sea (ICES) model organism for embryo/larval bioassays (Leverett & Thain, 2013). This guideline provides

detailed experimental environmental parameters developed to ensure robustness and consistency of resultant experimental outputs, with particular focus on extracting, handling, and combining gametes for experimental purposes. The modest space requirements required of *M. gigas* gamete exposures in combination with the species' fecundity also allow concurrent running of multiple exposures, enabling assessment of different scenarios or mechanistic endpoints to be assessed using the same broodstock.

Several studies have investigated propagation and survival of oyster larvae and spat with regards to salinity and other environmental factors (Falkenberg et al., 2019; Geffard et al., 2001; King et al., 2021; MacInnes & Calabrese, 1979; Y. Qin et al., 2018). There is also some existing context for responses of oysters to multiple-driver interactions involving salinity (Coglianese, 1982; Gamain et al., 2016; MacInnes & Calabrese, 1979; Nowland et al., 2019; C. Xu et al., 2020). A previous study has also examined responses of *M. gigas* to sound in a multiple driver context (Charifi et al., 2018), providing a knowledge base upon which to expand.

1.6 Thesis aims, objectives, and hypotheses

The principal aim of this thesis was to establish what, if any, interaction occurs between anthropogenic sound (as laboratory-based sound playbacks) and selected environmental drivers. To realise this aim, the following general objectives were identified, along with specific hypotheses where appropriate:

- To address knowledge gaps in responses of both invertebrates and early life stage organisms to anthropogenic sound, by collectively ascertaining effects on early life stage invertebrates

- To develop methodological approaches enabling expansion of anthropogenic sound impact assessment from a single-driver approach into a multi-driver approach
- Where possible and practical, attempt to provide a mechanistic explanation for any evidenced driver interactions

- To investigate how specific combinations of chemical drivers and anthropogenic sounds (as playbacks) relevant to marine (Chapter 3), freshwater (Chapter 4), and brackish (Chapter 5) environments respectively, influence the effects of anthropogenic sound, and vice versa

- To guide future studies by reflecting upon the challenges of undertaking multiple driver studies, particularly in the context of anthropogenic sound, highlighting experimental design choices/compromises likely to be necessary, and providing a critical appraisal of how such choices may affect study outcomes and interpretations

Specific objectives and hypotheses include:

- To assess how piledriving playbacks sound alters susceptibility of larval *N. norvegicus* to a known toxicant, cadmium (Chapter 3)

  Hypotheses:

  - ***N. norvegicus* larvae will demonstrate dose-dependent toxicity in response to cadmium**
  - **Piling sound playbacks will detriment *N. norvegicus* larval development**

- **Piling sound playbacks will increase the susceptibility of *N. norvegicus* larvae to chemical toxicity**

- To assess how ferry playbacks and availability of an essential macronutrient, calcium, impacts upon the fecundity and embryonic development of *L. stagnalis* (Chapter 4)

  Hypotheses:

  - **Greater calcium availability will promote *L. stagnalis* fecundity and embryonic development**
  - **Ferry sound playbacks will detriment *L. stagnalis* fecundity and embryonic development**
  - **Ferry sound playbacks will exacerbate the effects of calcium availability on *L. stagnalis* fecundity and embryonic development, particularly at low calcium concentrations**

- To assess whether acute ferry and piling playbacks differently affect the fertilisation rate and subsequent 48-hour development of *M. gigas* when occurring at different environmentally realistic salinities (Chapter 5)
- **Hypotheses:**
  - **Salinity will be progressively detrimental to fertilisation and development at increasingly extreme range limits**
  - **Anthropogenic sound playbacks will detriment *M. gigas* fertilisation and development**
  - **Anthropogenic sound playbacks will exacerbate the effects of salinity availability on *M. gigas* fertilisation and development**

1.7 Thesis structure

The complete structure of this thesis is described below, including a brief summary of each chapter's contents and specific objectives.

- **Chapter 1 – An overarching introduction**

  An introduction to the topics covered within this thesis, and providing the wider context and knowledge base required to interpret its findings

- **Chapter 2 – Methodology: Embracing opportunities and accepting limitations**

  All experimental studies are ultimately constrained by experimental design choices, often conscious compromises. As with all compromises, these inevitably engender a balance of gains and losses. This chapter outlines specific methodological considerations, and specific methods forming the core approach of this thesis, along with discussion and justification of these choices where appropriate.

- **Chapter 3 – Effects of pile driving sound playbacks and cadmium co-exposure on the early life stage development of the Norway lobster, *Nephrops norvegicus***

  This chapter features a first-author peer-reviewed journal article recently published in a Special Issue entitled '*Multiple Stressors in Marine Ecosystems*' hosted by *Marine Pollution Bulletin* (Stenton et al., 2022).

  The published study comprises two separate, yet complementary experiments assessing the effects pile driving sound playbacks and cadmium co-exposure on the larvae of *N. norvegicus*, a commercially and ecologically important decapod crustacean. The first experiment assessed phenomenological effects on

endpoints including survival, larval developmental duration, and juvenile behavioural fitness. The second experiment sought to ascertain whether oxidative stress was a driving mechanism of phenomenological observations, as hypothesised following the first experiment.

- **Chapter 4 – Effects of ferry noise on the embryonic development of the pond snail, *Lymnaea stagnalis*, at different waterborne calcium concentrations**

  Here, the model species *L. stagnalis* was exposed to a regime of anthropogenic sound in the form of sound playbacks mimicking that of a passenger ferry. Sound playbacks were additionally framed in the context of macronutrient availability, namely waterborne calcium.

  The study comprised two complimentary experiments, the first comprising an embryo assay assessing hatching success, developmental time, and key biometrics of successfully hatched snails. For the second experiment, an OECD reproductive test guideline (OECD, 2016) was adapted and modified for compatibility with assessment of aquatic sound playbacks, and adult fecundity and growth in the same experimental context as the embryo assay.

- **Chapter 5 – Effects of ferry and pile driving playbacks on the fertilisation and immediate development of the Pacific oyster, *Magallana gigas*, at different salinities**

  This chapter is the first known attempt to ascertain the effects of aquatic anthropogenic sound on gamete fusion and fertilisation in any species. The study takes a comprehensive approach, addressing effects of both pile driving and ferry sound playbacks on *M. gigas* gametes in several acute exposure

dynamics, across various environmentally relevant salinities. In addition to addressing fertilisation rates, the study also monitored 48-hour post-sound exposure development, noting occurrences of developmental abnormality and/or failure.

- **Chapter 6 – Discussion and synthesis**

This final chapter is a collective summary of the thesis findings and comprises two aspects. The first aspect is a reflective summary of the various experimental methods selected, their specific limitations, and the ramifications – tying in with the content discussed in the methodology chapter. This reflection also offers specific suggestions for future studies based upon experience gained during the process of building this thesis.

The second aspect is a comparative discussion of the major experimental findings of the thesis, framing them in a wider context than addressed in the individual chapters. In doing so, this synthesis seeks to uncover and discuss underlying similarities, which may be useful for extrapolating the results beyond the scope of the thesis itself.

Chapter 2
Contextualisation of Methods: Embracing Opportunities and Accepting Limitations

All experimental studies are inevitably constrained by some general combination of factors, key amongst which include space, equipment, time, and financial considerations. Additionally, biological studies may be further constrained by factors including seasonal availability of organisms, husbandry requirements, and practical limitations (such as size and handling concerns). Such considerations influence the design of experimental systems and implementation of data collection. In multi-factorial studies these challenges are often exacerbated by the necessity to appropriately incorporate disparate factors into a singular experimental system. Likewise, expansion into multi-factorial designs may have ramifications with regards to replication required for statistical power.

This chapter highlights some of the more specific constraints pertaining to sound research, and particularly the incorporation of sound into multiple driver studies. Here, the more prevalent design decisions taken are outlined, any key compromises raised, and context and explanation provided as how these choices bolster overall robustness to produce what was considered the strongest overall experimental approach.

2.1 Methods overview

2.1.1 Permits and Ethical Approval

Research for this PhD thesis on early life stage invertebrates (Project ID: 813843) was planned and conducted in compliance with Edinburgh Napier's Code of Research

Integrity, and the principles of the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3R's). As the three chosen invertebrate study species fall outside the remit of the Animals (Scientific Protection) Act 1986, no related licensing was required. Experimental designs were consented to and approved by the supervisory team/Director of Research, as well as the Health and Safety Advisor of the School of Applied Sciences at Edinburgh Napier University. Practice in relation to ethical animal experimentation was reviewed during regular progress monitoring in line with the Postgraduate Research Degrees Framework.

2.1.2 Sound exposures

All experiments were conducted under laboratory conditions for practical reasons. Principally, the small size of organisms studied throughout, the need to make frequent observations of known individuals, and the desire to specifically modify the environment of particular experimental subjects precluding field exposures.

Broadly, aquatic exposures were conducted in experimental systems comprising two principal components – 'exposure vessels', and 'exposure systems'. Exposure vessels were the units forming independent replicates. Specifications of exposure vessels varied between experiments according to experimental design and husbandry requirements and detailed in each chapter, but were of either plastics or borosilicate glass constructions which proved to have low impedance to sound transmission, established by comparison of received sound pressure levels within the water-bath tank and the exposure vessels in the presence of a point-source sound exceeding the noise floor. Exposure vessels were then arranged within an exposure system – a setup facilitating the experimental sound exposures and temperature control where necessary. Exposure systems took on two forms:

1) Larger tanks containing an underwater speaker providing sound playbacks. These tanks contained an 'acoustically transparent' platform on which exposure vessels were located. These exposure vessels were partially submerged within the exposure system such that approximately three-quarters of the working volume was submerged. In addition to providing a shared water interface for sound propagation, the temperature of the exposure system could be regulated by means of heater-chiller units and thus act as a water-bath to regulate the temperature of exposure vessels therein.

2) Vibrational platforms providing sound exposures onto which exposure vessels were placed direct. These systems did not require a water-based interface, instead delivering vibration directly. These offered select benefits where water interfaces were unfeasible (see conclusion), but did not enable temperature control independent of the prevailing laboratory conditions.

2.1.2.1 Playback tracks

Playback tracks were compiled from *in situ* field recordings of the focus sound sources, i.e. pile-driving and passenger ferry (detailed in appropriate chapters). Where possible, playback tracks were compiled from multiple source recordings to incorporate more realistic variability into the sound exposures.

Playback tracks were compiled in Audacity *ver. 2.3.3.* Elements of interest were identified from source tracks and looped to create tracks containing extended periods of each specific sound of interest. Where multiple source recordings were used, sound pressure level (SPL) of individual looped tracks were normalised to each other to ensure consistent sound exposure levels between source files. Normalised soundtracks were then combined randomly such that each element track contributed equally to the duration of the final track. Once finalised, soundtracks were exported as 32-bit floating WAV files to ensure maximum fidelity and dynamic range of the sound file.

2.1.2.2 Sound playback

Audio systems used throughout all experiments were built using commercially available products. Where possible, equipment capable of reproducing broadband frequency ranges up to approximately 20 kHz and demonstrating flat frequency responses were selected to maximise the fidelity of sound reproduction. Where this was not possible, as in the case of vibrational platforms which are more constrained in their frequency response ranges, units covering a frequency response range consistent with aquatic invertebrate sensory capability were used.

Where speakers were used, these were suspended within tanks to minimise physical points of contact between the speaker units and the surrounding tank to limit vibration propagating directly throughout the superstructure which could result in inconsistent acoustics. Speakers were located centrally within the bottom of the exposure system tanks and orientated perpendicularly towards to the water surface to maximise the direct sound field. Exposure vessels were similarly located as far from the speaker and walls to maintain a maximal direct sound field, and limit acoustic boundary and near-field effects. Use of vibrational platform precluded such mitigation methods given their design and functionality.

Playback sound exposure levels were chosen to approximate those found *in situ* based upon received sound pressures reported in literature and metadata of the original source recordings where sufficiently detailed. Exposure levels were set using sound pressure as the primary calibrator given a greater body of reported values exist in scientific and technical literature as compared to particle motion.

Sound control treatments were preferentially accomplished using playback of ambient environment recordings. This was in an attempt ensure that any key frequencies

characterising the natural environment, otherwise absent from the laboratory setting, would be represented in both the control and exposure sound treatments. Likewise, ambient playbacks ensure that electrical noise and electro-magnetic field effects intrinsic to the use of speakers were similarly present in both control and exposure tanks.

2.1.2.3 Sound characterisation

In all cases, both sound pressure and particle motion were measured within experimental systems. Sound pressure was measured using a manufacturer-calibrated HiTech HTI-94-SSQ hydrophone with inbuilt preamplifier (sensitivity -165 dB re: 1V/µPa). Particle motion, in the form of acceleration, was measured using a custom-built tri-axial accelerometer (sensitivity: -180 dB re 1V/µms^{-2}) validated against a manufacturer-calibrated Brüel & Kjær 4508B accelerometer (sensitivity: -160 dB re 1V/µms^{-2}). Signals from the hydrophone and accelerometer were recorded using a Roland R09 HR 24-Bit recorder using a 44.1 kHz sampling rate, calibrated at 1000 Hz using a signal generator and oscilloscope.

Where possible, measurements were taken within each exposure vessel to accurately quantify specific exposures to organisms. Where this was not possible (in cases such as the hydrophone and/or particle motion sensor being larger than the exposure vessels) these recordings were taken from within a larger proxy vessel of the same material as the exposure vessels placed in the specific location the exposure vessels would otherwise occupy. This method allowed robust estimates of sound exposure to organisms within the vessels, notwithstanding discrepancies resultant of the intervening materials composing the exposure vessels (which were observed to be negligible for the plastic and borosilicate containers utilised across the experiments when spot-checked.

Sound recordings were analysed using Matlab *ver. R2019b*. Sounds were characterised based on their waveform properties and definitions set out in ISO 12001:1996 (International Organization for Standardization, 1996). For the purposes of analysis and discussion, sounds defined by the standard as 'steady', 'non-steady', and/or 'fluctuating' are henceforth referred to as being 'non-impulsive'.

Sounds were quantified according to definitions stated in ISO 18405:2017 (International Organization for Standardization, 2017) with respects to both sound pressure and particle motion. Non-impulsive sounds were quantified by root mean square (RMS), sound exposure levels (SEL) and cumulative SEL (SELcum). Impulsive sounds were further characterised using peak-to-peak (pk-pk) values and single-strike SEL (SELss) calculated over the 90% cumulative energy window of the inter-strike period.

Sound pressures were analysed across a broadband frequency spectrum of 1-24,000 Hz. Analysis of particle motion was restricted to frequencies of 50-3000 Hz using a 3rd-order bandpass Butterworth filter given rapid attenuation of particle motion above this frequency range and the match the frequency range across which the tri-axial sensor was validated. Power spectral densities for each sound treatment were calculated using batch analysis of all respective recordings. Hanning and Hamming windows were used for pressure and particle motion respectfully, with 1-second and 0.1-second window lengths with 50-percent overlap used for non-impulsive and impulsive sounds respectfully.

2.1.3 Chemical exposures

Specifics of chemical exposure methods varied between studies dependent on experimental designs, target concentrations, and related concerns over accuracy and

precision. Detailed methods are stated in each experimental chapter, however underlying principles are stated below.

2.1.3.1 Materials selection and preparation

Experimental materials and equipment for individual studiers were selected on the basis of compatibility with experimental objectives and procedures. Prior to use, all materials were thoroughly cleaned and sterilised using deionised water and autoclaving. For studies concerning metal exposures at trace-level concentrations, equipment was additionally acid washed in 2M nitric acid to remove trace metal contamination. Correspondingly, all chemical reagents used for exposures and/or modifying husbandry conditions were all either analytical or commercial grade materials with full traceability sourced from reputable suppliers.

2.1.3.2 Dosing solutions and practices

Approaches to preparation and dosing of chemicals varied according to concentration, chemical properties, and working volumes.

Where chemical quantities were accurately and precisely measurable, concentration ranges linearly spaced, and working volumes practicable to handle, solutions were constituted separately using constituent chemicals and dilution media. In instances these criteria were not met, stock solutions were created from concentrated stocks and subsequent serial dilution with dilution media.

For each chemical, a Primary Stock solution was created in deionised water from which all other dosing solutions originated. Primary Stocks were made at disproportionately high concentrations enabling larger masses of solute to be utilised, and to reduce impacts of weighing error. Solutions were made in deionised water to aid chemical stability and using volumetric flasks to improve accuracy of measured volumes. Dosing

solutions were created by serial dilution of the Primary Stock using deionised water. Volumes were measured gravimetrically and/or using appropriate pipettes depending upon required volumes and associated errors. Dosing stocks were again made at disproportionately high concentrations – this reduced the required dosing volumes such that their addition to exposure vessels would not significantly affect either temperature or salinity within the replicates.

Prior to use, exposure vessels were 'conditioned' using solutions of target concentration to reduce potential impacts of chemical adsorption. Where possible, exposure vessels were dosed with organisms *in absentia* to prevent inadvertent exposure to excessive concentrations of dosing stocks prior to working solutions reaching homogeneity. Experiments were conducted under static-renewal conditions, with proportional re-dosing of chemicals at each water change.

2.1.3.3 Water sampling and quantification

Water samples were taken for retrospective confirmation of working solution concentrations. Water samples were pooled across replicates within each chemical treatment. Water samples consisted of ON and OFF samples; ON samples being taken immediately after dosing of working solutions, and OFF samples immediately prior to any subsequent water changes, and/or upon completion of the study. Where chronic studies were conducted, water samples were taken at regular time intervals to account for any losses from either the primary stocks, or discrepancies in the dosing solutions.

Water samples were preserved and stored appropriately before being analysed for the appropriate chemical target using an appropriately accurate and precise quantitative method. Where appropriate, these data were further used to calculate time-averaged exposure concentrations in each chemical treatment.

2.1.4 Replication and allocation

Treatment replication was driven by practical limitations in physical space, husbandry requirements, and availability of larvae. Where feasible, larvae originating from different broodstock were utilised, though this was not always possible or practical.

Larvae were allocated across experimental treatments sequentially, and replication subsequently being built incrementally (as opposed to batch-allocating replication by treatments). Larvae were allocated in this way to avoid biasing treatments with individuals of greater or lesser fitness consequent of their ease of capture (such as faster/slower swimming individuals). Likewise, where larvae were allocated at different times consequent of specific hatching times, such allocation accounted for potential variability in brood and/or batch-specific fitness.

2.1.5 Statistical approach

Data were modelled parametrically where possible, but where model residuals and equality of variance did not conform to assumed data distributions, parametric models were abandoned, and non-parametric analyses or data-resampling methods implemented instead.

Use of data transformations to meet data distribution assumptions were not applied due to the negative consequences this can have on error rates and interpretation (McArdle & Anderson, 2004; Schneider, 1992). Unless otherwise stated, statistical significance was conferred at $p < 0.05$.

Post-hoc analyses were often used to assess differences between specific treatment groups. Where multiple comparisons were made within a data set, statistical results were corrected for false detection rates (FDR) using the Benjamini-Hochberg Procedure (Benjamini & Hochberg, 1995).

2.1.6 Disambiguation of developmental terminology

This thesis makes common reference to gametes, embryos, larvae, and juveniles, yet the biological and ecological significance between these categories – particularly embryos and larvae – is sometimes unclear. For clarification, the following broad definitions are used when referring to experimental specimens pertaining to this thesis:

- **Gamete** – a sex-cell resultant of meiotic division.
- **Embryo** – a developmental stage following gamete fusion/fertilisation and preceding juvenility (see below), undertaken whilst encapsulated within a chorion-like structure and otherwise non-free-living
- **Larva** – as defined for embryo, except under non-encapsulated free-living
- **Juvenile** – a non-mature organism featuring a body-plan and ecological behaviours common to adult counterparts

2.2 Methodological justification

2.2.1 Laboratory vs field studies

Experimental studies can be broadly divided into two categories: those conducted in laboratories under artificially controlled conditions, and those conducted *in situ* under 'natural' conditions. Contrary to some ardently held beliefs, neither approach is inherently superior to the other, as each is subject to its own limitations. Rather, the appropriateness of each approach is heavily dependent on the specific research questions being addressed.

Field studies offer unqualified benefits in terms of realism given that observations conducted *in situ* are inherently representative of the conditions present in that

environment (Crane et al., 2007). This realism can aid with extrapolation from experimental results to potential population level impacts. Consequently, field studies are exceptionally suited to long-term monitoring studies assessing changes with regards to the introduction, removal, or flux of a driver from the environment. In these circumstances, a timeline of sufficient length provides a robust set of 'base-line' data against which comparisons can be drawn. Where long-term monitoring data is not available, comparisons are often drawn between different sites – one exposed to a driver of interest, and one free from such conditions which acts as a 'control' site; but this approach has implications. A major limitation in comparing between a limited number of sites is in achieving meaningful statistical power whilst avoiding pseudoreplication (Hurlbert, 1984). Furthermore, even where appropriate experimental design in implemented, field studies often suffer limited inference resultant of intrinsic environmental variability (Tilman, 1989). In addition to the variable(s) of interest (and additional variables that are quantifiable), there are countless potential factors present which may influence any observations being taken. For monitoring studies conducted at a single site, if observations (including baseline) cover a sufficient timescale, it may be possible to discount some transient extraneous factors, but this is less likely to be the case when contrasting different sites. Regardless of how carefully exposure and control sites are selected, there will always be environmental factors that could be affecting the observations and contributing to uncertainty in the resulting data, and accounting for long-term temporal trends will always be challenging. This uncertainty in exposure conditions, and therefore drivers, is of particular importance when trying to determine mechanistic effects.

In contrast to field studies, laboratory studies are ideally suited to mechanistic studies. Laboratory studies offer the potential to control and mitigate these extraneous factors,

thus increasing confidence that observed effects are directly contributable to the driver of interest (Tilman, 1989). The ability to directly control experimental variables also more readily enables assessment of specific threshold limits and contextual exposure effects. However, these stringent controls, whilst enabling high precision, come at the expense of environmental realism – making extrapolation between laboratory observations and real-world impacts challenging (Crane et al., 2007). An increasingly popular compromise is the adoption of the so-called lab-field approach – one that incorporates aspects of both laboratory and field components. For example, this might involve taking organisms exposed to drivers *in situ* being transported to a laboratory and subsequently raised and observed under more controlled conditions, as in Day et al. (2016) . This may also allow access to additional analytical equipment and techniques that are not possible or practical in the field. Of course, the specific choice of study approach may ultimately be influenced by more practical concerns.

Every experiment undertaken as part of this thesis was conducted under laboratory conditions. The justification for this decision is three-fold:

1. It has previously been stated that a lack of mechanistic understanding is one of the main limitations in our current understanding of multi-driver interactions (Griffen et al., 2016; Hom Carole L.;Cochran, 1991; R. Williams et al., 2015). Thus, the ability to reduce all extraneous factors to a minimum will bolster confidence in the quality of data produced, and any observed cause-effect relationships observed.
2. Many invertebrate larvae are small and planktonic. Attempting to rear and continually track/monitor individual organisms over time with confidence in an expansive and changeable environment was not considered practical. Moreover, the ability to rear larvae under controlled, ‘ideal’ conditions was

selected to promote better base-line survival, which was considered paramount to assist statistical robustness and confidence in any resulting inferences.

3. Exposing organisms to sounds, particularly of anthropogenic origin, in the field is often complicated and expensive due to a lack of easy and consistent accessibility to appropriate sound sources. Using playbacks of field-derived sound recordings offers better reproducibility of sound exposures, both in laboratory and field settings. This further reinforces the concepts stated above, whilst also offering more control over the acoustic exposure levels, be this at the expense of realism (discussed in detail in Section2.2.2).

2.2.2 Sound playback

A common criticism of laboratory studies of environmental and anthropogenic sounds pertains to a lack of environmental realism; namely an inability to accurately recreate sounds of interest in a laboratory setting using speaker playbacks, and issues pertaining to 'small-tank acoustics'. Natural aquatic environments are known to contain many sounds comprising a large low-frequency (< 100 Hz) component. These are often abiotic in nature, characteristic of wave action and other geophysical processes such as such as tectonic activity (Haver et al., 2017; Hildebrand, 2004). Similarly, anthropogenic sounds such as those generated by ships and activities including pile driving can contain a substantial sub-100 Hz component (Hildebrand, 2004). Unfortunately, these low frequency noises are extremely difficult to recreate artificially via speaker playback due to fundamentals of speaker design (Kyriakakis, 1998). In order to generate these frequencies, speakers would require acoustic drivers with large physical dimensions, inertial mass, and power requirements, all of which would preclude their use in tanks. Whilst speakers capable of producing these low

frequencies at sufficient amplitudes exist, they are either of bespoke design, prohibitively expensive, or can only recreate a very limited range of frequencies.

Submersible speakers utilised throughout experiments in this thesis are all commercially available units, capable of reproducing broadband frequency ranges, and demonstrating flat frequency responses. Although these speaker models share a limitation in producing low-frequencies, their ready availability and certified specifications are conducive to reproducibility. Similarly, their otherwise expansive frequency range is considered to offer the best overall compromise for playbacks. Another potential limitation in using speaker playback is the possible presence of a sound-cone – a non-uniform distribution of sound consequential of differing proximity to an acoustic point-source, and associated differences in sound attenuation (Kinsler et al., 1963). Unless a very specific and consistent sound level is essential to the research question (such as attempting to define response threshold limits), sound-cone effects can be accounted for with appropriate distribution of treatments and replicates within the sound field. Within this thesis, experimental treatments were arranged within the centre of the sound-cone to minimise any variability in sound levels, or otherwise distributed throughout the sound-field such that the average sound exposure levels between treatments were consistent.

In addition to limitations related to speaker playbacks, there are also constraints arising from ‘small-tank acoustics’; a term encompassing a range of phenomena constraining audio signals in confined spaces. Predominantly, these are attenuation and amplification effects of specific frequencies resultant of constructive and destructive waveform interference resultant of boundary effects. The term ‘small’ in the context of ‘small-tank acoustics’ is somewhat ambiguous and flexible, given it applies to a tank with physical dimensions smaller than those of wavelengths of sound

frequencies of interest (Rogers, Hawkins, Popper, Fay, Gray, et al., 2016). For context, in the case of frequencies of 100 Hz, 'small' would constitute a tank with any physical dimension less than 15 metres. Ultimately, one impact of small-tank acoustics is a shift towards predomination of frequencies whose wavelengths match, harmonise, or are otherwise smaller than the physical dimensions of the tank. Practically, this leads to sounds of lower frequencies (with longer wavelengths) being constrained by the physical dimensions of the tank, driving a shift towards predomination of shorter wavelengths, and thus higher frequencies. Boundaries also have the impact of disproportionately reducing sound pressure compared to particle motion, resulting in disproportionately large particle motion relative to pressure (Rogers, Hawkins, Popper, Fay, Gray, et al., 2016). Similarly elevated particle motion relative to pressure also results from close proximity to the sound source and so-called near-field effects, occurring within any distance less than the equivalent wavelength (Kinsler et al., 1963).

Intuitively, effects of small-tank acoustics can be lessened by use of larger tanks – both reducing the extent to which low-frequency sounds are attenuated and enabling greater separation of exposure vessels from the tank walls and resultant boundary effects. Correspondingly, sound exposures were conducted in the largest tanks practical, however it is not possible to eliminate these effects entirely. Indeed, these same effects are actively present in ocean environments where similar constraints are imposed by the likes of the seabed, coves etc, albeit at different magnitudes of scale where these effects are arguably less evident. Resultantly, there is little that can be done to overcome speaker design limitations and potential near-field effects beyond those already mentioned and maximising the distance between the sound source and experimental organisms as much as possible. However, whilst it is not possible to

eliminate these effects, it is possible to quantify the resultant sound fields and place them in their correct context.

Whilst it can be categorically proven that sound power levels at various frequencies differ between the playbacks and the sounds as recorded in the field, the actual exposure is nonetheless a known quantity. Likewise, it is possible to show how exactly exposure treatments compare to their respective sound controls (Figure 2.1).

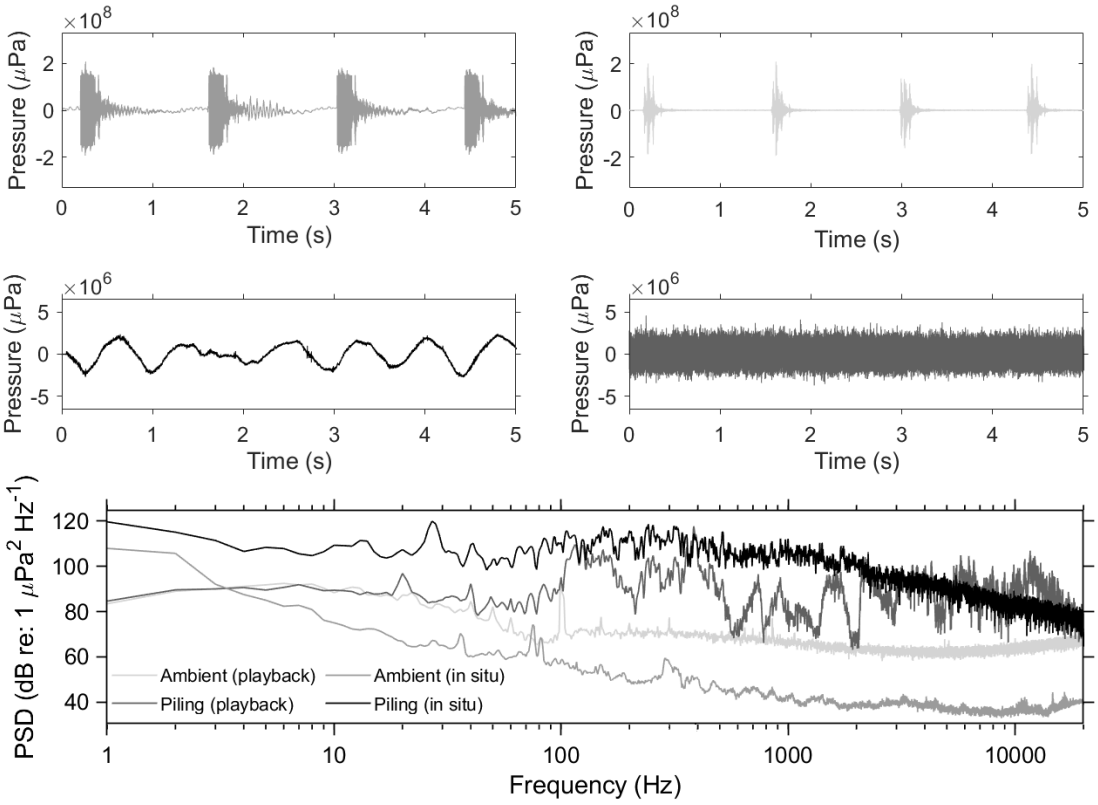

Figure 2.1: Comparison of amplitude-normalised in-situ recordings and tank playbacks. *Top: Waveforms of ambient sound as recorded in-situ (left) and in experimental playback (right). Mid: Waveforms of piling sound as recorded in-situ (left) and in experimental playback (right). Bottom: RMS power spectral density plot of ambient and piling sounds as recorded in-situ and as played back.*

Moreover, it is currently asserted that marine invertebrates are most sensitive to the particle motion component of sound in a frequency range of 20-3000 Hz (Lovell et al., 2005; Mooney et al., 2010; Popper et al., 2001). Therefore, discrepancies in acoustic power outwith these frequencies may ultimately prove irrelevant for studies involving invertebrates. However, assertions regarding invertebrate frequency sensitivities are

based on limited research. Thus, where possible it was decided to expose organisms to broadband sounds and to quantify both particle motion and pressure to provided additional context in the event these assertions change in the future.

Despite these discrepancies between *in situ* recordings and playbacks, it was deemed more appropriate to use these field-derived sounds than alternatives such as white-noise, pink-noise, or pure tones. Sounds approximating characteristics of authentic sounds likely share more environmental realism than completely artificial sounds. Similarly, whilst environmental realism may be diminished, it can still be ascertained whether 'additional' sound exposure has effects relative to 'ambient' sound levels, and as long as it is clearly communicated that these sounds were achieved via 'playback' of a given sound, is neither misleading nor diminishes the results.

2.2.3 Chemical exposures

Chemical exposures were conducted according to ecotoxicological principles (Harris et al., 2014). Larval exposures were preferentially carried out under either static or static-renewal conditions depending upon exposure durations. The choice not to aerate exposure vessels where feasible was taken for practicality reasons, namely to avoid challenges of rigging multiple airlines, minimising complications associated with aerosolisation of chemicals including potential cross-contamination, and to avoid risking stressing larvae through introduction of bubbles. Additionally, aeration of water is known to produce considerable sound (Hildebrand, 2004), which would be extremely difficult to standardise across treatments and replicates and may influence the results of the studies.

Dosing processes were designed specifically to maximise the likelihood of achieving and maintaining nominal concentrations in the exposure vessels. Deionised water was used as a carrier wherever possible to prevent any cross-reactivity between chemical

species. Once in solution, chemical ions are free to interact with other ions within the solution, which can result in the formation of compounds that may precipitate out of solution, or otherwise become biologically unavailable (R. Lewis, 2011). Likewise, factors including temperature and pH are known to affect equilibria between ionic and molecular forms of various chemicals. Although these chemical interactions are unavoidable and inevitable once introduced to more chemically complex experimental media, preparing dosing solutions in deionised water maximises the potential that initial dosing achieves the desired nominal concentrations. In addition to chemical losses through chemical interactions and aerosolisation, many chemicals are prone to losses through adsorption – chemicals becoming weakly bonded to surfaces such as glass and plastics (Struempler, 1973). To limit these potential losses, exposure vessels were conditioned prior to use. Conditioning entails the rinsing of exposure vessel surfaces using chemical solutions of the target concentration, such that any adsorption that can/may occur does so prior to experimental exposures.

Water samples were taken immediately following spiking and immediately prior to water changes, the target chemical quantified, and time-weighted mean exposure concentrations calculated. Water samples were pooled across replicates at each chemical concentration ensuring replicates contributed equally to the final pooled samples. Water samples were also pooled across replicates in different sound treatments. This decision, though not as robust as quantifying concentrations in each individual treatment, was made for pragmatic reasons. Firstly, all solutions were dosed using the same solutions, so all initial exposures concentrations should be consistent. Secondly, whilst sonication can affect the physicochemical properties of some chemicals (R. J. Wood et al., 2017), this is only evidenced at ultrasonic frequencies exceeding 20 kHz, suggesting that sound at the frequencies used throughout this thesis

should have no impact on the physicochemical properties of any of the chemicals investigated. Thirdly, whilst it is conceivable that different behavioural and/or physical responses between sound treatments may influence *effective* doses through differences in uptake/internalisation of the chemicals, it was concluded that any such variation in chemical flux would be quantitatively indistinguishable from losses resultant of previously mentioned processes, especially given the comparative size of test organisms used throughout.

The assumed minor chemical fluxes attributed to biological uptake mean it is also likely that stated time-averaged exposure concentrations are still an overestimation, albeit it less-so than relying solely on nominal concentrations. Chemical losses to adsorption and cross-reactivity likely occur relatively quickly after dosing, resulting in a rapid decrease in available chemical. After this, a more linear decrease through uptake would be expected. However, to properly determine this, a chemical fate study with high temporal resolution would be required, which was not considered cost-effective given the research questions of interest.

2.2.4 Replication and allocation

Whilst it is understood that methods such as power analysis exist for estimating appropriate sample sizes for statistical purposes, these were not utilised within this thesis. To be effective, power analyses require estimates of potential outcomes, both with regards to magnitudes and variability (Cohen, 1992). Given the novel nature of the research presented, there was insufficient data available to attempt such estimates. Instead, it was decided to allocate sample sizes as large as possible whilst working within the constraints of organism, space, and time availabilities alluded to throughout Section 2.1. Given full factorial designs were prioritised, and the exponential increases of replication requirements characteristic of multi-driver studies,

this required compromises on the number of factor levels, and sample sizes therein. Ideal sample sizes varied depending on specific experiments and research questions and were informed by replication in studies sharing similar response parameters. Where organism availability was a potential limitation, sample-sizes were prioritised at the expense of factor levels, excepting circumstances where the research question fundamentally required a given set of factors (such as repeat/follow-up experiments), in which case sample sizes were determined strictly by organism availability.

2.2.5 Statistical analysis

2.2.5.1 General approach

Statistical analyses can be thought of as tools that exist to help rationalise a set of data. Because of data variety, differing research questions, and an ongoing pursuit of refinement, a whole suite of options now exists for data analysis. However, picking the most suitable analysis can be a daunting task – especially when trying to operate within the various constraints of statistical models and of given data sets. Likewise, it is entirely possible that multiple analyses may be able to handle a data set, albeit with varying levels of appropriateness. The challenge therefore becomes a matter of 'choosing the right tool for the job'.

Underlying data distribution, or rather the distribution of resultant model residuals and equality of variance, have a profound impact on the selection of appropriate analyses – namely whether the data are suited to parametric analyses. Parametric models work on the assumption that the sample data conform to a given distribution (commonly a normal distribution). Resultantly, the models rely on a set number of coefficients which describe the specific 'shape' of these distribution curves, and how they differ. These analytical coefficients, being consistent and directly comparable,

means parametric analyses are considerably more powerful and robust. However, many biological data sets do not conform to standard distributions – either consequent of a sampling method, or the nature of the specific variable being observation. When these data do not meet the underlying assumptions of parametric models, non-parametric analyses should be considered.

Non-parametric models are less reliant on underlying assumptions of data distribution. Because the distribution of the data set is not assumed, non-parametric analyses must define both the distributions themselves, as well as how they differ. Consequently, non-parametric analyses tend not have a constrained number of coefficients making them more flexible, but the requirement for additional coefficients often erodes statistical power as any data variation will also be spread more broadly across these same variables. Non-parametric analyses also differ to parametric analyses in that they typically contrast sample medians rather than means given medians are often more representative of non-normally distributed data.

Another key difference between non/parametric analyses is in their respective ability to incorporate multiple independent variables. Common parametric analyses such as ANOVA-based models are capable of estimating effects attributable to individual factors, as well as any interactions. In contrast, many of the more common non-parametric equivalents (e.g. Kruskal-Wallis) are univariate, meaning the factors have to be condensed down into ‘treatment groups’ which can then be contrasted against each other, and thus cannot be used to directly assess effects of individual factors or indeed interactions.

The increasing accessibility of high-power computing is enabling ever more flexible statistical approaches. One such example is the ability to apply data resampling techniques such as interpolation and bootstrapping to traditional models. These

approaches, though implemented in different ways, both effectively act to address and remove non-normality in data sets, allowing more flexible application of existing parametric approaches. However, increased computing power also facilitates the use of more advance models, such as generalised linear models (GLM), linear mixed models, and generalised additive models (GAM) which are better able to handle non-normal data distributions, and even non-linear effects.

2.2.5.2 Analysis selection and chosen approaches

All statistical approaches used were selected on the basis of data distributions and underlying model assumptions. Correspondingly, no data transformation techniques were used in order to meet data distribution assumptions of otherwise inappropriate statistical models. In the cases below where highly comparable model options were available, the following justification for model selections apply.

Kaplan-Meier Survival Analysis vs Cox Proportional Hazard

Time-dependent binary variables (e.g. mortality data and developmental rate data) were assessed using Kaplan-Meier Survival Analysis. Though univariate, the Kaplan-Meier model was chosen over the multivariate Cox Proportional Hazard model for two reasons. Firstly, The Cox model assumes a proportional hazard risk with respect to time; an assumption that was not guaranteed to be met by the data, and a constraint not imposed by the Kaplan-Meier model. Secondly, the Cox model incorporates multiple drivers to produce an estimated hazard posed by the combination of drivers, which is not optimal for addressing how two or more drivers interact. A key research question was to ascertain differences between sound treatments across additional driver levels, which would require estimations of hazard/risk for factor and level within the model. This can be achieved using a Stratified Cox Proportional Hazard model, but

ultimately a Kaplan-Meier model applied to discrete treatments achieves this same outcome whilst being more transparent, and more closely utilising the raw data.

Fisher's test vs Chi-squared test

Where overall count data were ascertained within a study, but independent replication was not feasible, differences between treatments were assessed using Fisher's Exact Test. Fisher's Exact Test was chosen over the more common Chi-squared as it is accepted to be more accurate when replication is limited, but to converge with the Chi-Squared Test as replication increases (Kim, 2017). Therefore, despite Fisher's Exact Test being slightly conservative, it is considered the more robust analytical choice.

Logistic regression vs Probit

For binary data conforming to sigmoidal dose-dependent responses, it was possible to model the data at given timepoints using logistic regression (logit). Logit was chosen over the similarly capable probit regression given that the mathematical operations underpinning logit are fewer and less abstract than those of probit, allowing for more intuitive interrogation of the model if desired.

Logit models enabled interpolation of the data to produce curves from which data including EC_{50} values can be estimated. Similarly, the models could be stratified by sound treatments and the curves compared in context across the continuous range of a driver.

Permutation vs bootstrapping

Permutation methods are highly suited to contrasting *observed* data again null-distributions, and thus highly suited to comparative hypothesis testing. Consequently, where data resampling methods were desirable, permutation-based analyses were

selected over bootstrapping which is otherwise better suited to estimating *expected* data distributions, and thus producing data estimates such as standard error and confidence intervals.

2.2.5.3 Multiple comparisons

In addition to simply ascertaining whether the drivers of interest elicited differences in response parameters, it was often desirable to know between which specific treatment groups these occurred given that not all treatment pairings may be experimentally or ecologically relevant. For example, a dual-driver design with two and four levels respectively, results in eight treatments which can be contrasted between each other in 28 pairings. However depending upon ones research question, some of these pairings may not provide meaningful context (Figure 2.2). Thus, in the context of this thesis, comparisons were limited to these pairings.

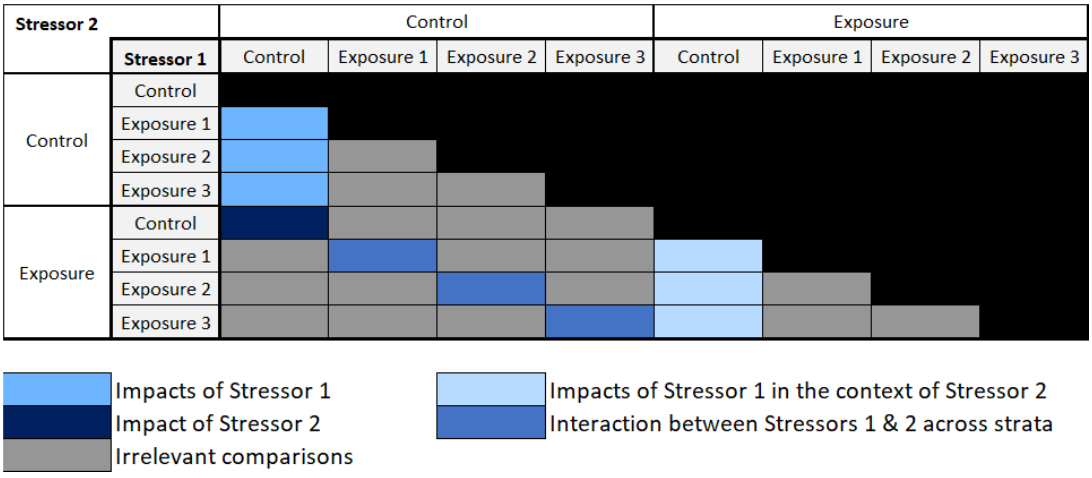

Figure 2.2: Table outlining all potential comparisons in a full-factorial two-by-four dual driver study. Colours signify context provided by treatment comparisons

In all cases, *post-hoc* comparisons address specific questions and ascertain key data values such as the no observed effect concentration (NOEC), lowest observed effect concentrations (LOEC) of a chemical, or to compare between sound treatments across chemical driver levels to ascertain whether an interaction was evident. However,

undertaking multiple comparisons within a dataset inflates the likelihood of encountering Type-I errors. To account for this, false detection rate (FDR) was corrected for using the Benjamini-Hochberg Procedure (Benjamini & Hochberg, 1995). Benjamini-Hochberg was selected as it maintains a good representation of the specific data set given that corrections are applied based upon the rank-order p values. It is also less conservative than other common alternatives, such as Bonferroni corrections, which are applied uniformly to all values but grow increasingly conservative as the numbers of comparisons increase.

Given experiments were deliberately designed full factorially to allow contrast of specific treatments and the conservative nature of FDR corrections, both corrected and uncorrected p values are presented. Consequently, uncorrected p values <0.05 between specific treatment pairs are considered and discussed as being statistically significant, with those with corresponding FDR corrected p values also < 0.05 considered with greater confidence.

2.2.6 Addition, antagonism, and synergism

Whilst the terms additive, antagonistic, and synergistic are accepted lexicon in scientific literature, interpretation and use of these terms varies between studies and authors. Such discrepancies are seemingly resultant of contextual subjectivity, and directional aspects of specific drivers (J. J. Piggott et al., 2015). Many of the interpretations are commonsensical and readily understandable within the context of their respective studies, however there are also examples within the literature where the terminology is misused, with assertions that are both fundamentally unsubstantiated and/or incorrect. These more troublesome issue arises from a disparity between use of the terms according to their *stricto sensu* definitions and applying them as a description of an outcome.

Strictly speaking, antagonism and synergism mean a combination of drivers having respectfully a lesser and greater effect than each of the drivers individually. Thus, the terms are a mathematical description of how the drivers combine vectorially, irrespective of the absolute outcome. These definitions require a quantitative understanding of how each individual driver affects the dependent variable relative to the control group, and thus can only be used in instances where full-factorial designs have been implemented. This usage of the terms has the benefit of requiring no contextual qualification, and being applicable regardless of whether drivers demonstrate double-positive, double-negative, or opposing effects (Crain et al., 2008). However, this definition but does not intuitively lend itself to understanding the biological or ecological implications of the results.

Despite their *stricto sensu* definitions, the terms are also commonly used to signify a combination of drivers resulting in a magnitudinally different result with respect to a comparative frame of reference (e.g. a control group or single-driver response equivalent), with antagonism or synergism being applied to describe and signify directionality. For example, where data values are mutually exclusive as in the case of mortality data, terminology will depend on whether results are discussed in terms of mortality or survival – after all, something that is antagonistic to mortality could equally be described as being synergistic to survival. Despite relying on contextual qualification in the reporting, these 'outcome based' terms are often more intuitive to understand and better signify 'bottom-line' impacts, albeit at the expense of describing precisely how each of the drivers combine. Piggott et al. (2015) suggested a notation system that would incorporate this context into the nomenclature, however a two-variable response generates 45 potential classifications, and therefore is arguably less comprehensible despite its transparency.

For the purposes of this thesis, interactions are discussed using ‘outcome-based’ definitions unless otherwise stated or further qualified, given that phenomenological observations herein are considered more robust than accompanying mechanistic interpretations – an approach also suggested by Boyd et al (2018). Similarly, the methodological approach taken throughout is heavily based on ecotoxicological principles which are tailored towards providing ecological context – something more accessible using the outcome-based interaction definitions.

Chapter 3

Effects of Pile Driving Sound Playbacks and Cadmium Co-exposure on the Early Life Stage Development of the Norway Lobster, *Nephrops norvegicus*.

The majority of this chapter is presented in the peer review article:

Stenton, C.A., Bolger, E.L., Michenot, M., Dodd, J.A., Wale, M.A., Briers, R.A., Hartl, M.G.J., Diele, K. 2022. Effects of pile driving sound playbacks and cadmium co-exposure on the early life stage development of the Norway lobster, *Nephrops norvegicus*. Marine Pollution Bulletin 179, 113667. https://doi.org/10.1016/j.marpolbul.2022.113667

Some minor additions have been made to the introduction and discussion of this chapter for the benefit of this thesis.

CRediT authorship contribution statement

C.A. Stenton: Conceptualization, Methodology, Formal analysis, Data curation, Investigation, Writing – original draft, Visualization. **E.L. Bolger:** Investigation, Methodology. **M. Michenot:** Investigation. **J.A. Dodd:** Formal analysis. **M.A. Wale:** Investigation. **R.A. Briers:** Supervision, Writing – review & editing. **M.G.J. Hartl:** Conceptualization, Supervision, Writing – review & editing. **K. Diele:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Project administration.

3.1 Abstract

There is an urgent need to understand how organisms respond to multiple, potentially interacting drivers in today's world. The effects of the pollutants of anthropogenic sound (pile-driving sound playbacks) and waterborne cadmium were investigated across multiple levels of biology in larvae of the Norway lobster, *Nephrops norvegicus* under controlled laboratory conditions. The combination of pile-driving playbacks (170 dB_{pk-pk} re 1 µPa) and cadmium combined synergistically at concentrations > 9.62 $µg_{[Cd]}$ L^{-1}, resulting in increased larval mortality, with sound playbacks otherwise antagonistic to cadmium toxicity. Significant delays in larval development were caused by exposure to 63.52 $µg_{[Cd]}$ L^{-1}, dropping to 6.48 $µg_{[Cd]}$ L^{-1} in the presence of piling playbacks. Pre-exposure to piling playbacks and 6.48 $µg_{[Cd]}$ L^{-1} led to significant differences in swimming behaviour of the first juvenile stage. Biomarker analysis suggested oxidative stress as the mechanism of deleterious effects, with cellular metallothionein (MT) being the predominant protective mechanism.

3.2 Introduction

Many marine environments and the species therein are facing unprecedented pressure resultant of anthropogenic activities. To date, many studies have considered the effects of individual drivers, however in reality environments are complex with multiple drivers co-occurring or interacting (Boyd, Gattuso, et al., 2018; Griffen et al., 2016). The need to better understand the impacts and implications of multiple drivers is emphasised by inclusion as an objective of the United Nations Decade of Ocean Science for Sustainable Development (Leinen, 2019). Though climate change is arguably at the forefront of people's minds when discussing environmental drivers in our oceans, there are many other drivers including pollution in the form of chemical

loading (Bocchetti et al., 2008; Matlock et al., 2002; Rider et al., 2014), and sound pollution caused by shipping, marine construction and other anthropogenic activities (Bocchetti et al., 2008; Matlock et al., 2002; Rider et al., 2014; Solan et al., 2016). Whilst many studies have evidenced impacts of chemical pollutants on marine life, and a growing body of work addressing the biological impacts of sound pollution exists (Wale et al., 2021), few studies have investigated these drivers in combination, despite their potential co- occurrence.

To assess whether interaction between anthropogenic sound and chemical pollution occurs, the toxicity of waterborne cadmium was assessed in combination with simulated pile driving sound achieved via playback of *in situ* recordings. Pile driving was selected as a sound driver given the expected prevalence of offshore construction in the coming years, particularly in the energy sector (Gourvenec et al., 2022). Cadmium was selected given its common use as a reference toxicant, but also due to its legacy prevalence in marine sediments. The specific combination of pile driving sound and cadmium exposure was considered environmentally plausible as sediment disturbances during construction activities can result in dissolution of sediment-associated minerals and chemicals (Eggleton & Thomas, 2004; Gutiérrez-Galindo et al., 2010).

Pile driving, or piling, is the process of driving supports known as ‘piles’ deep into bedrock to provide solid structural foundation for construction. This often involves percussively hammering metal piles through the ground, the acoustic profile of which varies depending on the installation specifics, with source sound pressure levels exceeding 250 dB re 1 μPa @ 1 m recorded for installations in UK waters (Nedwell et al., 2007). Based upon recordings of piling derived in the North Sea and specific sound propagation modelling, it has been estimated that pile driving sound exposure levels

remain at 168 dB re 1 μPa^2 s even at 2 km distance from the source (Bolle et al., 2012; Hazelwood & Macey, 2021). Piling also produces impulsive sounds, so in addition to typically producing high amplitudes of sound pressure and particle motion, these shifts in pressure and particle motion occur extremely rapidly (Hastie et al., 2019).

Cadmium is a heavy metal predominantly produced as a by-product of zinc refinement (Shiel et al., 2010) and is widely used in industry, including use in batteries, pigments, and as an alloying material (Hasanuzzaman & Fujita, 2013). Cadmium is also a well-established and highly potent environmental toxicant. At a molecular level, cadmium promotes the creation of reactive oxygen species (ROS), which stress can both damage DNA and inhibit its repair (Zhang & Reynolds, 2019). These effects on DNA contribute greatly to cadmium's known carcinogenic and teratogenic properties (Witeska et al., 2014) which have been widely researched across a variety of taxa (Bohra et al., 2015; Marettová et al., 2015; Okocha & Adedeji, 2011). In early life stage invertebrates specifically, cadmium-induced oxidative stress has been shown to drive cellular apoptosis in larvae of the fruit fly, *Drosophila melanogaster* (Yang et al., 2022) and induce damage and structural changes in sperm of the of freshwater crab, *Sinopotamon henanense* (Ma et al., 2013). Cadmium exposure led to dose-dependent changes in protein expression in larvae of the sea urchin *Paracentrotus lividus* which persisted beyond the exposure period, delayed and/or inhibited embryonic development, and impaired skeletal development (Roccheri et al., 2004). Cadmium can also have endocrine disruptive effects in early life stage organisms, affecting expression of regulatory hormones (Takiguchi & Yoshihara, 2006), with retardation of growth and development linked to altered expression of key growth hormones (Abidi et al., 2016; Jones et al., 2001; Rodríguez Moreno et al., 2003; Zou, 2005).

Although environmental discharge is now regulated in much of the world, cadmium of historic origin present in sediments remains a persistent contaminant (Ei Tun et al., 2009; Kühn et al., 1992), especially in estuarine and marine settings where it is likely to deposit given its physicochemical properties (Jiann & Ho, 2014; Stephenson et al., 1996). For example, following construction of the Morowali district's mining port in Indonesia, concentrations of cadmium in the local seawater spiked at over 100 µg L^{-1} (Delly et al., 2021), compared to mean open-ocean surface concentrations of cadmium range between 0.001-0.1 µg L^{-1} (Neff, 2002). Given a plausible co- occurrence of sound-producing construction activity and heavy metal enrichment of waters, the aim of this study was to evaluate the potential interactive effects of the combination of pile driving sound playbacks and cadmium exposure. The study focused on early life stage organisms given these are often more sensitive to stressors than mature counterparts (Braunbeck et al., 2014). Hence, early life stage exposures can lead to population 'bottlenecks' (J. Pineda et al., 2010).

The model species chosen for this study, the Norway lobster, *Nephrops norvegicus* (henceforth *Nephrops*), is a decapod crustacean common to the shallow-water regions of the North-East Atlantic and North Sea regions (Fisheries Global Information System (FAO-FIGIS), 2016). *Nephrops* has a biphasic life-cycle — undergoing planktonic larval development, followed thereafter by a benthic existence excavating and occupying burrows in muddy sediments. Adult female *Nephrops* produce a clutch of eggs annually, typically fertilised during summer months. The egg clutch is carried on the female's pleopods for around nine months before hatching in the following spring. Newly hatched pelagic Zoea larvae (Zoea I) are dispersed into the water column, and over a six- week period undergo two moults (to stages Zoea II and Zoea III) before

metamorphosing to the first juvenile stage and commencing a benthic existence (Powell & Eriksson, 2013).

The conservation status of *Nephrops* is currently considered as 'least concern' by the International Union for Conservation of Nature (IUCN); nonetheless the species faces a variety of pressures as the result of human activities. *Nephrops* are highly sought after across NE Europe for their commercial value, representing the third largest target-species fishery in Scotland by mass, valued at £86 m for 2019 (The Scottish Government, 2020). In addition to fishing pressure, the North Sea habitat range of *Nephrops* overlaps with a region of high utilisation for energy production, with a high density of existing and/or planned fixed structures including windfarms and fossil fuel platforms, the construction of which usually requires some degree of pile driving.

This study aimed to ascertain (i) whether anthropogenic sound playbacks and/or chemical pollutants affect the early life stages of *Nephrops*, (ii) whether these two drivers combine and interact, and (iii) the potential mechanism contributing to any such interactions.

3.3 Methods

The study comprised two complementary experiments conducted in a controlled laboratory setting. Experiment 1 focused on impacts of individual and combined sound and cadmium exposure on mortality, growth and development of *Nephrops* larvae and behavioural fitness of the first *Nephrops* juvenile stage. Experiment 2 addressed a potential mechanistic link to the phenomenological observations from Experiment 1, focusing on quantification of oxidative stress biomarkers.

3.3.1 Animal husbandry

Berried female *Nephrops* were procured from DR Colin & Sons Ltd. of Eyemouth, Berwickshire, UK during July 2018 (Experiment 1) and June 2019 (Experiment 2). All *Nephrops* were trawl-caught, landed, sorted, and held in refrigerated seawater before being transported to the St Abbs Marine Station, Berwickshire, UK on the same day they were caught. Healthy-appearing berried females with eggs in an advanced stage of development were selected and placed individually in 15 l conical upwelling hoppers (30 cm top-diameter, 15 cm bottom-diameter, 30 cm depth) with 1.5 mm mesh-covered outflows to enable retention of larvae of known maternity upon hatching. Hoppers contained segments of PVC pipes to provide shelter and were covered by 75% shade netting to reduce light intensity. The females were fed cooked blue mussel (*Mytilus edulis*) *ad libitum*. Flow-through conditions were maintained using raw, ambient-temperature seawater. On a weekly basis, each berried female was carefully transferred to a freshly cleaned hopper. Hoppers were visually inspected each morning for presence of newly hatched larvae. If present, these were collected into a 2-litre beaker, and subsequently transferred individually to experimental conditions using a 10 ml pipette. Larvae were maintained in UV sterilised seawater dosed to a designated cadmium concentration (detailed below). Water temperature was maintained at 12 ± 1 ˚C, and 75% shade netting used to reduce light intensity and minimise disturbance. Vessels were cleaned and received a 95% water change twice weekly, at which time larvae were fed *ad libitum* with *Artemia* sp. nauplii.

3.3.2 Experimental system

Two identical exposure systems, each facilitating a different sound treatment, were set up to allow concurrent co-exposure of sound and cadmium under controlled conditions. Each system comprised a 750- litre fibreglass tank (internal dimensions

(LWD): 152 cm × 94 cm × 60 cm) acting as a water bath, containing a Clark Synthesis Diluvio AQ339 Aquasonic underwater speaker (frequency response: 20–17,000 Hz) suspended centrally above the tank floor and orientated upwards towards the water surface, such that the speaker cone was 14 cm above the floor of the tank, and a minimum of 40 cm from the base of the closest exposure vessels (Figure A1). Speaker suspension was employed to minimise extraneous vibration transfer between the speaker body directly into the tank superstructure and to reduce the potential for additional, diffuse sound pressure and/or particle motion sources. Each speaker was coupled to a Samson Audio Servo 300 Power Amplifier with signal input facilitated via a laptop computer and M-Audio M-Track QUAD Audio Interface. Each fibreglass tank was filled with raw seawater (34 ppt salinity) maintained at 12 ± 1 °C using a Teco TK-2000 heater- chiller unit fed by an Eheim Universal 3400 pump operating at a flow rate of 775 l/h, forming a static recirculating system. Both the pump and heater-chiller unit were externally isolated from the fibreglass tank to prevent transfer of additional sound and vibration, and all necessary contact points between in/outflow piping dampened by 4 mm-thick rubber sheeting placed between their interfaces. Water levels within the fibreglass tank were maintained where necessary by addition of deionised water, accounting for losses to evaporation and maintaining consistent seawater density (and associated sound propagation) conditions. Each fibreglass tank contained a table positioned centrally above the speaker and isolated from the tank floor using anti-vibrational rubber sheeting, providing a surface onto which exposure vessels could be placed. Table height maximised the distance of the exposure vessels from the speaker to reduce the impacts of potential sound cone and near-field sound effects, whilst ensuring the working volume of the exposure vessels remained

submerged. This resulted in a water-based interface for sound propagation, and effective temperature regulation of the exposure vessels.

3.3.3 Sound exposures

Exposure to ambient and pile driving sounds was simulated using playbacks of field-derived sound recordings made available by Rick Bruintjes (Defra, UK), Sophie Nedelec (University of Exeter, UK) and Irene Voellmy (University of Berne, CH). Piling playback tracks were compiled from multiple recorded strikes of a 1.2 m diameter monopole being driven approximately 25 m into the seabed in a water depth of 6.5 m, recorded at distances between 87 and 200 m from the sound source using a Hi Tech Inc. HTI-99HF hydrophone with inbuilt preamplifier (manufacturer calibrated sensitivity − 204 dB re 1 V μPa^{-1}, 20–125,000 Hz frequency range) and a RTsys EASDA data logger using a 44.1 kHz sampling rate. Ambient playback tracks were compiled from comparable recordings made in the absence of any evident anthropogenic sound, taken using a HiTech HTI-96-MIN hydrophone with inbuilt preamplifier, and an Edirol R09-HR 24-Bit recorder (44.1 kHz sampling rate). All sound files for experimental playback were compiled in Audacity 2.2.2 and output as 24-bit WAV files. The ambient sound treatment comprised a 4:00 h looped ambient recording repeated continuously. The piling playback sound treatment comprised four pile driving tracks of varying length (1:00 h, 1:15 h, 1:30 h, 2:15 h) interspersed with four ambient tracks (3:30 h, 4:00 h, 4:30 h, 6:00 h duration). Pile driving and ambient tracks were alternated such that no single track was repeated within a 24-hour period, amounting to a pseudorandomised sound regime, whilst maintaining a known, consistent sound exposure each day. Received sound pressure level (SPL) in exposure vessels was targeted at 118 dB_{RMS} re 1 μPa in ambient playback phases to approximately match the noise- floor of the tanks, and at 170_{pk-pk} re 1 μPa for piling playback phases. Received sound exposure levels were

measured within each exposure vessel. Sound pressure was measured using a manufacturer-calibrated HiTech HTI-94-MIN hydrophone (sensitivity: − 165 dB re 1 V μPa^{-1}) coupled with a calibrated Roland R-26 2-channel Portable Recorder. Particle motion (as three-dimensional magnitudinal acceleration) was measured using a calibrated custom-built triaxial accelerometer (STMicroelectronics LIS344ALH) potted within epoxy resin and suspended within the exposure vessels using 1 mm diameter elastic cord (Wale, 2017).

3.3.4 Cadmium exposures

All equipment and exposure vessels were acid washed using 2 M nitric acid prior to use to remove trace-metal contamination. Exposure vessels were then subsequently conditioned with a cadmium solution of their respective designated nominal concentration. In each experiment, larvae were exposed to one of four cadmium treatments with nominal waterborne cadmium ion concentrations (Cd^{2+}) of 0 $\mu g\ L^{-1}$, 1 $\mu g\ L^{-1}$, 10 $\mu g\ L^{-1}$, and 100 $\mu g\ L^{-1}$ – henceforth referred to as $Control_{[Cd]}$, $Low_{[Cd]}$, $Medium_{[Cd]}$ and $High_{[Cd]}$ respectively. All chemical exposures were conducted under semi-static renewal conditions, with twice-weekly (Experiment 1) and daily (Experiment 2) 95% water changes and full cadmium renewal. In each experiment, replication was fulfilled using conspecific larvae originating from a single berried female. Detailed cadmium dosing regimens for both Experiment 1 and Experiment 2 can be found in the supplementary materials (Table A6.1).

Time-averaged waterborne Cd^{2+} concentrations were quantified from paired water samples taken immediately after dosing and immediately preceding water changes pooled from each replicate and preserved by acidification to pH < 2 using addition analytical grade nitric acid. Cadmium quantification was conducted by GEOMAR Helmholtz Centre for Ocean Research, Germany using solid-phase extraction ICP-MS.

3.3.5 Experiment 1: phenomenological observations

For Experiment 1 a total of 160 *Nephrops* Zoea I larvae were evenly distributed between treatment groups, resulting in 20 independent replicates (i.e. larvae) per treatment. Larvae were allocated over a two- day period due to timing of hatching, with 80 larvae evenly allocated across treatment groups on each of the two days. Larvae were maintained individually in 330 ml BPA-free, food-grade virgin polypropylene plastic cups containing 250 ml of cadmium-dosed UV sterilised seawater. Treatment replicates were arranged in a 14 × 6 Latin-square array to account for environmental factors and sound gradient effects (Figure A2).

Larvae were concurrently exposed to sound and chemical treatments for the duration of their planktonic development (Zoea I, II, III), and for an additional three days following metamorphosis to the benthic juvenile.

3.3.5.1 Mortality

All larvae were observed daily. In the event of a mortality, this was noted and the carcass collected, labelled, and frozen in a − 20 °C freezer for later biometric analysis.

3.3.5.2 Development

In the event of a successful moult, the day of moulting was recorded relative to the date each individual larva initially hatched.

3.3.5.3 Behavioural fitness

Nephrops that successfully transitioned to juveniles were subjected to a behavioural fitness assessment three days post metamorphosis. Their tail-flick escape response was assessed using methods similar to those described in (Kellie et al., 2001) and (Bolger, 2022). The fitness assessment was conducted in a circular ‘arena’ (19 cm

diameter, height 12 cm), filled with 250 ml of fresh, temperature acclimated UV sterilised seawater. The contents of individual exposure vessels, juvenile *Nephrops* included, were carefully poured into the arena. Following a five-minute acclimation period, the first-stage juvenile *Nephrops* were provoked by vertically lowering a small plastic rod onto the arena floor directly in front of them (0.5 cm from the rostrum) at a speed of approximately 10 cm s^{-1}. For each provocation, the presence/absence of an induced escape response was recorded on video at 720p resolution and 60 fps using a DSLR camera suspended above the arena for later analysis using ImageJ Fiji (Schindelin et al., 2012). Where a tail-flick response was provoked, the total number of tail flicks within that response was counted, along with the distance the larva had travelled and its average swimming speed of each component tail flick within that response. This generated a hierarchical data set detailing each aspect of the swimming dynamics across all provocations. Fitness assessment and reviewing of the video files were both conducted blind to reduce potential bias.

3.3.5.4 Biometrics

Following completion of the behavioural fitness experiment, carapace length (mm) of first-stage juveniles (rostrum to anterior carapace) was measured using digital calipers and whole organism wet- and dry- weight (mg) (dried at 60 °C to constant mass) determined using a gravimetric balance.

3.3.6 Experiment 2: biomarker assays

For Experiment 2 a total of 672 *Nephrops* Zoea I larvae were evenly distributed across seven replicates of each treatment. Each replicate constituted a 1000 ml borosilicate glass beaker, containing 800 ml of cadmium-dosed UV sterilised seawater, and 12 larvae. Communal allocation of larvae within replicates was required to provide sufficient tissue quantities to facilitate biomarker analyses. Full allocation was

conducted over a 12-day period over which the larvae hatched, with specimens hatching on any given day being evenly distributed across all treatments. Exposure vessels were randomly allocated to one of 16 positions (arranged in a 4 × 4 square within the central portion of the exposure system (Figure A3) where sound levels were most consistent, and randomly reallocated to one of these 16 positions following each water change to minimise the influence of any environmental variation.

Following the 5-day exposure period, surviving larvae from each replicate were removed from the vessels, gently dried with absorbent tissue, and all individuals within a replicate collected into a single cryovial before being flash-frozen in liquid nitrogen and stored at − 80 °C. Replicate whole-organism samples were homogenised in 800 μl Tris- HCl (50 mM, 0.15 M KCl, pH 7.4) buffer solution using a motorised pestle, and spun at 10,000 RPM for 3 min in an Eppendorf Mini Spin centrifuge. The resulting supernatant was split into aliquots for each of the oxidative stress assays, and re-frozen at − 80 °C until required. Quantitative assays were normalised against total protein content (Bradford, 1976). Superoxide dismutase (SOD) inhibition was quantified using the Sigma-Aldrich SOD Determination Kit (19160). Catalase (CAT) activity was quantified using the Cayman Chemical Catalase Assay Kit (707002). Glutathione (GSH) concentration was determined according to methods outlined by (C. J. Smith et al., 2007) adapted from (Owens & Belcher, 1965). Glutathione peroxidase (GpX) was quantified using the Cayman Chemical Glutathione Peroxidase Assay Kit (703102). Thiobarbituric acid reactive substances (TBARS) were quantified following the protocol of Al-Shaeri et al. (2013) adapted from Smith et al. (2007). Metallothioenein (MT) was quantified in accordance with the methods derived from Viarengo et al. (1997) and Cenov et al. (2018) assuming 18 Cys residues per

metallothionein residue (Cenov et al., 2018; Zhu et al., 1994). Detailed methods for all biomarker assays can be found in the supplementary materials.

3.3.7 Treatment characterisation and quantification

Sound analyses were conducted using the Signal Processing Toolbox in MATLAB R2021b (The MathWorks Inc., 2021). Sound pressure was analysed over a broadband frequency range of 1–24,000 Hz from 10 s recordings. Sound particle motion was analysed over a 50–3000 Hz range using a 3rd-order Butterworth bandpass filter. Ambient sound treatments were characterised as continuous sounds, and therefore quantified as root-mean-square (RMS) sound pressure. Piling playback treatments were characterised as impulsive sounds, and thus primarily quantified using peak-to-peak sound pressure, with RMS being calculated for comparative purposes only.

3.3.8 Statistical analyses

All statistical analyses were performed using the R version 4.1.0 (R Core Team, 2021). Analyses were selected based on underlying analytical assumptions and data conformity. Where multiple comparisons were undertaken, statistical significance ($\alpha = 0.05$) was conveyed according to direct pairwise comparisons (p), with secondary values controlling for false discovery rate (p_{FDR}) using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995) providing robustness of interpretation.

3.3.8.1 Mortality

Total larval mortality was modelled using logistic regression. Model significance was reported as deviance compared to the null model. Treatment mortality rates were also compared using Kaplan-Meier survival analysis and *post-hoc* log-rank Mantel-Cox test.

3.3.8.2 Developmental rate

Developmental duration of *Nephrops* larvae was assessed by the timing of the transition (through moulting) between each zoeal stage up to-and-inclusive of the first juvenile stage. Day of transition was compared using Kruskal-Wallis test, with *post-hoc* analysis via Dunn's test.

3.3.8.3 Biometrics

Carapace length of both larvae and juveniles was compared using a two-way ANOVA with cadmium concentration and sound treatment as factors. Dry tissue weight was compared using a Kruskal-Wallis test across discrete treatment groups.

3.3.8.4 Behavioural fitness

Behavioural fitness of the first-stage juveniles was analysed using a hurdle model approach. Firstly, it was assessed whether the simulated threat provoked an escape response, and secondly the dynamics (average speed, distance travelled, duration, number of responses, flick per response) of resulting tail-flick escape responses. The total number of induced escape responses and non-responses to provocation, and the proportional response rate for juveniles were analysed using a Kruskal- Wallis test. For elicited escape responses, principal component analysis (PCA) was conducted combining data duration, distance travelled, and the average swimming speed for each tail flick within each response. For both the primary and secondary components of the PCA, variation in scores between treatments was analysed using a Kruskal-Wallis test with *post-hoc* analysis via a Dunn's test.

3.3.8.5 Oxidative stress

Biomarkers for GPx, GSH and SOD were each analysed using a two-way ANOVA. CAT, TBARS and MT were each analysed using a Kruskal- Wallis test, with *post-hoc* analysis via a Dunn's test. Collective biomarker responses were assessed using PCA, with scores for both the primary and secondary components being analysed seperately using a two-way ANOVA with *post-hoc* analysis via Dunn's test.

3.4 Results

3.4.1 Sound exposure

Measured SPLs in all sound treatments were in line with nominal target exposures and broadly consistent between the two experiment set-ups (Table 3.1).

Table 3.1: Received sound levels in exposure vessels.*Sound pressure measurements were conducted across a 1-24,000 Hz frequency range. Particle motion measurements were conducted across a 50-3000 Hz frequency range using a third-order Butterworth bandpass filter.*

Mean (± SD) received sound						
Pressure						
Expt.	**Sound Playback Treatment**	**SPL (dB re 1 µPa)**		**SEL (dB re 1µPa^2 s)**		
		pk-pk	**RMS**	**SELss**	**SEL**	**SELcum**
Expt. 1	Ambient	-	118.0 ± 0.3	-	156.5 ± 0.3	195.8 ± 0.3
	Piling	169.2 ± 1.9	136.8 ± 1.7	185.8 ± 1.7	175.4 ± 1.7	217.0 ± 1.7
Expt. 2	Ambient	-	115.9 ± 0.2	-	154.5 ± 0.2	193.8 ± 0.2
	Piling	171.1 ± 1.3	141.2 ± 1.5	184.9 ± 3.1	175.0 ± 2.7	216.6 ± 2.7
Particle motion						
Expt.	**Sound Playback Treatment**	**PM (dB re 1 µm s^{-2})**		**SEL (dB re 1 (µm s^{-2})^2 s)**		
		pk	**RMS**	**SELss**	**SEL**	**SELcum**
Expt. 1	Ambient	-	56.6 ± 0.2		106.4 ± 0.2	139.8 ± 0.2
	Piling	82.7 ± 3.4	62.7 ± 2.2	118.8 ± 3.3	109.1 ± 2.2	154.1 ± 3.3
Expt. 2	Ambient	-	56.7 ± 0.2	-	106.6 ± 0.2	139.9 ± 0.2
	Piling	84.6 ± 2.6	64.0 ± .2.0	120.0 ± 2.7	110.4 ± 2.0	155.9 ± 2.7

Ambient playback SPLs were mostly consistent regardless of location within the experimental system, but a sound cone effect was present during the piling playback consistent with proximity to the speaker (Figure A4). There were also some discrepancies in the sound frequency distribution between the piling sound as recorded *in situ* and recreated via experiment playbacks (Figure A5). The marginally higher sound levels during piling playback phases of Experiment 2 compared to Experiment 1 were a consequence of exposure vessels being more confined within the sound cone. However, the 24-hour cumulative sound exposure levels (SEL_{cum}) in each sound treatment were highly comparable between experiments.

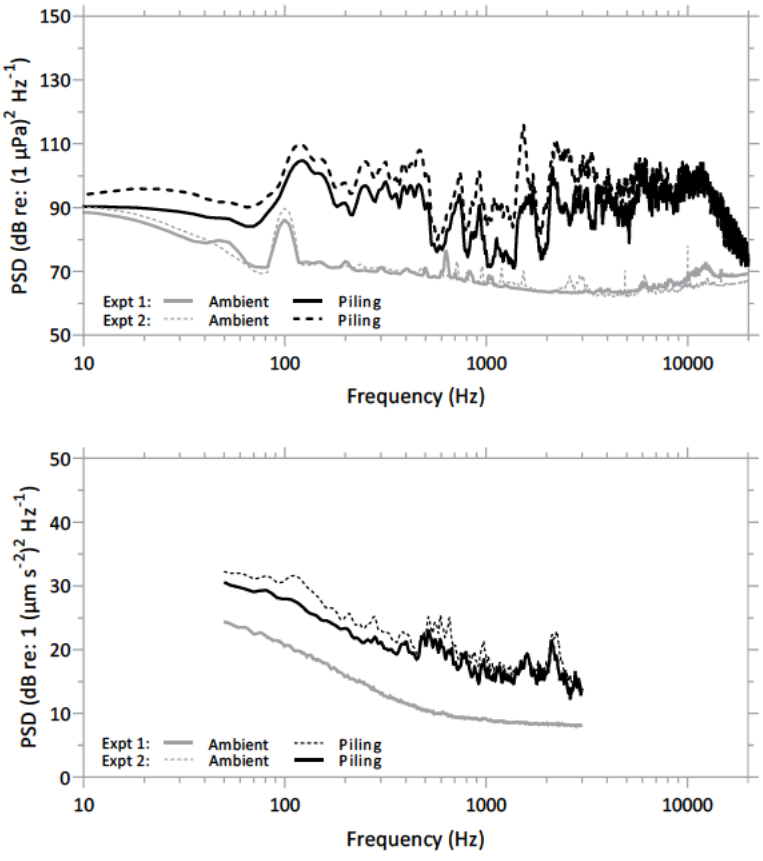

Figure 3.1: Power spectral density of sound playback exposures. *RMS power spectral density of received ambient- and piling playback in each experiment as recorded in exposure vessels. Top: sound pressure; 0.1 second Hann window and 50-percent overlap. Bottom: particle motion; 0.1 second Hamming window and 50-percent overlap.*

Differences in ambient playback sound levels were likely a consequence of variation in background laboratory noise between the times of measurement. Power spectral analysis confirmed that the received sound levels during piling playback phases were consistently greater than ambient playback treatments across all calculated frequencies for both pressure and particle motion (Figure 3.1).

3.4.2 Cadmium exposure

Time-averaged Cd^{2+} concentrations of experimental media were approximately 65% of nominal dosage across both experiments (Table 3.2). Paired water samples showed no consistent evidence of Cd^{2+} depletion in the media between dosing and subsequent water change.

Table 3.2: Mean ± SD time-averaged waterborne Cd^{2+} concentrations of experimental media. *n=6 samples (n=3 paired samples) per average.*

Cadmium treatment	Nominal Cd^{2+} concentration	Time-averaged Cd^{2+} concentration (µg L^{-1})	
		Expt 1	Expt 2
Control	0 µg L^{-1}	0.08 ± 0.02	0.07 ± 0.02
Low	1 µg L^{-1}	0.71 ± 0.11	0.71 ± 0.06
Medium	10 µg L^{-1}	6.48 ± 0.13	6.31 ± 0.14
High	100 µg L^{-1}	63.52 ± 2.55	62.47 ± 1.56

3.4.3 Phenomenological observations

3.4.3.1 Total larval mortality

Under ambient sound playbacks, larval mortality rates were 35%, 50%, 40% and 75% in the $Control_{[Cd]}$, $Low_{[Cd]}$, $Medium_{[Cd]}$ and $High_{[Cd]}$ treatments, respectively. This compared with mortality rates of 25%, 15%, 25% and 100% in the corresponding cadmium

treatments under piling playbacks. Both sound treatment and cadmium concentration significantly affected larval mortality rates with a significant interaction also occurring (logistic regression: $\chi^2_{=-}$ 31.748, df = 3, $p < 0.001$) (Figure 3.2and Table A6.2).

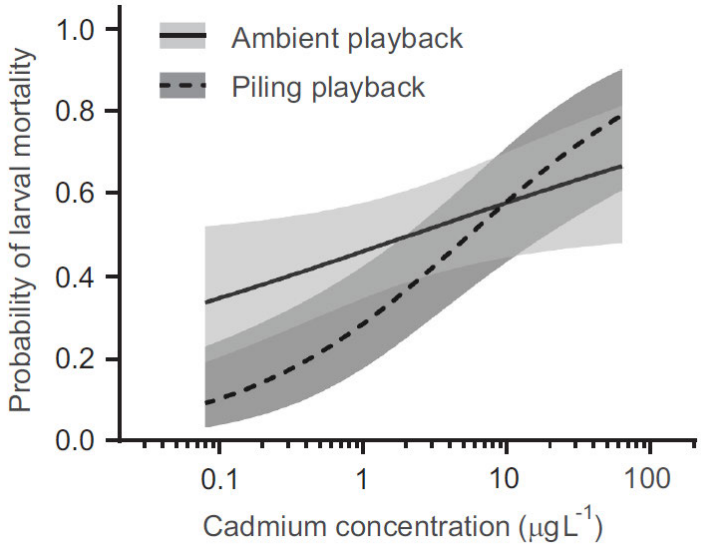

Figure 3.2: Modelled probability of N. norvegicus larval mortality. *Logistic regression model of predicted probability of larval mortality of N. norvegicus exposed to ambient and piling playbacks, and waterborne cadmium concentrations. Shaded areas represent 95% CI. Model fit using N = 20 observations for each sound treatment at each cadmium concentration of 0.08 µg L^{-1}, 0.71 µg L^{-1}, 6.48 µg L^{-1},63.52 µg L^{-1}.*

The modelled data demonstrate that survival was higher in the piling playback treatments at waterborne cadmium concentrations of <9.62 µg L^{-1} compared to equivalent concentrations in ambient playbacks but reduced at concentrations exceeding this. This corroborates comparisons of the raw count data (Figure A6), evidencing a mechanistically antagonistic interaction between piling playback in the $Low_{[Cd]}$ and $Medium_{[Cd]}$ treatments, and a synergistic interaction in the $High_{[Cd]}$ treatment with respects to mortality.

3.4.3.2 Temporal patterns of mortality

There were significant differences in mortality curves (Figure 3.3) between treatments (Kaplan-Meier: $\chi^2_{=}$ 49.2, df = 7, $p < 0.001$). *Post-hoc* log-rank Mantel-Cox analysis

determined lowest observed effect concentration (LOEC) of cadmium to be 63.52 μg L^{-1} in both ambient (Z = 2.017, df = 1, p = 0.043, p_{FDR} = 0.100) and piling (Z = 4.464, df = 1, p < 0.001, p_{FDR} < 0.001) playback treatments, and significant interactions with piling playbacks at cadmium concentrations of 0.71 μg L^{-1} (Z = − 2.310, df = 1, p = 0.002, p_{FDR} = 0.051), and 63.52 μg L^{-1} (Z = 2.632, df = 1, p = 0.005, p_{FDR} = 0.015). No other treatment groups differed significantly (Table A6.3).

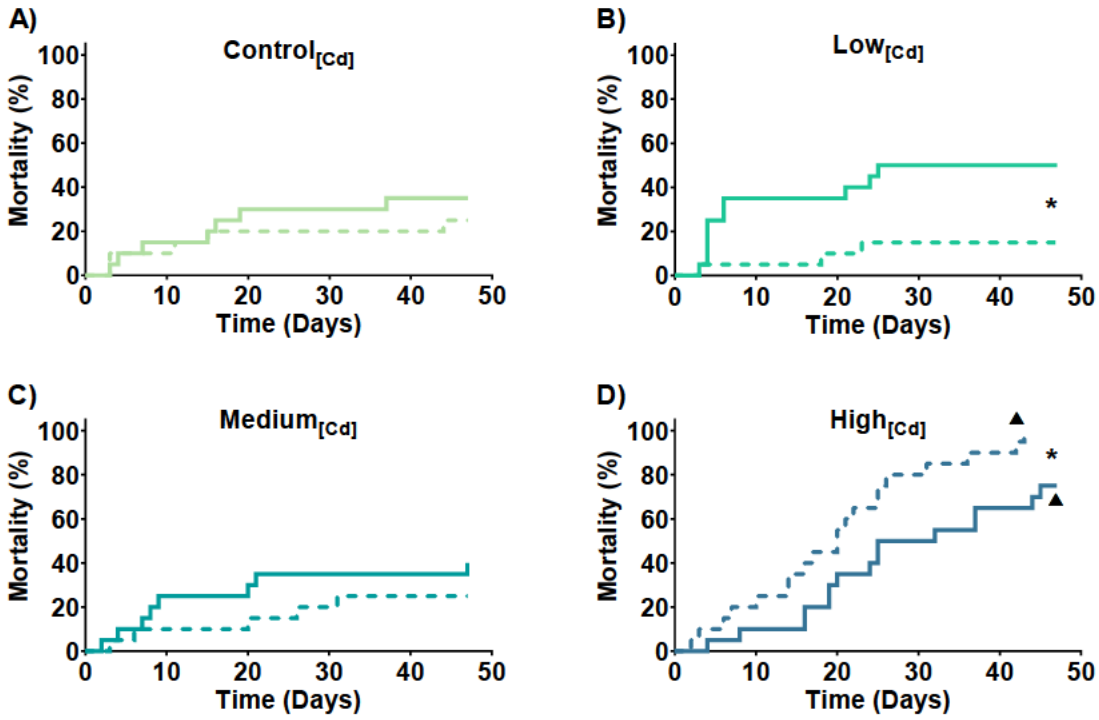

Figure 3.3: Mortality curves of N. norvegicus larvae. *Kaplan-Meier plots of cumulative mortality between sound treatments in each cadmium treatment: A) Control$_{[Cd]}$; B) Low$_{[Cd]}$; C) Medium$_{[Cd]}$; D) High$_{[Cd]}$. Control$_{[Cd]}$, Low$_{[Cd]}$, Medium$_{[Cd]}$, and High$_{[Cd]}$ represent Cd^{2+} ion concentrations of 0.08 μg L^{-1}, 0.71 μg L^{-1}, 6.48 μg L^{-1}, 63.52 μg L^{-1} respectively. Solid lines = ambient playback sound treatment, dashed lines = piling playback sound treatment. *significant difference (p <0.05) between sound treatments; ▲significant difference (p <0.05) between cadmium treatment and Control$_{[Cd]}$ in respective sound treatment.*

3.4.3.3 Developmental duration

The timing of transition from Zoea I to Zoea II was unaffected by exposure treatments, but significant differences in timing were observed in the transitions to Zoea III (χ^2 = 22.342, df = 7, p = 0.002) and juvenile (χ^2 = 15.129, df = 6, p = 0.019) (Figure 3.4).

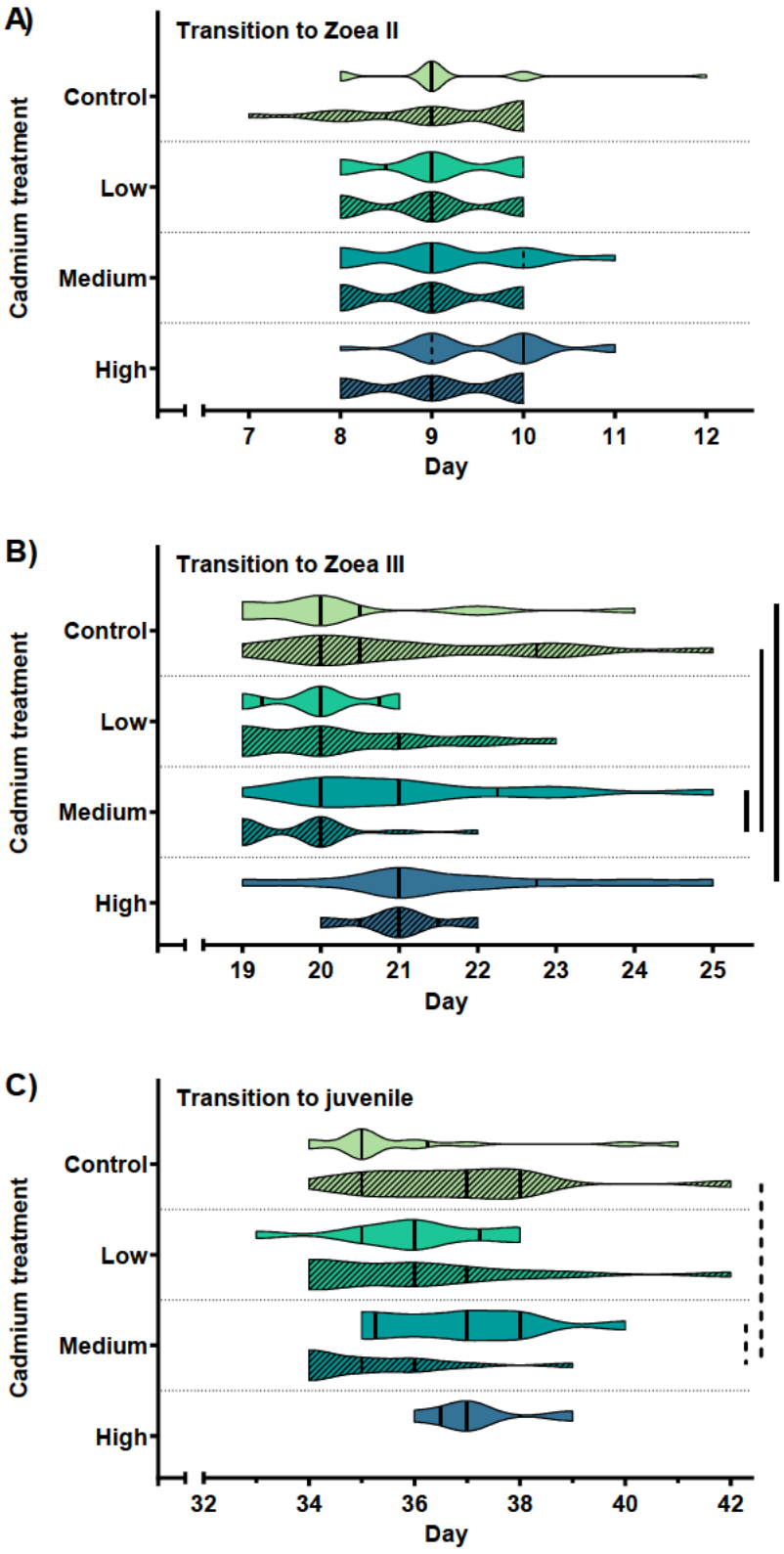

Figure 3.4: Timing of N. norvegicus moulting. *Violin plot showing timing of transition moults between zoeal stages of N. norvegicus larvae as measured from day of hatching. A) Zoea I to Zoea II; B) Zoea II to Zoea III; C) Zoea III to juvenile. Control$_{[Ca]}$, Low$_{[Ca]}$, Medium$_{[Ca]}$, and High$_{[Ca]}$ represent Cd^{2+} ion concentrations of 0.08 µg L^{-1}, 0.71 µg L^{-1}, 6.48 µg L^{-1}, 63.52 µg L^{-1} respectively. Absent violin in High$_{[Ca]}$ piling playback treatment is a consequence of no larvae surviving to metamorphosis. Solid and hatched plots represent ambient and piling playback sound treatments respectively. Solid and dashed vertical bars within plots represent median and quartile values respectively. Vertical markers beside violins denote significant differences between groups (Dunn's test, dashed lines uncorrected $p < 0.05$, solid lines corrected $p < 0.05$).*

Post-hoc analysis (Table A6.4) showed that under ambient sound playback conditions, $High_{[Cd]}$ exposures caused significant delays in transition to Zoea III (Dunn's test, Z = $2.616_{(14, 12)}$, $p = 0.009$, $p_{FDR} = 0.042$), however these did not persist with regards to transition to juvenile. Conversely, larvae exposed to piling playbacks transitioned to Zoea III significantly earlier in the $Low_{[Cd]}$ treatment relative to $Control_{[Cd]}$ (Z = $-2.630_{(16, 17)}$, $p = 0.009$, $p_{FDR} = 0.042$), with this trend persisting through to metamorphosis (Z = $-2.744_{(15, 15)}$, $p = 0.006$, $p_{FDR} = 0.050$). Larvae in the $Medium_{[Cd]}$ treatment also showed consistently earlier development when exposed to piling playbacks relative to ambient playbacks at transition to both Zoea III (Z = $-2.837_{(14, 17)}$, $p = 0.005$, $p_{FDR} = 0.042$) and juvenile (Z = $-2.823_{(12, 15)}$, $p = 0.005$, $p_{FDR} = 0.050$).

3.4.3.4 Behavioural fitness of the first stage juveniles

When considered independently, no statistical differences were observed in the total number of provoked escape responses, the number of non-responses to provocation, or the relative proportion of responses (Figure A7and Table A6.5). Analysis of tail-flick escape response dynamics by PCA (Figure 3.5) implied the presence of two key axes of response behaviour. The primary principal component (PC1), representing 48.3% of variance within the data (Table 3.3), broadly corresponds to an axis of physical swimming dynamics, contrasting swimming speed against swimming duration and total distance travelled. The secondary principal component encompasses a further 23.1% of data variance, seemingly forming an axis contrasting the total number of responses against their magnitude (number of tail-flicks). Comparison of PCA scores by Kruskal-Wallis evidenced significant differences in both PC1 ($\chi^2 = 349.91$, df = 6, $p < 0.001$) and PC2 scores ($\chi^2 = 114.08$, df = 6, $p < 0.001$) between experimental treatments.

Table 3.3: Eigenvalues and proportion of variance PCA of juvenile N. norvegicus tail-flick escape response

Principal component	Eigenvalue	Proportion of variance
PC1	2.415	48.31%
PC2	1.156	23.12%
PC3	0.820	16.40%
PC4	0.467	9.33%
PC5	0.142	2.84%

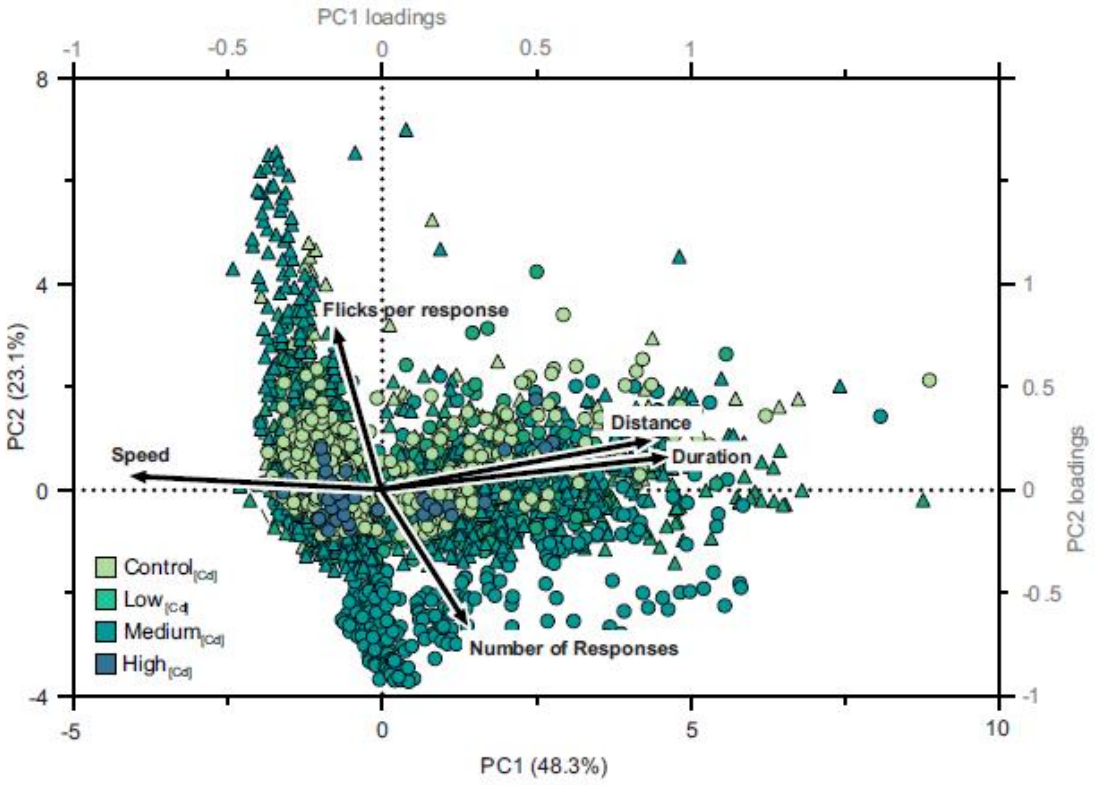

Figure 3.5: Juvenile N. norvegicus escape behaviour dynamics. *Biplot of loadings and scores generated by principal component analysis of escape behaviour dynamics (number of responses, flicks per response, duration of escape response, speed of escape response, distance travelled during escape response. $Control_{[Cd]}$, $Low_{[Cd]}$, $Medium_{[Cd]}$, and $High_{[Cd]}$ represent Cd^{2+} ion concentrations of 0.08 µg L^{-1}, 0.71 µg L^{-1}, 6.48 µg L^{-1}, 63.52 µg L^{-1} respectively. Circles = ambient playback sound treatment; triangles = piling playback sound treatment.*

Under ambient sound playback conditions, *post-hoc* analysis of PCA scores (Table A6.6) for behavioural response dynamics found the LOEC of cadmium to be 6.31 µg L^{-1}, which led to a noticeable and significant shift towards prioritising swimming distance and duration at the expense of speed, with individual responses comprising fewer tail-

flicks. Similarly, juveniles from the $Low_{[Cd]}$ ambient playback treatment also demonstrated fewer tail-flicks per individual response, but did not display any specific response in relation to speed or distance. Piling playback in the $Control_{[Cd]}$ treatment also led to a significant shift in juvenile behaviour towards more responses of fewer tail-flicks, maximising total distance and duration. Disparities in escape responses were also seen upon co-exposure to cadmium and piling playback. Whilst the general trend of elevated cadmium resulting in escape responses of lesser magnitude and greater frequency persisted, under ambient playback conditions this paired with juveniles covering an overall greater distance, whereas those exposed to piling playbacks had faster swimming speeds at the expense of distance and duration.

3.4.3.5 Biometrics

Neither carapace length (two-way ANOVA: sound: $F = 0.228_{(1, 80)}$, $p = 0.634$, cadmium: $F = 0.241_{(3, 80)}$, $p = 0.868$; sound × cadmium: $F = 0.699_{(2, 80)}$, $p = 0.500$) nor dry tissue weight (Kruskal Wallis test: $\chi^2 = 4.609$, df = 6, $p = 0.565$) of juvenile *Nephrops* differed significantly between treatment groups. *3.4. Experiment 2: biomarker assays*

3.4.4 Oxidative stress biomarkers

One replicate sample from the $Low_{[Cd]}$ ambient playback treatment exhibited spurious results across multiple biomarkers and was thus censored from all analysis as an outlier. Biomarker quantities were highly variable (Figure 3.6), with only MT varying significantly between treatment groups (Kruskal-Wallis test, $\chi^2 = 14.565$, df = 7, $p = 0.032$), with significantly lower quantities present in larvae exposed to $Medium_{[Cd]}$ piling playback treatments relative to both their respective $Control_{[Cd]}$ treatments (Dunn's test, $Z = -2.718_{(7, 7)}$, $p = 0.007$, $p_{FDR} = 0.092$) and larvae experiencing ambient

playback ($Z = -3.669_{(7, 7)}$, $p < 0.001$, $p_{FDR} = 0.007$). Full details of statistical outputs can be found in Table A6.7 and Table A6.8.

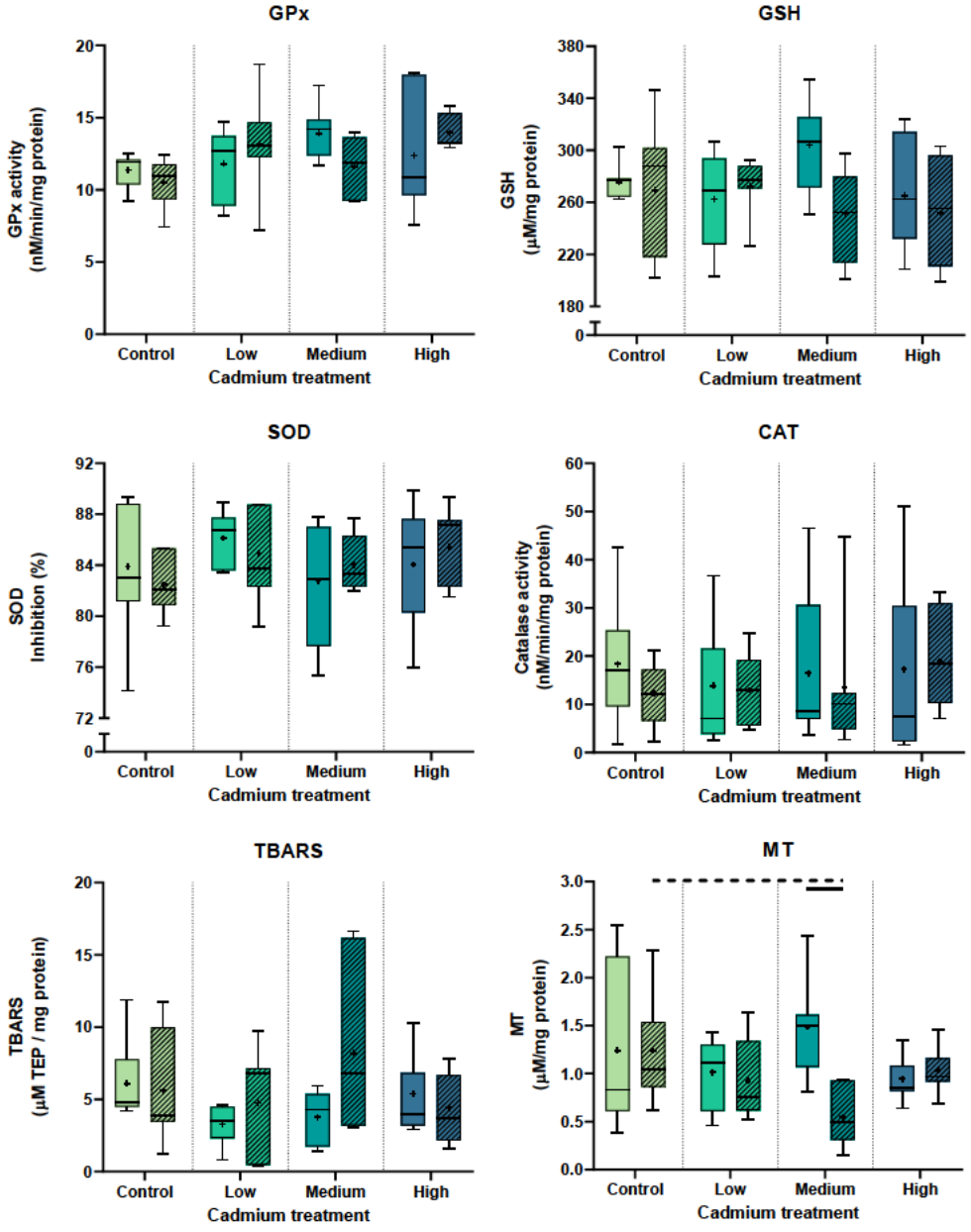

Figure 3.6: Larval N. norvegicus oxidative stress biomarkers. *Box-and-whisker plots of biomarker responses for A) GPx; B) GHS; C) SOD; D) CAT; E) TBARS; F) MT. Plots shows range, interquartile range, and median. Mean denoted by +. Solid and hatched bars represent ambient and piling playback sound treatments respectively. $Control_{[Ca]}$, $Low_{[Ca]}$, $Medium_{[Ca]}$, and $High_{[Ca]}$ represent Cd^{2+} ion concentrations of 0.07 µg L^{-1}, 0.71 µg L^{-1}, 6.31 µg L^{-1}, 62.47 µg L^{-1} respectively. Horizontal markers above plots denote significant differences between groups (Dunn's test, dashed line uncorrected $p < 0.05$, solid line corrected $p < 0.05$).*

Principal component scores were consistent with trends of the individual results (Figure 3.7). The primary principal component (PC1) accounted for 37.0% of variance within the data, primarily aligning with GPx and CAT activities, whilst the secondary principal component (PC2), representing a further 21.1% of data variance, seemingly aligned primarily to MT. When contrasted using ANOVA, PC1 scores did not differ significantly between treatment groups (Table A6.9), whereas PC2 scores were significantly driven by both cadmium ($F_{[1,46]} = 6.104$, $p = 0.017$) and an interaction between sound and cadmium ($F_{[3,46]} = 5.830$, $p = 0.002$).

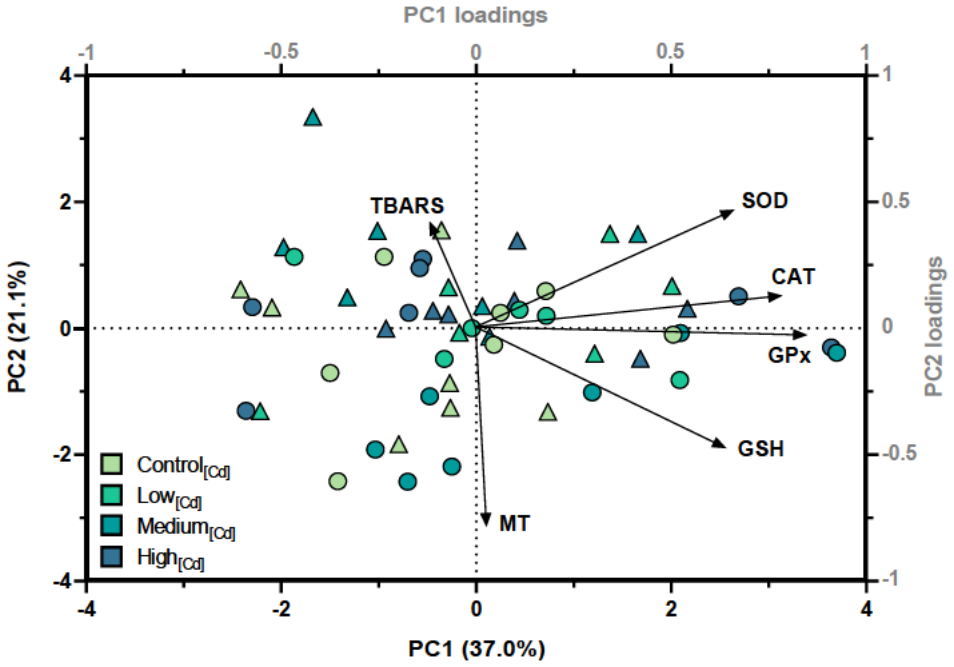

Figure 3.7: Oxidative stress Principal Component Analysis. *Biplot of loadings and scores generated by principal component analysis of oxidative stress biomarkers. $Control_{[Cd]}$, $Low_{[Cd]}$, $Medium_{[Cd]}$, and $High_{[Cd]}$ represent Cd^{2+} ion concentrations of 0.07 µg L^{-1}, 0.71 µg L^{-1}, 6.31 µg L^{-1}, 62.47 µg L^{-1} respectively. Circles = ambient playback sound treatment; triangles = piling playback sound treatment.*

Further *post-hoc* analysis (Table A6.10) attributed these differences to the $Medium_{[Cd]}$ treatments, with differences occurring in the $Medium_{[Cd]}$ piling playback treatment relative to $Control_{[Cd]}$ piling playback treatment ($Z_{[7, 7)} = 2.361$, $p = 0.018$, $p_{FDR} = 0.102$) and between the sound treatments ($Z_{[7, 7)} = 3.890$, $p < 0.001$, $p_{FDR} = 0.003$), mirroring

statistical significance attributed to the individual biomarkers and reinforcing assertions of PC2 being driven by MT.

3.5 Discussion

This study is the first to address the effects of cadmium and anthropogenic sound playback co-exposure in crustaceans, and likewise the first study of this pairing of drivers to focus on early life stage organisms. It is also currently the only study addressing this combination of these drivers using a full-factorial study design, and thus the only study enabling a true insight into how the drivers interact mechanistically.

The combination of pile-driving playbacks (170 dB_{pk-pk} re 1 µPa) and cadmium combined synergistically at concentrations > 9.62 $µg_{[Cd]}$ L^{-1}, resulting in increased larval mortality, with sound playbacks otherwise being antagonistic to cadmium toxicity. Significant delays in larval development were caused by exposure to 63.52 $µg_{[Cd]}$ L^{-1}, dropping to 6.48 $µg_{[Cd]}$ L^{-1} in the presence of piling playbacks. Pre-exposure to piling playbacks and 6.48 $µg_{[Cd]}$ L^{-1} led to significant differences in swimming behaviour of the first juvenile stage. Biomarker analysis suggested oxidative stress as the mechanism of deleterious effects, with cellular metallothionein (MT) being the predominant protective mechanism.

3.5.1 Sound exposures

Speaker playbacks and acoustic properties of tanks are unlikely to enable realistic recreation of sounds as experienced in the ocean. All presented sound metrics reflect values as recorded within the exposure vessels at each occupied location within the exposure system. As such, values are fully representative of the received sound levels

within the waterbody of the exposure vessels, accounting for inherent transmission losses and tank acoustic effects.

Currently, only Germany imposes legal limits on sound produced by pile driving, with SPL limits 190 dB_{pk-pk} re 1 μPa and 160 dB_{SELss} re 1 μPa^2 s as measured at 750 m from the piling source (Müller et al., 2019). Whilst SPL_{pk-pk} levels in this study were well below the 190 dB_{pk-pk} re 1 μPa limits, accompanying SELss levels far exceed the limits. A comparison of the relative power spectral density (PSD) of sound pressure between the piling as recorded *in situ* and as received as playback (Figure A5) clearly demonstrates that playbacks result in lesser proportion of sound at frequencies <2000 Hz and an increase in the proportion above this. The larger proportion of sound at frequencies exceeding 2000 Hz (Figure A5) in the current study have also inflated SELss and SEL values compared to those of natural environments. Sound levels in the sub-3000 Hz range in the current study are however comparable with similar studies (Nedelec et al., 2014; Nedelec, Mills, et al., 2017). Despite some inevitable discrepancies between *in situ* piling sound and experimental playbacks, some frequency dependent features were nonetheless maintained between the *in situ* and playback signals in the lower frequency domain, and discrepancies in the upper frequency domain may be irrelevant in the context of *Nephrops* sensory capability.

There is clear evidence that some decapod larvae can utilise sound as an orientation and settlement cue, and hence are auditorily capable (C. A. Radford et al., 2008; Stanley et al., 2010). To date, it is unknown whether the sensory capabilities of *Nephrops* larvae are comparable with those of mature specimens. Assessments of behavioural responses of mature specimens suggest that *Nephrops* is only responsive to the particle motion aspect of sound, with a displacement response threshold of 0.888 μm independent of the assessed frequency range of 20–200 Hz (Goodall et al.,

1990), but an audiogram has not yet been established for the species. Mature *Nephrops* nonetheless have an array of mechanoreceptive structures associated with both physical orientation and sound reception, including cuticular setae widely distributed across their exoskeleton and statocysts located in the basal portion of the sensory antennules (Katoh et al., 2013).

Given the uncertainties in the sound perception capabilities of *Nephrops* larvae, and the discrepancies in tank-based sound studies, it is impossible to adequately assess the realism of *in situ* vs playback sounds as experienced by the organisms, nor was this the principal aim of the study. Nonetheless, both sound pressure and particle motion in the present study were consistently higher during piling playback than in the ambient playback sound treatment regardless of sound frequency. At a minimum, the study should therefore be considered in the context of exposure to additional sound sharing characteristics to that resultant of pile driving.

3.5.2 Cadmium exposures

Quantified cadmium concentrations were approximately 65% of nominal concentrations. Such discrepancies are expected given losses associated with adsorption and cross-reactivity with other chemical species. These losses do not impact the study as quantified values were used for all modelling and metric purposes. Waterborne cadmium concentrations in the $Control_{[Cd]}$ treatment were consistent with background levels of British coastal waters (Neff, 2002). $Low_{[Cd]}$ and $Medium_{[Cd]}$ treatment concentrations were consistent with those reported of coastal regions featuring discrete and or point-sources of cadmium (Abe, 2007; Delly et al., 2021). $High_{[Cd]}$ treatment concentrations are in the range of those observed at heavily contaminated sites (Delly et al., 2021).These concentrations are considered to be

unrealistic of most environments, but provide useful context for modelling and risk assessment purposes.

Uptake of cadmium by the *Nephrops* larvae is thought to have predominantly occurred from the waterborne fraction via the cuticle, which is poorly calcified and permeable in larval-stage *Nephrops* (Eriksson & Baden, 1997). Absorption via the gills, a known uptake site of toxic metals in aquatic crustaceans (Henry et al., 2012), is unlikely in this instance given that gills are absent or rudimentary in larval-stage *Nephrops* (Spicer & Eriksson, 2003). Direct ingestion of cadmium bioaccumulated within their *Artemia* prey is also considered to have been minor given the frequency of water changes and *Artemia* replacement. Bioaccumulation of cadmium within the *Nephrops* larvae was not assessed due to tissue availability being considered insufficient to meet limits of detection of available analytical methods.

3.5.3 Mortality

Modelled data show dose-dependent increases in mortality rate with regards to cadmium in both sound treatments. Mortality in the $Control_{[Cd]}$ treatment was lower in larvae exposed to piling playbacks, but increased at a greater rate with increasing cadmium concentration compared to treatments experiencing ambient playbacks, leading to a switch in predominating mortality. Notably, whilst overall mortality below the 9.62 $\mu g_{[Cd]}\, L^{-1}$ equivalence concentration was lower for larvae exposed to piling playback, they showed greater sensitivity to elevating cadmium relative to larvae in the ambient playback sound treatment. This context is important, as it suggests survival of larvae in cadmium-contaminated water may be enhanced by anthropogenic sound exposure, whilst larvae commonly exposed ro anthropogenic sound in otherwise more pristine environments may be more susceptible to chemical pollution events.

Observed mortality rates across experimental treatments are hypothesised to reflect differences in oxidative stress responses between treatments (discussed in detail below). Results suggest that the $Low_{[Cd]}$ concentration was sufficiently high to cause toxicity, but not to invoke a suitably strong antioxidant response. Piling playbacks meanwhile triggered a larger oxidative stress response, which consequently offered additional protection from the cadmium. Correspondingly, the switch in the driver interaction from initially antagonistic to synergistic, modelled to occur above 9.62 $\mu g_{[Cd]}$ $L_{,}^{-1}$ equates to the concentration above which the cadmium toxicity protection afforded by the oxidative responses to piling playback is seemingly overwhelmed, with piling-induced oxidative stress not only ceasing to be beneficial but actually increasing the burden.

Scrutiny of the raw mortality data (Figure A6) shows that Zoea I larvae reared in ambient playback sound conditions and exposed to $Low_{[Cd]}$ were significantly more susceptible to cadmium toxicity than those exposed to piling playbacks. This pattern was also mirrored, albeit to a lesser extent, in the $Medium_{[Cd]}$ treatment. Furthermore, larval mortalities during the study coincided with moulting, suggesting that the process of moulting increases susceptibility to acute metal toxicity; possibly reflecting the stressful and energetically intensive undertaking of moulting itself (Bacqué-Cazenave et al., 2019; E. S. Chang & Mykles, 2011). Whilst tissue limitations of the *Nephrops* larvae and exuviae precluded a similar analysis of metal distribution in the present study, the commonality of metal distributions between the investigations of Bergey and Weis (2007) and Perugini et al. (2014) suggests that rather than being selectively sequestered into the exuviae prior to moulting, cadmium may actually have been reabsorbed into the soft tissues along with calcium, as also seen in the grass shrimp *Palaemonetes pugio* (Keteles & Fleeger, 2001). If this was the case, it would suggest

that chronic exposure led to continued accumulation of cadmium in *Nephrops* with limited ability to depurate. Permeability to, and absorption of cadmium is also likely greater in recently moulted individuals as seen in the shore crab *Carcinus maenas* (Bondgaard & Bjerregaard, 2005). Correspondingly, it may be that the process of moulting effectively results in short-term concentration of cadmium in soft tissues, which are known to accumulate metals in *Nephrops*. Perugini et al. (2014) found that approximately 85% of the total cadmium loading within *Nephrops* was distributed in the 'brown meat' (including the gills and hepatopancreas), with the remainder distributed between the exoskeleton, legs, and claws. These observations are consistent with the role of the hepatopancreas in the detoxification of metals in decapod crustaceans (Ahearn et al., 2004), and evidence of the hepatopancreas being the primary location of cadmium accumulation even in the case of waterborne exposures (Canli et al., 1997).

3.5.4 Developmental and biometrics

Following metamorphosis, carapace length and wet-weight of juveniles were consistent across treatment groups, demonstrating little variation.

Piling playbacks experienced by *Nephrops* larvae in the absence of cadmium caused developmental delays of two days compared to the ambient playback treatment. This delay was not statistically significant, however this is believed to be a Type II error resulting from two notable outliers in the ambient playback sound $Control_{[Cd]}$ group. This assertion is supported by results demonstrating significant delays in timings of *Nephrops* metamorphosis in response to ship sound playbacks (Bolger, 2022) and developmental delays observed in scallops (*Pecten novaezelandiae*) exposed to seismic pulse playbacks (de Soto et al., 2013).

Delays in development may result, at least in part, from differences in metabolic rates and/or energy budgeting. Metal exposures can induce dose-dependent increases in metabolic rate and developmental duration in several taxa including crustaceans (Lyla & Khan, 2010; Vernberg et al., 1974). Such variations in developmental rate in response to environmental conditions and stressors are not uncommon. Metabolic consequences have been evidenced in response to osmotic stress (Curtis & McGaw, 2010), and temperature is well known to impact metabolism and energy assimilation of invertebrates, influencing their growth and development (Chiasson et al., 2015; Dickey-Collas et al., 2000; Z. Han & Li, 2018; Xiao et al., 2014).

Metals such as cadmium can also have teratogenic and endocrine- disrupting effects (Takiguchi & Yoshihara, 2006), adding another facet to potential impacts where complex, hormonally driven processes such as crustacean moulting are concerned. Moulting in crustaceans is predominantly mediated by the negative regulation of moult-inhibiting hormone (MIH) produced by the X-organ, which inhibits the release of ecdysteroid hormones produced by the Y-organ (Devaraj & Natarajan, 2006). Cadmium at concentrations of 3–900 $\mu g\ L^{-1}$ elevated the levels of ecdysteroid in embryos of the amphipod *Gammarus fossarum* (Abidi et al., 2016), suggesting low concentrations of cadmium could alter the balance of these hormones mediating growth and development. Disruption of ecdysteroid receptors in the Y-organ can also disrupt moulting (Zou, 2005), which may explain observations of moult inhibition in the crab *Chasmagnathus granulata* induced by cadmium despite consistent ecdysteroid levels (Rodríguez Moreno et al., 2003). Nevertheless, in the current study, endocrine-related effects of cadmium on growth and development are not thought to be the primary mechanism driving the observed differences in developmental rate. Firstly, post- moult feedback between MIH and crustacean hyperglycemic hormone levels is proposed to

effectively reset moult cycles after each moult-phase (Techa & Chung, 2015), which would explain inconsistencies in intermoult periods at different developmental stages. This also suggests acute exposure to endocrine disruptors may have limited impacts on continued development of larvae as MIH is rapidly cleared from haemolymph, and therefore episodic releases of the hormone are unlikely to contribute to a critical threshold (Chung & Webster, 2005). Secondly, only modest deviations in moult timing were observed in the current study (around 5% compared to controls). In comparison, targeted inhibition of MIH in the prawn *Fenneropenaeus indicus* reduced intermoult period to around 50% of that of controls (Devaraj & Natarajan, 2006).

Although the mechanisms controlling the moult process itself are relatively well studied (E. S. Chang & Mykles, 2011), a consensus on triggers and timing of ecdysis events is apparently lacking, adding to the difficulties in identifying the mechanism driving the observations of this study. It is accepted that multiple factors relating to bioenergetics such as food availability, temperature, behaviour, as well as endogenous aspects such as 'biological clocks' impact reproduction, growth, and development (Sardà, 1991). Despite this, under consistent and favourable conditions, other crustacean larvae have been observed to have similarly consistent and predictable biometrics following ecdysis, albeit at variable ages (Anger, 1998). This, and the timing of ecdysis in *Nephrops* larvae in the present study, suggest a certain plasticity that would be beneficial — enabling energetic prioritisation to body condition preceding the energetically intense moult process under sub- optimal conditions, but also expediting development in more favourable conditions. It is important to state that even the relatively minor developmental delays observed represent a protracted period in the planktonic phase of *Nephrops* life-cycle, and could therefore have consequences on larval dispersion and susceptibility to predation pressures.

Resultantly, developmental delays could prove ecologically significant despite lacking statistical significance.

3.5.5 Behavioural fitness

Differences in behavioural responses to simulated threats were observed in first stage juvenile *Nephrops* following experimental exposures. Principal component analysis evidenced that the provoked tail- flick responses can be considered in terms of two axes of variation: an axis that is considered to represent the 'sprint-marathon' continuum, and a second interpreted as reflecting whether individual reactions comprise a greater number of responses of lesser magnitude, or fewer responses of greater magnitude.

Maximal swimming speed of the juveniles appeared to be highly constrained and showed little variation, undoubtedly reflecting the consistency in the size of the juveniles assessed, given swimming speed is constrained by maximal displacement of water and therefore correlates with carapace size (Newland et al., 1988). Total distance travelled was highly correlated with the duration of the tail-flick response, both of which were negatively correlated to swimming speed. Therefore, a reduction in swimming speed must result from a reduction in muscular contraction power. It is however uncertain whether these differences between 'sprinters' and 'marathoners' are mediated by physiological limitations in musculature condition, energy partitioning, behaviour choices, or a combination of these factors. Irrespective of the causal drivers, significant differences in the composition of tail-flick responses were evident between treatments. Both response axes demonstrated a LOEC of 6.48 $\mu g_{[Cd]}\, L^{-1}$ in both ambient- and piling playback treatments. Likewise, significant interactions between piling playback and cadmium were seen at all concentrations, though there was little consistency in terms of magnitudes and/or directionality of effects. In the

context of swimming dynamics, it is difficult to assess the ecological implications of how such differences are likely to impact the long-term survival prospects of individual *Nephrops* given this would be context- and threat-specific. For example, those prioritising speed may have an advantage against ambush predators, but at the detriment of resilience against more persistent foraging predators, and vice versa.

The ability to perceive and respond to potential threats, primarily predators, is of undoubted importance to long-term survival, as even slight changes in response rate to perceived threats could have serious consequences. Observations on response rates were consistent with the experimental exposures potentially having had energetic consequences during development. However, where responses were provoked, PCA analysis implied no correlation between swimming dynamics (speed and distance), and response dynamics (number of responses and tail-flicks per response), implying responses may better reflect a continuum of either behavioural boldness or corresponding reaction to threat, rather than being indicative of energetic budgeting.

Variation in response dynamics could also theoretically reflect differences between non/reflex-reactions, however this seems less likely given different nervous pathways are responsible for initiation and continuation of tail-flick responses (Faulkes, 2009). Tail-flick responses are a low-latency reflex reaction mediated by medial giant (MG) and lateral giant (LG) nerves in response to mechanosensory stimulation of the anterior cephalothorax and abdomen respectively (D. J. Jackson & MacMillan, 2000). Whilst a similar non-reflex swimming response with higher latency mediated by non-giant nervous pathways has previously been characterised in *Nephrops* (Newland et al., 1988), only the MG pathway is considered of relevance to the assessment methods used here. Crayfish (*Procambarus clarkia*) exposed to predators showed survival rates of 50% when MG mediated responses were triggered, compared to just 20% for non-

reflex responses due to the difference in response latency (Herberholz et al., 2004). As such, if piling playback has impacted on the ability of the MG pathway to function in the current study, this would also likely have a considerably detrimental impact on long-term survival potential.

Alternatively, observations may reflect a reduction in sensitivity to vibratory stimulus given high-intensity impulsive sound has been observed to cause mechanical damage to statocysts in both lobsters and scallops (Day et al., 2016, 2017, 2019, 2020). Reduction in synaptic transmission between sensory and motor neurons resultant of habituation to stimuli could also be a factor (Zucker, 1972), as could cross- modal sensory reduction in response to sound and vibration, postulated by Roberts and Laidre (2019).

3.5.6 Oxidative stress biomarkers

TBARS is a group of biomarkers indicative of lipid peroxidation (LPO), being the final product of other similar LPO biomarkers including malondialdehyde (MDA) (Camejo et al., 1998; Tsikas, 2017), and the only biomarker assessed that is directly indicative of pathology. The PCA analysis indicated that TBARS were predominantly mediated against by MT and GSH, whilst, SOD, GPx, and CAT were not strongly correlated with reducing pathology of cadmium and sound exposure. Glutathione (GSH) directly scavenges free radicals by acting as a hydrogen ion donator (Fanucchi, 2014), suggesting not only were free radicals the predominant cause of LPO, but that direct scavenging of these free radicals was the primary method for regulating against such ROS. This is further supported by the consistently high degree of SOD inhibition rates observed between treatments given that SOD catalyses the conversion of superoxide ions to hydrogen peroxide, which is then further catalysed into water and oxygen by both GPx and CAT (Fanucchi, 2014). The weak correlation shared between TBARS and

both GPx and CAT further suggests that the presence of hydrogen peroxide influences LPO, albeit to a much lesser extent. This is consistent with the role of hydrogen peroxide as a non-radical oxidising agent (Phaniendra et al., 2015), and with observations by Badisa et al. (2007) who demonstrated that presence of cadmium can further potentiate the production of radicals from hydrogen peroxide. It is also possible that the weak correlation of TBARS to other biomarkers is indicative of a mechanism of cadmium toxicity unrelated to LPO.

Mitochondrial respiration inherently generates ROS as a by-product of ATP synthesis (Andreyev et al., 2015). Consequently, several highly conserved cellular antioxidant defences have evolved for mediating against oxidative stress, with commonly identified mechanisms in crustacea including the antioxidant GSH, and the enzymes GPx, CAT, and SOD (Fanjul-Moles & Gonsebatt, 2011). Where substantial and/or prolonged exposure to oxidative stress results in antioxidant capacity being exceeded, oxidative damage will occur. Cadmium exposure results in the production of free radical ROS — directly driving oxidative stress (Singh et al., 2017). Such exposures in crustacea can result in multifaceted effects including causing gill damage, altering metabolic activity, inducing differences in gene expression, and cause cellular apoptosis and necrosis (Torreblanca et al., 1989; J. Wang et al., 2013).

To date, the study of Charifi et al. (2018) is the only one having assessed oxidative stress in relation to co-exposure to anthropogenic noise and cadmium stress in a marine invertebrate, the Pacific oyster (*Magallana gigas*). Waterborne concentrations of approximately 14 $\mu g_{[Cd]}\, L^{-1}$ led to a significant positive correlation in expression of genes associated with SOD and GPx, whilst genes for CAT and MT were also positively correlated, though not significantly. However, when *M. gigas* was also exposed to ship noise of 150 dB_{RMS} re 1 μPa, no significant correlations in gene expression were

identifiable. Blue mussels (*Mytilus edulis*) exposed to ship noise peaking at 150–155 dB re 1 $\mu Pa^2\,Hz^{-1}$ exhibited significantly elevated TBARS in comparison to the control group, yet SOD, GSH and GPx were unaffected (Wale et al., 2019). The present study did not observe similar increases in quantity of TBARS in response to sound, potentially due to the significantly lower RMS sound levels, but could equally reflect differences in species/taxa specific responses. Juvenile *Nephrops* that had been exposed to peak ship noise of 122 dB re 1 $\mu Pa^2\,Hz^{-1}$ throughout their larval development, showed no significant difference in SOD, GSH, GPx, CAT or TBARS (Bolger, 2022). This may evidence sound-specific differences in biological response, i.e. between 'continuous' ship noise versus 'impulsive' pile driving noise, but more likely reflects differences between life-stages. Even temporally close life stages can exhibit considerably different physiological responses, with disentanglement of differences resultant of natural development being fraught with difficulty (Rato et al., 2017; Styf et al., 2013). Differences in life-stage responses also likely contribute to the lack of a consistent trend between the oxidative stress data and mortality data in the present study. This will be further compounded by survivorship bias inherent of the oxidative stress protocols which precluded the assessment of organisms which had died during exposures. Regardless, oxidative stress being a driver of larval mortality supports many of the observed trends in the data of the present study. The mortality data show that piling playback had an antagonistic effect to total larval mortality in the presence of cadmium at concentrations ≤6.48 $\mu g\,L^{-1}$. Given that the current data suggest that piling playback alone results in limited stress, this antagonistic effect is consistent with assertions that exposure to low-level stress promotes an antioxidant response resulting in enhanced defence capacity (Niki et al., 2005). Similar observations in *Nephrops* embryos have been noted in response to ocean acidification, where

oxidative stress was significantly higher in control groups compared to acidified treatments (Styf et al., 2013).

Biomarker assay results not only support the assertions advanced above in relation to life-stage specific physiological responses, but also suggest that oxidative defences in response to sound exposure either develop earlier than those of metals such as cadmium, or more likely, that responses to the piling playbacks during this developmental stage were more acute than those to cadmium. As concentrations of ROS and other oxidants were not quantified in either the water or tissues samples from the present study, there remains an assumption that biomarker responses correlate directly with oxidative loading. There is also mechanistic uncertainty in whether the presumed increased oxidative loading originates in the water, tissues, or both – though the origins ultimately do not alter the observed outcomes.

3.6 Conclusion

Exposure to piling playbacks and cadmium caused a wide range of physiological effects on larval *Nephrops*, with the drivers each having individual effects, but also demonstrating various interactions when co-occurring. The multifaceted nature of these effects makes direct assessment of risk and harm of these drivers on the species difficult to judge. In some scenarios, exposure to piling playbacks could be considered beneficial, promoting larval survival and growth rates in cadmium- contaminated waters, however the opposite is also true for more pristine environments. Extrapolation between laboratory-based findings and real-world environmental impacts should be approached with caution, especially given the known discrepancies in the characteristics of *in situ* sounds vs experimental playbacks. Nonetheless,

evidence of synergism leading towards net-negative impacts on larval survival at environmentally plausible cadmium concentrations of 9.62 $\mu g\ L^{-1}$ highlights that consideration should be given to how the combination of metal pollutants and sound exposure modifies the risks posed as compared to each driver occurring in isolation. Future studies directly addressing current uncertainties regarding exposure dynamics, including if and how sound perception directly affects heavy metal kinetics in decapod crustaceans, would contribute greatly to the understanding of this driver combination.

Ultimately, the results of this study simultaneously support both the adage "*what doesn't kill you makes you stronger*", and that of "*the straw that broke the camel's back*" — highlighting the need for more integrative and case-specific consideration of anthropogenic impacts in ecological contexts.

Chapter 4

Effects of Ferry Noise on the Embryonic Development of the Pond Snail, *Lymnaea stagnalis*, at Different Waterborne Calcium Concentrations

4.1 Abstract

All organisms are shaped by their environments, with nutrient availability being among the most fundamental of drivers of health and fitness of a population. Despite the known importance of macronutrient availability on these aspects, it is currently unknown how macronutrient provisions influence susceptibility to anthropogenic sound. To address this knowledge gap, a pair of complementary experiments were undertaken, respectively assessing the fecundity and developmental success of the pond snail, *Lymnaea stagnalis*. In both experiments, snails were concurrently exposed to either low (20 mg L^{-1}) or high (100 mg L^{-1}) levels of waterborne calcium and exposed to an environmentally realistic temporal regime of ferry sound. Calcium was a significant driver of fecundity, with high calcium availability resulting in fewer eggs compared to the low calcium treatment. Anthropogenic sound playbacks had no effect on fecundity. There was an interaction between ferry playbacks and high calcium concentrations leading to a significant reduction in growth of adult snails during the fecundity study, suggesting that fecundity was being maintained through energetic plasticity at the expense of growth. A significant interaction between anthropogenic sound and calcium concentrations was also seen with regards to embryonic development of snails, with ferry playbacks promoting failed hatching of snails in high calcium treatments in comparison to low calcium.

4.2 Introduction

Physiological systems are extremely complex, being the culmination of countless biochemical processes. To facilitate these processes, all organisms require access to nutrients – chemical compounds providing the necessary constituents to maintain biological function. Nutrients fall into two broad categories – micronutrients and macronutrients. Micronutrients, as the name suggests, are chemicals required in small quantities or for very specific processes. Examples of micronutrients include metals such as copper and cadmium, which while essential in small quantities, are also well-established toxicants when available in excess (Barpete et al., 2021). In comparison, macronutrients are those chemicals utilised en masse in a wide variety of functions, such as metabolic function and/or as a key component in structural tissues (Nassar, 2018), and as such limitation in their availability is known to have widespread biological consequences (McCue, 2012).

Despite the acknowledged need to better understanding the impacts of anthropogenic sound on invertebrates (Morley et al., 2013), very little is understood – especially with regards to freshwater invertebrates, with only two such studies identified. Villalobos-Jiménez et al. (2017) identified that exposure to playbacks of canal boat playbacks depressed feeding in larvae of the damsel fly, *Ischnura elegans*., whilst Yağcılar and Yardımcı (2021) observed reduced fecundity and infertility in the water fleas (*Daphnia magna*) exposed to 432 Hz and 440 Hz sound frequencies. Correspondingly, it was decided to focus on sublethal reproductive endpoints in a freshwater model species – the great pond snail *Lymnaea stagnalis* – to address the existing knowledge gaps. Calcium was selected as the macronutrient driver given the calciphilic nature of *L. stagnalis* (Boycott, 1934) and the fact that calcium availability often can be a biologically limiting factor in freshwater environments (Weyhenmeyer et al., 2019).

L. stagnalis is a freshwater pulmonate gastropod common to north-western Europe and North America (Kuroda and Abe, 2020) which preferentially inhabits freshwater waterways with low flow (Kuroda and Abe, 2020). Despite demonstrating tolerance to a range of environments through a wide degree of behavioural and physiological plasticity (Côte et al., 2015; Hermann & Bulloch, 1998; Salo et al., 2017). The species is used as a model organism across many biological disciplines given its geographical abundance, ease of culture under laboratory conditions, and its specific biology making it well-suited to a diversity of scientific research (Fodor et al., 2020; Kuroda and Abe, 2020). Among the most well-studied aspects of *L. stagnalis* is its reproductive biology, with both its fecundity and embryonic development having widely been used to assess effects of pollutants and/or environmental factors (Gomot, 1998; Janse et al., 1996, 1989; Piggott and Dussart, 1995; ter Maat et al., 1989). Consequently, *L. stagnalis* is one of only two OECD guideline species for assessing chemical reproductive toxicity in molluscs (OECD, 2016). Several methods have also been proposed for embryo assays using this species (Gomot, 1998; Mazur et al., 2016; Munley et al., 2013), each assessing hatching success in response to different waterborne chemicals, and taking advantage of the species high fecundity to provide high replication and statistical power. Moreover, embryo assays often provide better sensitivity to environmental factors than equivalent studies using adult organisms (Braunbeck et al., 2014; Pineda et al., 2012), further improving limits of detection and resolution (Lange et al., 1995; K. S. Wang et al., 2011). This ability to conduct both fecundity and embryo assays with *L. stagnalis* allows an expanded understanding of how environmental conditions may impact upon the species.

Calcium is fundamental in cellular biology, contributing to a variety of biochemical functions, including respiration and ATP production (Fink et al., 2017), inter- and intra-

cellular signalling (Kudla et al., 2018), and the establishment and discharge of cellular electro-potential required for nervous transmission (He et al., 2018) to name but a few. Calcium signalling has established roles in modulating reproductive biology of aquatic invertebrates, regulating the onset of germinal vesicle breakdown (GVBD) in bivalves (Whitaker, 2006), and with similar large releases of intracellular calcium observed during fertilisation of the sea urchin, *Lytechinus pictus* (Steinhardt et al., 1977). In *L. stagnalis*, both egg production and laying behaviours are controlled by the release of various neuropeptides, however the secretion of these peptides by neuroendocrine caudodorsal cells is initiated and mediated via calcium signalling (Kits et al., 1997; Roubos, 1988), and thus might be particularly susceptible to calcium availability. Calcium is also a key component in many structural minerals – including shell-forming calcareous molluscs (Bevelander, 1952; Clark et al., 2020; Weiss et al., 2002) – with its importance to the growth and development of *L. stagnalis* embryos having been established (Ebanks et al., 2010a, 2010b).

Of the existing multiple driver studies addressing aquatic nutrient availability, most relate to nutrient loading of waterbodies resulting from agricultural run-off and other anthropogenic sources (Ban et al., 2014; Ellis et al., 2017; Piggott et al., 2015; Townsend et al., 2008; van de Waal & Litchman, 2020). Resultantly, the focus of these studies tends to either focuses directly on plants and algae or the wider concerns of eutrophication, which is in itself multifaceted (Dorgham, 2014; Hutchins & Hitt, 2019). Beyond these more established scopes, comparatively little is known about the wider multiple driver interactions between other key environmental nutrients, and specific to this thesis, no known studies have yet assessed how nutrient availability and anthropogenic sound combine in a multiple driver context. To address this, two experimental assays were conducted, each addressing a different aspect of its

reproduction – namely adult fecundity, and embryonic development – with snails exposed to playbacks of the Bowness passenger ferry (detailed in Section 4.3.3) and one of two calcium concentrations (detailed in Section 4.3.4). Sound levels and calcium concentrations both mimic environmentally relevant exposure levels. Sound exposures are used to broadly represent sounds emitted by smaller motorised watercraft operating on similar waterways such as rivers and canals, whilst selected calcium concentrations (20 mg L^{-1} and 100 mg L^{-1}) represent the lower and ideal tolerance levels of *L. stagnalis,* respectively. It was predicted that greater calcium availability would promote *L. stagnalis* fecundity and embryonic development, that both *L. stagnalis* fecundity and development would be detrimented by ferry playbacks, and that the combination of low calcium availability and ferry playbacks would prove additionally detrimental to both fecundity and development.

4.3 Methods

4.3.1 Animal Husbandry

Experimental exposures and animal culture were both conducted in a temperature-controlled laboratory maintaining 20 ± 1 °C. The laboratory was lit using natural daylight 6500K lamps controlled by using IKS AquaStar Simmod 0-10 volt controllers, and operated a 16:8 hours day:night cycle, with light intensity at the surface of all occupied aquaria/test vessels within the range of 250-500 Lux, in accordance with culture conditions stipulated for *L. stagnalis* reproductive assays (OECD, 2016). Snails were cultured in constantly aerated husbandry media based upon American Society for Testing and Materials (ASTM) reconstituted hard water media (ASTM, 1975) with nominal calcium ion (Ca^{2+}) concentrations modulated to 60 mg L^{-1} using calcium sulphate dihydrate (Table 4.1).

An F_0 culture of *L. stagnalis* comprising 12 sexually mature organisms was established at Edinburgh Napier University in January 2021 from an isogenic, lab-cultured population donated by Angus Davison of the University of Nottingham, UK. The culture was maintained in a 10-litre aquarium (internal dimensions (LWD): 30 cm x 20 cm x 22 cm) containing aerated husbandry media. Full water-changes were conducted every other day to maintain water quality and encourage egg laying.

An F_1 culture of approximatively 150 sexually mature *L. stagnalis* was bred from the F_0 culture. The F_1 culture was seeded in February 2021 using approximately 20 F_0 egg clutches initially placed into a 10-litre aquarium, with juvenile snails later transferred into a 100-litre aquarium (internal dimensions (LWD): 80 cm x 34 cm x 40 cm). Both tanks contained constantly aerated husbandry media.

Adult and juvenile snails were fed *ab libitum* on round lettuce twice washed in culture media, and having had the hard leaf stems removed as these are known to be unpalatable to the snails and to accelerate decline in water quality (Crabb, 1929). Embryonic snails are fully sustained by perivitelline fluid provisions within their respective eggs and thus required no feeding prior to hatching (Nagle et al., 2001).

4.3.2 Experimental system

Two identical exposure systems were created to allow co-exposure of sound and calcium under controlled conditions, each facilitating one of the sound treatments and allowing all exposures for each experimental assay to be run concurrently.

Each system comprised a 300-litre plastic water butt (internal dimensions (bottom diameter, top diameter, height): 48 cm × 65cm × 88 cm)) containing a DNH Aqua-30 underwater speaker (frequency response: 80-20,000 Hz). Speakers were suspended within a high density polyethylene cylindrical support, placed on 6mm-thick foam

matting located centrally on the base of the water butt floor, with speakers orientated upwards towards the water surface, with a minimum of separation of 84 cm from the base of the closest exposure vessel, and 2.5 cm from the base of the water butt (Figure 4.1). Suspension of the speaker and foam matting were used to minimise extraneous vibration transfer from the speaker to the water butt super structure. Each speaker was coupled to a Pioneer A-10-K amplifier, with signal input from a laptop computer and M-Audio M-Track QUAD Audio Interface. This implementation enabled experimental sound outputs to be switched between the experimental systems as desired. Resultantly, experimental sound treatments could be relocated between exposure systems to account for environmental variation between the two exposure system locations.

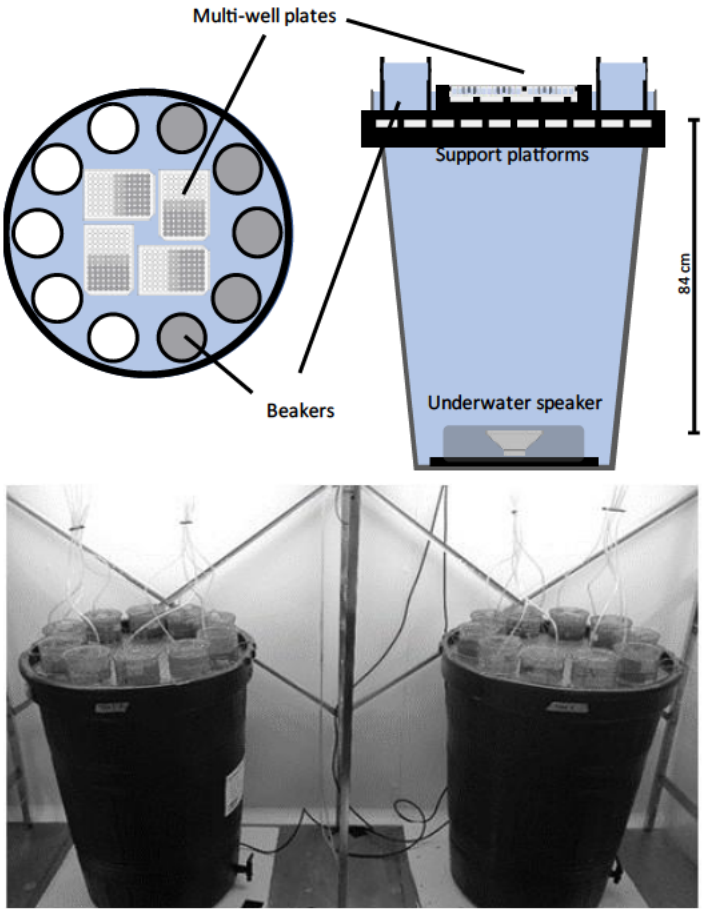

Figure 4.1: Cross-sectional schematic and photograph of exposure systems in situ. *Top: Plan and longitudinal cross-sectional schematic of the exposure systems demonstrating positioning of exposure vessels (beakers and multi-well plates) as located for each experimental assay. Bottom: Photograph showing exposure vessels (beakers) within an exposure system.*

4.3.3 Sound exposures

Exposure to ambient and ferry sounds were simulated using playbacks of field-derived sound recordings as taken by, and featured in, Bolgan et al (2016). Ferry playback tracks were compiled from 10-minute recordings capturing the arrival and departure of the Bowness ferry at Lake Windermere, with ambient recordings compiled from recordings made from the nearby Bowness foot walk in the absence of any ferry activity. Both ferry and ambient sounds were recorded using an Aquarian H2a hydrophone (sensitivity -180 dB re 1V/Pa; frequency response 10 Hz-100 kHz), calibrated using a sine wave of known voltage (100 mV_{RMS} @1 kHz; Welleman Instruments HPG1) connected to a Zoom H1 16-bit recorder using a 44.1 kHz sampling rate. All sound files were compiled in Audacity 2.2.2 and output as 24-bit WAV files.

The ambient sound treatment comprised of a 2:00 hour looped ambient recording repeated continuously. The ferry sound treatment comprised eight tracks of 2:00 hr duration with ferry arrival and departure playbacks interspersed with the ambient playbacks, such that sound exposure occurrences, timings and durations were consistent with those of the ferry's known daily operational regime. An additional four tracks of the 2:00 hr looped ambient recordings were appended to the ferry track series to complete each 24-hour period, and the whole playlist repeated daily for the duration of the experiment.

Received SPLs of ambient playbacks were targeted at approximately 118 dB_{RMS} re: 1 µPa to match the noise-floor of the tanks. Received sound pressure levels of ferry playback were targeted at 145 $dB_{0\text{-}pk}$ re: 1 µPa, consistent with sound levels of the original Bowness ferry recording, and realistic exposure levels that may result from other ecologically relevant watercraft (Barlett & Wilson, 2002; Parsons et al., 2021).

Targeted sound levels also mimic fall far below source levels or larger Sound pressure was measured using a manufacturer-calibrated HiTech HTI-94-MIN hydrophone (sensitivity: -165 dB re 1V mPa^{-1}) coupled with a calibrated Roland R-26 2-channel Portable Recorder. Particle motion was measured using a calibrated custom-built triaxial accelerometer (STMicroelectronics LIS344ALH) potted in epoxy resin and suspended within the exposure vessels using a 1.0 mm elastic cord (Wale, 2017). Received sound exposure levels were measured within the exposure vessels for the fecundity assay. For the embryo assay, where exposure vessel dimensions prohibited direct internal sound measurements, recordings were conducted within a proxy vessel (100 ml polypropylene sample pot; diameter 5.5 cm, height 5.5 cm).

4.3.4 Calcium exposures

In each assay of the current study, snails were exposed to one two of calcium treatments with environmentally relevant nominal calcium ion concentrations (Ca^{2+}) of 20 mg L^{-1} (low calcium) and 100 mg L^{-1} (high calcium) – representing the lower limit at which *L. stagnalis* can passively uptake calcium from the environment, and a calcium concentration intermediate of the species' calcium environmental tolerance (Dalesman & Lukowiak, 2010). Calcium concentrations for the media of the breeding culture was targeted at 60 mg L^{-1} as the intermediate of the experimental test media.

The selected experimental calcium concentrations reflect potential concentrations in waterways of different underlying geology as well as realistic fluctuations within environments due to flood and drought events, with *L. stagnalis* populations in Somerset, U.K. having been observed to experience 8-fold changes in waterborne calcium concentrations from 23 mg L^{-1} to 185 mg L^{-1} (Dalesman & Lukowiak, 2010), and pulmonate snail populations elsewhere in the world also noted to experience comparably large fluctuations (McKillop & Harrison, 1972; N. V. Williams, 1970).

Experimental test media were all based upon ASTM reconstituted hard water media (ASTM, 1975) with Ca^{2+} concentrations achieved through modulation of calcium sulphate dihydrate (Table 4.1). Media were made by creating a stock solution of calcium sulphate, and a second stock solution containing the other salts, before combining these. This aided the dissolution of the sparingly soluble calcium sulphate dihydrate, and limited cross-reactivity of the constituent chemicals and formation of insoluble compounds including calcium carbonate.

All equipment and exposure vessels were thoroughly cleaned and sterilised via autoclaving prior to use.

Table 4.1: Chemical constituents of breeding culture and experimental media.*Modified from ASTM hard water media (ASTM, 1975)*

Media	Nominal Ca^{2+} concentration	Quantities of salts (mg L^{-1})			
		$CaSO_4.2H_2O$	$NaHCO_3$	$MgSO_4$	KCl
Standard ASTM (hard water)	27.9 mg L^{-1}	120	192	120	8
Breeding culture media	60 mg L^{-1}	258	192	120	8
Experimental media	20 mg L^{-1}	86	192	120	8
	100 mg L^{-1}	430	192	120	8

Experimental media were created in bulk once per week, aerated vigorously for 12 hours prior to use to allow equilibrium of the media, and stored in 25 litre polypropylene carboys (fecundity assay) or 2 litre Duran bottles (embryo assay) until use. During storage media was constantly aerated to maintain oxygen saturation and homogeneity.

All exposures were conducted under semi-static renewal conditions, with twice-weekly (fecundity assay) and daily (embryo assay) full water changes, with calcium concentrations of fresh media verified via colorimetric titration using commercially available tests (Salifert Calcium Profi-Test Kit).

4.3.5 Fecundity assay

A total of 100 mature F_1 *L. stagnalis* snails were evenly distributed between five replicates within each treatment group. Each replicate comprised a 1-litre squat-form

borosilicate glass beaker (exposure vessel) containing 1000 ml of experimental test media gently aerated using glass Pasteur pipette airlines, and five adult snails of recorded shell length (26 ± 3 mm) ensuring comparable size-class distribution between all treatment groups. Shell length was measured linearly from the apex of the shell to the anterior-most edge of the shell aperture as in Figure 4.2 using digital callipers.

Figure 4.2: Representative image showing shell length measurement as applied to L. stagnalis adults. *Snail as viewed from left-ventral side. Scale bar included for reference only.*

Exposure vessels were randomly distributed around the perimeter of the exposure system (Figure 4.1) where the sound cone of sound playbacks was most consistent exposures. Prior to commencing sound playbacks, snails were acclimated to experimental conditions for four days to account for the oogenesis period of the species (Janse et al., 1989), ensuring that any following oviposition occurred whilst experiencing experimental calcium concentrations and not those of the husbandry media.

Full water changes were conducted twice-weekly at alternating 3- and 4-day intervals. During water changes, any egg clutches were removed and both clutches and eggs therein counted with the aid of a dissection microscope. Any un-grazed lettuce was also removed, air dried for one-hour, then weighed and recorded, to enable calculation of average food consumption rate during the previous water change

interval. In the event of any mortalities, dead individuals were removed, and the experimental test media volumes and feed allocation proportionately reduced for subsequent water changes to maintain stocking densities. Replicates were then provisioned for the upcoming water change interval with 0.5 ± 0.05 g snail^{-1} day^{-1} round lettuce twice-washed in the corresponding experimental media, and the precise portioning recorded. Lettuce allocation was measured as 'wet-weight' of lettuce which had been dabbed dry using absorbent tissue, and further air-dried for one hour to remove excess surface water content.

Prior to commencement of sound exposures following water changes, sound playbacks were switched between exposure systems, and corresponding exposure vessels randomly redistributed around the perimeter of the appropriate exposure system to better account for any spatial variation between and within exposure systems.

Following the 28-day exposure period, surviving snails were removed and their shell length measured and recorded as previously. Snails were then euthanised by chilling in a -20 °C freezer.

4.3.6 Embryo assay

A total of 200 eggs originating from five egg clutches laid by the F_0 culture over a one-week period were utilised for the embryo assay. Eggs from each egg clutch were distributed evenly between experimental treatment groups resulting in n = 50 replicates per treatment. Eggs were excised from egg clutches known to be < 24 hours old. Egg clutches were first removed from the breeding culture tank and transferred to a dry glass petri dish. Egg clutches were then quadrisected transversely and longitudinally using butterfly scissors to allow extraction of the individual eggs. Eggs were carefully teased from the egg clutch matrix using a pair of small metal spatulas and carefully manipulated within the petri dish to aid removal of the extracellular

gelatinous material contained within the egg clutches. Excised eggs were then allocated to individual wells Merck Millipore MultiScreen® 96-well mesh filter plates with a 100 µm pore size containing 300 µl of the experimental test media (Figure 4.3). Replication and timing of egg production necessitated the use of two parallel 96-well plates per sound treatment. Eggs originating from any given egg case were allocated to the same plate replicate.

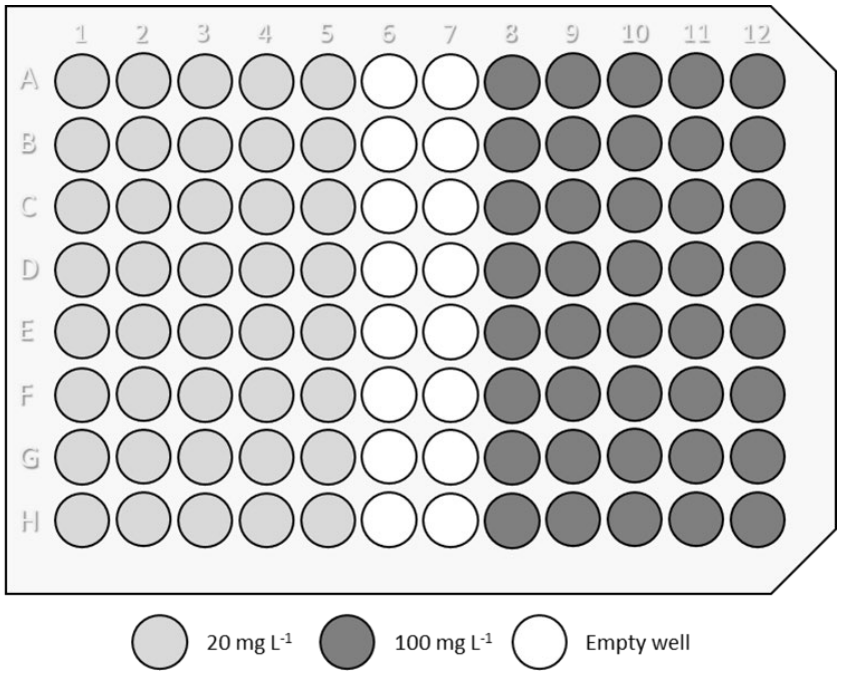

Figure 4.3: Schematic of calcium treatment layout within 96-well plates. *Schematic shows arrangement of wells designated for each calcium treatment. Not all designated wells were used during experimental exposures.*

Developing embryos were observed daily, with developmental stage, any developmental abnormalities, and mortalities recorded. In all instances, practicalities of the experimental logistics precluded blind observations and data collection, however all observations were conducted according to well-defined parameters by a single, experienced observer, and thus the impacts of any subjectivity or observational bias are considered negligible. Developmental stage was characterised according to five-stage (morula, trochophore, veliger, hippo, hatched) outlined by Bandow and

Weltje (2012). Embryos were considered morula from the moment of oviposition until transitioning to trochophore, with trochophore being defined by the embryo demonstrating clear and distinct rotary independent of any inertial movement of the egg. Embryos were considered veliger upon development of a velum, evident by a departure from a spherical form and transition to a classically kidney-bean shape. Finally, embryos were designated as hippos upon the development of eyespots and a clearly identifiable rhythmic heartbeat. Successful hatching was defined as snails which fully excised themselves from their respective egg chorion. Upon observation, any embryos seemingly in a transitional phase between the developmental stages as defined were considered as being in the less-developed stage to avoid bias or subjectivity. During development, any occurrences of physiological abnormalities were recorded. Abnormalities were broadly delineated into two categories:

1) Malformations – embryos displaying visually identifiable physiological traits clearly departing from the established body plan for any given developmental stage.

2) Cessations – embryos displaying no outward signs of abnormal development which nonetheless fail to progress across developmental stages.

Mortalities were defined differently according to developmental stage. For morula stages which lack more distinctive indicators, mortalities were defined by a loss of physical integrity. For trochophore and veliger stages, the definition was expanded to include an obvious and persistent cessation of previously identified rotary movement. For hippo stages, mortality was ascertained by the obvious and persistent lack of a previously observed heartbeat.

Snails observed to have successfully hatched had their shell length and heart rate recorded (see below). Measurements were taken in-plate using microscopy to prevent

causing inadvertent physical harm to the snails. Shell length was measured using a calibrated reticule eyepiece, ensuring the snail was on the base of the filter-mesh insert for consistency. Shell length measurement was taken from the anterior-most point of the shell to the posterior most point along the medial line as viewed either laterally or dorsoventrally (Figure 4.4).

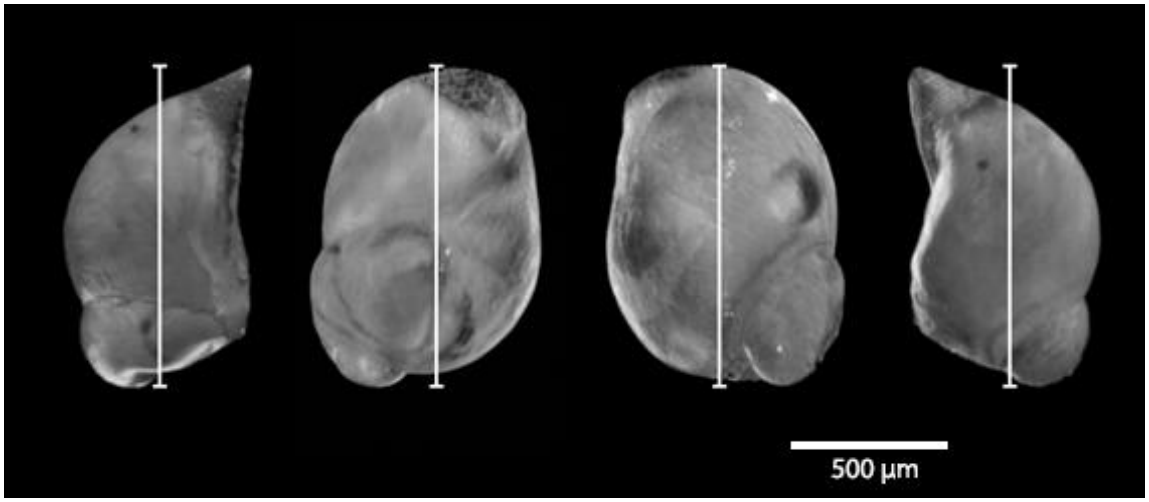

Figure 4.4: Representative image showing shell length measurement line as applied to L. stagnalis hatchlings. *Snail as viewed from right-lateral, ventral, dorsal, left-ventral sides (left to right). Scale bar included for reference.*

Heart rate was ascertained by visual observation of the heart through the semi-transparent shell. In all cases, the heart was observed 30 seconds, and the number of beats occurring during this time recorded. Wherever possible, heart rate was measured prior to shell length to ensure any necessary manipulation of the snail did not influence heart rate. In instances where snails had to be manipulated to facilitate heart observations, snails were allowed a 30-seconds recovery period before heart rate measurements were conducted.

The embryo assay was conducted for 21 days. After this time, any unhatched snails not displaying physical malformation were considered to have ceased development. Remaining snails and embryos were euthanised by chilling in a -20 °C freezer.

4.3.7 Statistical analyses

Statistical analyses were performed using R Statistical Software (version 3.6.1). Analyses were selected based on underlying analytical assumptions and data conformity. Where multiple comparisons of data were undertaken, statistical significance ($\alpha = 0.05$) is stated as both uncorrected values, and as corrected values controlling for false discovery rate (FDR) utilising the Benjamini-Hochberg procedure (Yekutieli & Benjamini, 1999).

4.3.7.1 Adult fecundity

Fecundity data was corrected for mortalities by use of the metric 'clutches/eggs per snail-day', accounting for the cumulative number of days those snails were extant prior to mortalities. If the exact date of mortality was unknown, mortality was assumed to have occurred at the median time-point between observations.

Number of clutches and eggs laid over the study duration, and the number of eggs per clutch were assessed using generalised linear mixed-effect models (GLMM) assuming gaussian data distribution, fit using Residual Maximum Likelihood (REML), with sound and calcium treatments included as fixed factors, and individual beakers as a random factor. Satterthwaites's method was used to provide *p*-values estimates for additional context.

4.3.7.2 Adult biometrics

Growth, food consumption, and growth-dependent fecundity were assessed using two-way Permutation Analysis of Variance (PERMANOVA), with post-hoc analysis via one-way PERMANOVA contrasts between treatments where applicable.

4.3.7.3 Juvenile hatch success

Overall hatching success was assessed using a GLMM model assuming binomial data distribution, fit using REML with calcium and sound treatments as fixed factors, and originating egg clutch as a random factor.

4.3.7.4 Embryonic mortalities and developmental abnormalities

Principal cause of unsuccessful hatching was assessed with Fisher's exact test.

Occurrence and timing of events leading to unsuccessful hatching were assessed using log-rank Mantel-Cox contrasts of Kaplan-Meier survival curves.

4.3.7.5 Duration of embryonic development

Duration of embryonic development post-oviposition was assessed using two-way Permutation Analysis of Variance (PERMANOVA), with post-hoc analysis via one-way PERMANOVA contrasts between treatments.

4.3.7.6 Juvenile biometrics

Shell length and heart rate were assessed using two-way Permutation Analysis of Variance (PERMANOVA).

4.4 Results

4.4.1 Sound exposure

Mean peak received sound pressure levels during ferry passage playbacks were approximately 3-4 dB re: 1 µPa higher than the 145 dB re: 1 µPa target but did not exceed 150.0 dB re: 1 µPa as desired (Table 4.2). Comparing sound exposures between the two assays, sound pressure levels and particle motion during ferry playbacks were broadly consistent, albeit marginally higher during the embryo assay compared to the

fecundity assay; presumably resultant of their central placement in the speaker sound cone. Higher sound pressure levels present during ambient playbacks in the fecundity assay were contributable to the aeration required within these experimental replicates. Increases in broadband RMS particle motion attributable to ferry playbacks were also modest compared to increases in pressure, but nonetheless higher than those in observed in ambient playbacks across all calibrated frequencies (50-3000 Hz).

Table 4.2: Received experimental playback sound levels during each experiment.*Fecundity assay measurements were taken from within test beakers; embryo assay measurements were taken within the vicinity of 96-well plates.*

Mean (± SD) received sound						
			SPL (dB re: 1 µPa)		SEL (dB re: 1 µPa² s)	
	Assay	Sound Treatment	RMS	Peak	SEL	SELcum
Pressure	Fecundity	Ambient	126.2 ± 3.2	-	177.3 ± 2.3	216.6 ± 2.3
		Ferry	142.9 ± 2.3	147.2 ± 2.3	192.8 ± 2.3	232.1 ± 2.3
	Embryo	Ambient	119.4 ± 0.2	-	169.4 ± 0.2	208.8 ± 0.2
		Ferry	145.4 ± 0.8	148.9 ± 0.8	196.6 ± 3.5	235.9 ± 3.5
			PM (dB re: 1 µm s^{-2})		SEL (dB re: 1 (µm s^{-2})² s)	
	Assay	Sound Treatment	RMS	Peak	SEL	SELcum
Particle motion	Fecundity	Ambient	59.8 ± 1.4	-	109.6 ± 1.4	139.4 ± 1.4
		Ferry	67.6 ± 3.1	81.6 ± 3.8	117.4 ± 3.1	149.0 ± 3.1
	Embryo	Ambient	58.6 ± 0.1	-	108.5 ± 0.1	138.3 ± 0.1
		Ferry	74.1 ± 6.8	89.5 ± 7.7	123.9 ± 6.8	155.6 ± 6.8

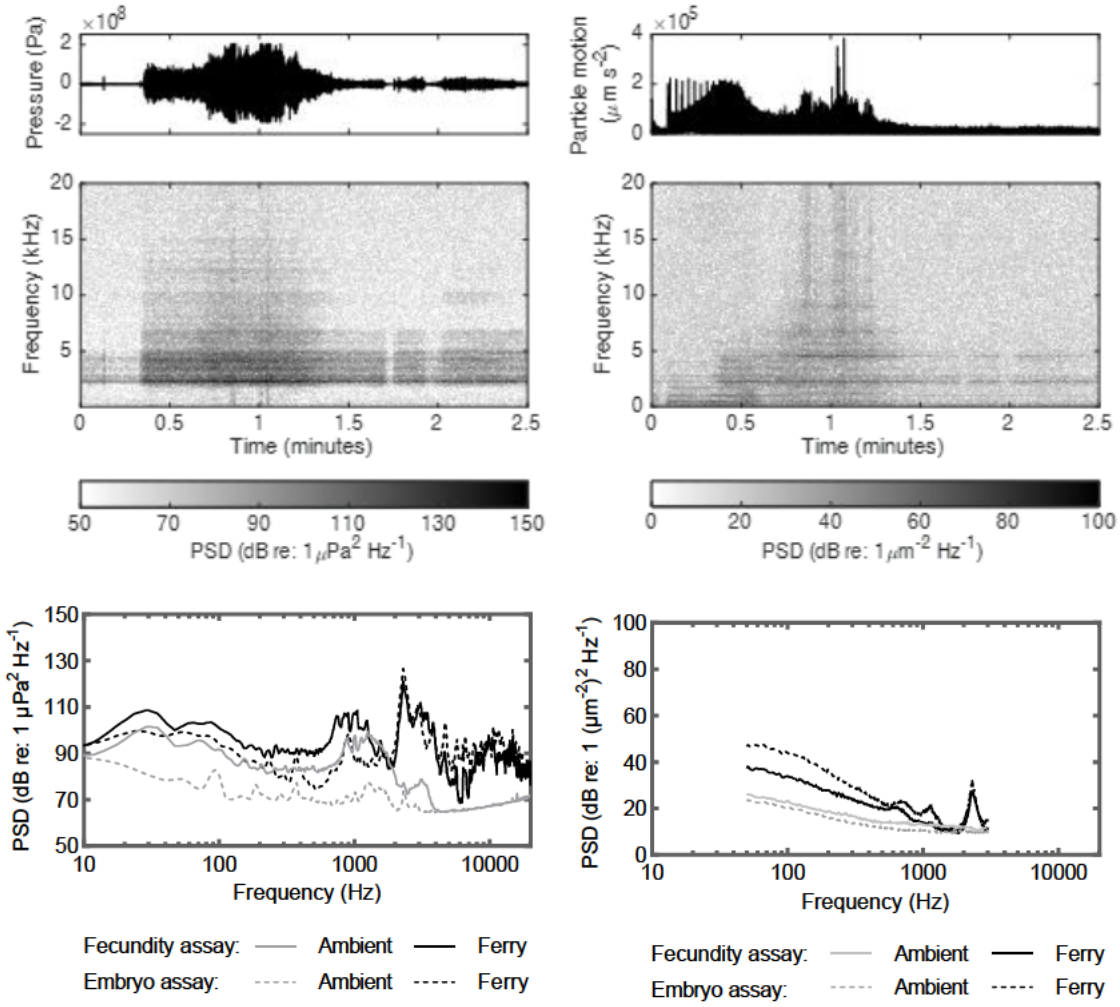

Figure 4.5: Received sound levels during a single ferry playback. *Sound pressure (left) and magnitudinal particle motion (right). Top: waveform. Middle: spectrogram. Bottom: power spectral density comparing ambient and ferry sound treatments. Pressure PSDs calculated using 1 second Hann window and 50-percent overlap. Particle motion PSDs calculated using 1 second Hamming window and 50-percent overlap.*

Some discrepancies between pressure and particle motion waveforms were evident, which appear to be contributable to a higher prevalence of sub-2 kHz particle motion as compared with sound pressure. This may in part have been resultant of electrical noise within the recording system as evidenced by consistent, rhythmic signal peaks visible in the first 30-seconds of the spectrogram. Otherwise, there was reasonable consistency in pressure and particle motion with regards to dominant frequencies and their occurrence with regards to time (Figure 4.5).

4.4.2 Calcium treatments

Table 4.3: Water quality traits of experimental media. *Mean ± SD*

Assay	Calcium treatment	Measured Ca^{2+} (mg/L)	pH	Hardness ($CaCO_3$)	Dissolved O_2 (%)
Fecundity	20 mg/L	20 ± 1 mg/L	8.36 ± 0.02	119.6 ± 1.8	94.1 ± 0.7
	100 mg/L	100 ± 1 mg/L	8.34 ± 0.02	119.6 ± 1.8	94.2 ± 0.8
Embryo	20 mg/L	20 ± 1 mg/L	8.35 ± 0.02	119.6 ± 1.7	94.4 ± 0.6
	100 mg/L	100 ± 1 mg/L	8.35 ± 0.02	119.5 ± 1.8	94.7 ± 0.6

Measured water quality traits were consistent between treatments and assays. No meaningful variation in water parameters was evident with respects to time, nor solutions made from different stocks (Table 4.3).

4.4.3 Adult fecundity

Fecundity was unaffected by the ferry playbacks, but high calcium treatments led to a significant reduction in both number of egg clutches and individual eggs produced compared to the low calcium treatment (Table 4.4 and Table 4.5). Within each individual treatment, fecundity rate was temporally consistent over the duration of the study (Figure 4.6, and Table 4.6). Neither sound nor calcium treatments significantly affected the number of eggs per clutch.

Table 4.4: Statistical summary of fecundity mixed effect model terms and effects

	Random effects		Fixed effects		
	Variance	SD	Estimate	SE	t
Clutches					
Beaker (intercept)	0.000	0.000			
Residual	0.013	0.113			
(Intercept)			0.1587	0.0178	8.892
Sound			0.0063	0.0252	0.248
Calcium			0.0704	0.0252	2.790
Sound:Calcium			-0.0159	0.0357	-0.444
Eggs					
Beaker (intercept)	0.0000	0.000			
Residual	29.650	5.445			
(Intercept)			7.0248	0.8609	8.160
Sound			0.3239	1.2175	0.266
Calcium			3.7059	1.2175	3.044
Sound:Calcium			-0.8433	1.7219	-0.490
Eggs per clutch					
Beaker (intercept)	12.260	3.501			
Residual	130.800	11.437			
(Intercept)			43.311	2.516	17.216
Sound			2.743	3.540	0.775
Calcium			4.332	3.526	1.229
Sound:Calcium			-2.260	4.937	-0.455

Table 4.5: Summary of fecundity mixed-effect model statistical significance. *Satterthwaite approximation. Bold values signify statistical significance $p < 0.05$*

		Sums Sqs	Mean Sqs	Df	DenDf	F	p
Clutches	Sound	0.0001	0.0001	1	156	0.0088	0.926
	Calcium	0.1562	0.1562	1	156	12.2570	**<0.001**
	Sound:Calcium	0.0025	0.0025	1	156	0.1973	0.658
Eggs	Sound	0.3800	0.3800	1	156	0.0129	0.910
	Calcium	431.40	431.40	1	156	14.5522	**<0.001**
	Sound:Calcium	7.1100	7.1100	1	156	0.2398	0.625
Eggs per clutch	Sound	55.064	55.064	1	15.471	0.4210	0.526
	Calcium	216.83	216.83	1	15.471	1.6577	0.217
	Sound:Calcium	27.021	27.021	1	15.471	0.2066	0.656

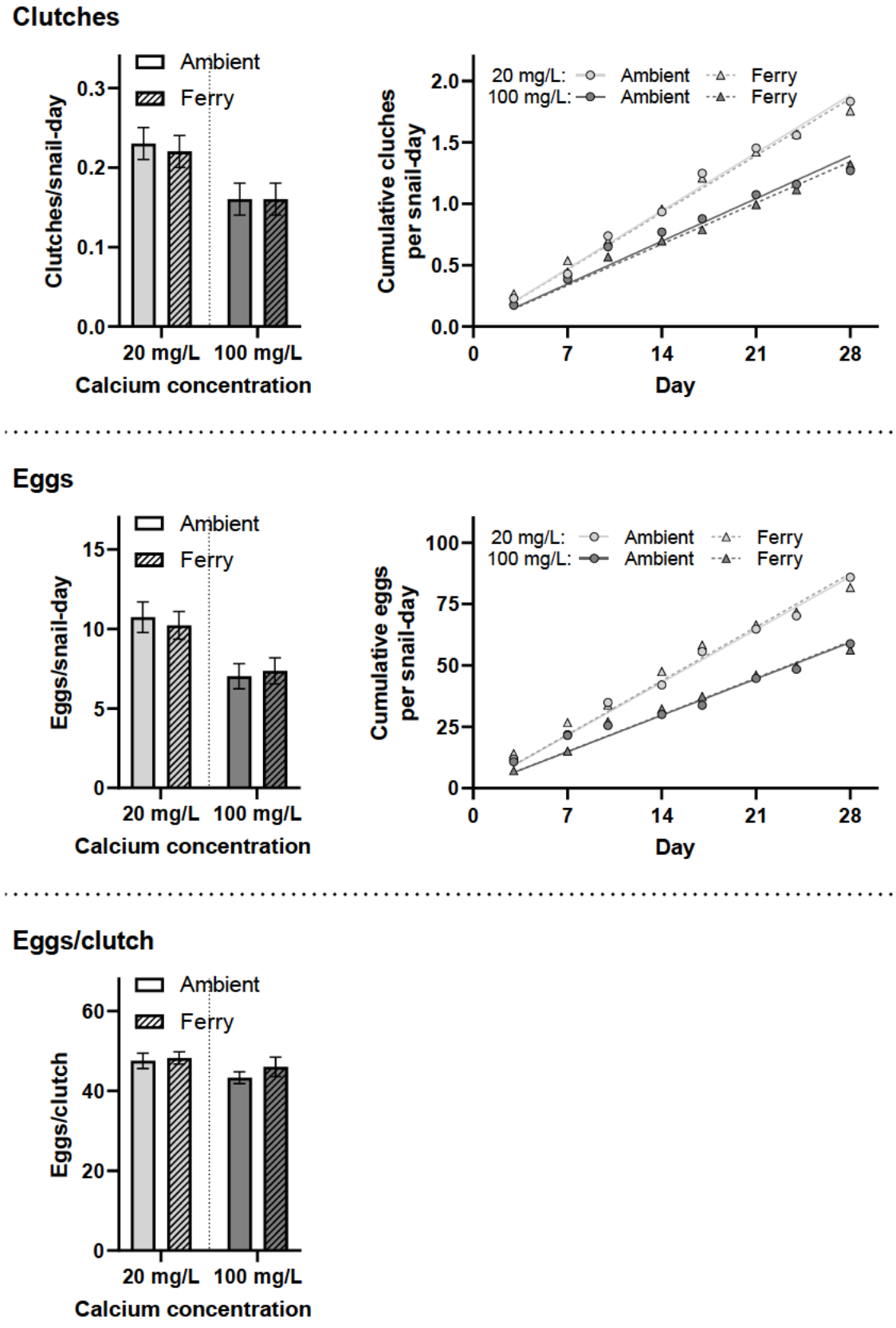

Figure 4.6: Summary of fecundity data. *Top: mean number of egg clutches; Middle: mean number of individual eggs; Bottom: mean number of eggs per clutches during the study. Bar charts display mean ± SE. Regressions show cumulative values over the exposure duration. All values and fits calculated using n=40 observations per treatment, though regressions show only mean values for each time-point observation to aid visual clarity*

Table 4.6: Linear regression equations and goodness-of-fit details for fecundity data. *Regressions fit using n=40 observations per treatment and constrained to pass through the origin. Bold values signify statistical significance $p < 0.05$*

	Treatment	N	Equation	R^2	F (DFn, DFd)	p
Cumulative Clutches	Ambient – Low calcium	40	y = 0.067x	0.99	3612.96 (1,39)	**<0.001**
	Ferry – Low calcium	40	y = 0.066x	0.98	1775.09 (1,39)	**<0.001**
	Ambient – High calcium	40	y = 0.050x	0.92	420.47 (1,39)	**<0.001**
	Ferry – High calcium	40	y = 0.048x	0.95	825.25 (1,39)	**<0.001**
Cumulative Eggs	Ambient – Low calcium	40	y = 3.076x	0.99	2785.19 (1,39)	**<0.001**
	Ferry – Low calcium	40	y = 3.121x	0.98	1574.79 (1,39)	**<0.001**
	Ambient – High calcium	40	y = 2.134x	0.88	293.09 (1,39)	**<0.001**
	Ferry – High calcium	40	y = 2.119x	0.97	1338.86 (1,39)	**<0.001**

Food consumption of adult snails during the study was marginally, but significantly lower in the 100 mg L^{-1} calcium treatments than in the low calcium treatments, but unaffected by ferry playbacks. Neither sound nor calcium treatments independently resulted in differences in growth of adult snails during the study, however there was a significant interaction between the drivers resulting in significantly reduced growth of snails exposed to ferry playbacks in the high calcium treatment. A mirroring interaction was evident in growth-dependent fecundity, with snails exposed to ferry playbacks in the high calcium treatment producing significantly more eggs relative to their growth (Table 4.7).

Table 4.7: Two-way PERMANOVA of growth-related factors of adult L. stagnalis*. N=1000 permutations. Bold values signify statistical significance p < 0.05*

		Df	Sums Sqs	Mean Sqs	F	R^2	p
Length gain	Sound	1	0.0542	0.0542	1.901	0.0836	0.180
	Calcium	1	0.0281	0.0281	0.984	0.0433	0.355
	Sound:Calcium	1	0.1387	0.1387	4.861	0.2137	**0.027**
	Residuals	16	0.4279	0.0285	0.659		
Food consumed	Sound	1	0.0003	0.0003	0.306	0.00865	0.588
	Calcium	1	0.0161	0.0161	17.179	0.48537	**0.004**
	Sound:Calcium	1	0.0018	0.0018	1.909	0.05393	0.202
	Residuals	16	0.0150	0.0009	0.452		
Clutches per mm growth	Sound	1	0.0483	0.0483	1.044	0.0512	0.334
	Calcium	1	0.0290	0.0290	0.627	0.0308	0.543
	Sound:Calcium	1	0.1257	0.1257	2.716	0.1332	0.078
	Residuals	16	0.7404	0.0463	0.785		
Eggs per mm growth	Sound	1	0.0559	0.0559	1.257	0.0579	0.272
	Calcium	1	0.0455	0.0455	1.023	0.0471	0.348
	Sound:Calcium	1	0.1527	0.1527	3.431	0.1580	**0.041**
	Residuals	16	0.7122	0.0445	0.737		

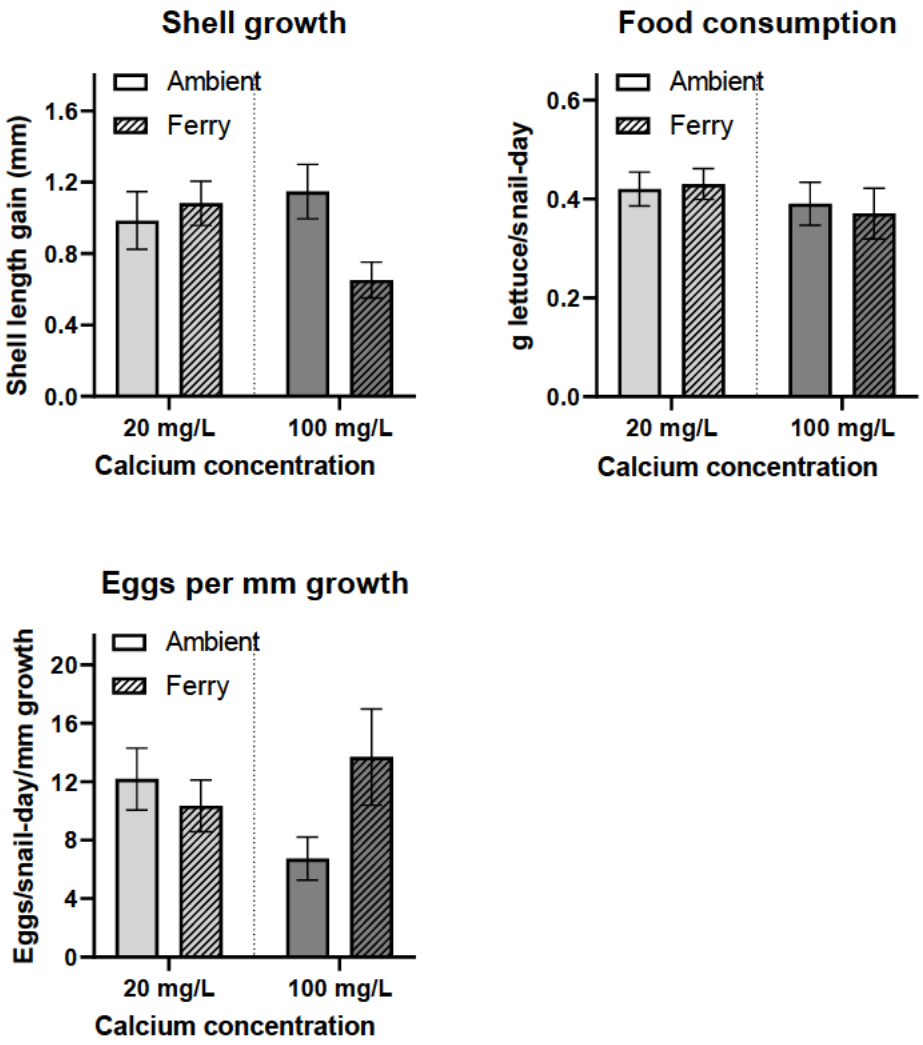

Figure 4.7: Comparison of growth, food consumption, and fecundity relative to growth following 28-day exposures to ferry sound playbacks and different calcium concentrations. *Values given as mean measurements of each replicate (N = 5) replicates per treatment All values given as mean ± SE. during over study duration. Growth of adult snails Values calculated from values obtained upon conclusion of the study relative to values ascertained at the beginning of the acclimation period.*

4.4.4 Juvenile hatching success

Hatch success was relatively high and consistent regardless of experimental treatment (Figure 4.8). Sound exposure was nonetheless shown to be a statistically significant driver of hatching success, leading to a slight reduction in hatching success when accounting for variation between the originating egg clutches (Table 4.8).

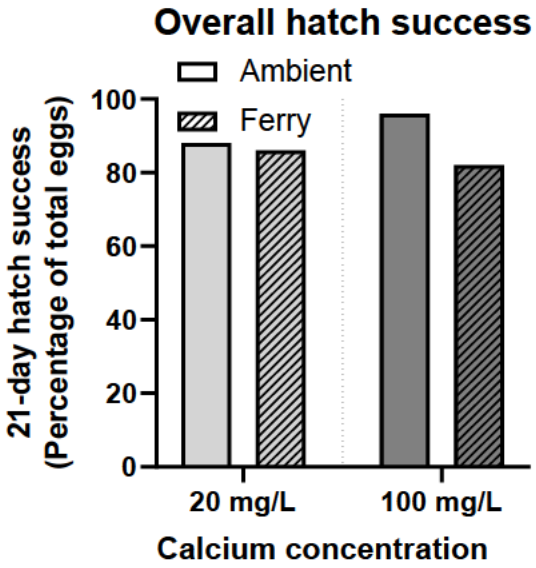

Figure 4.8: L. stagnalis 21-day hatch success

Table 4.8: Statistical summary of juvenile hatching success mixed effect model terms and effects.Bold values signify statistical significance $p < 0.05$

	Random effects		Fixed effects			
	Variance	SD	Estimate	SE	t	p
Egg clutch (intercept)	0.000	0.000				
(Intercept)			3.178	0.721	4.404	< 0.001
Sound			-1.662	0.810	-2.051	**0.040**
Calcium			-1.186	0.843	-1.407	0.160
Sound:Calcium			1.485	1.006	1.476	0.140

4.4.5 Embryonic mortalities and developmental abnormalities

The majority of unsuccessful embryos resulted either from morula-stage mortalities or malformations occurring in pre-hippo stages. In all instances, malformations ultimately led to mortality of the developing embryo within the 21-day observation period. Additionally, there were three instances of cessation among hippo-stage embryos, which otherwise appeared to develop normally but were seemingly unable to hatch (Figure 4.9).

There were no significant differences in the principal cause of unsuccessful hatching between experimental treatments (Fishers' exact test, p = 0.737), however significant differences in the timing and cumulative total of events leading to unsuccessful hatchings were evident (Table 4.9), corroborating linear hypotheses projections of the GLMM model.

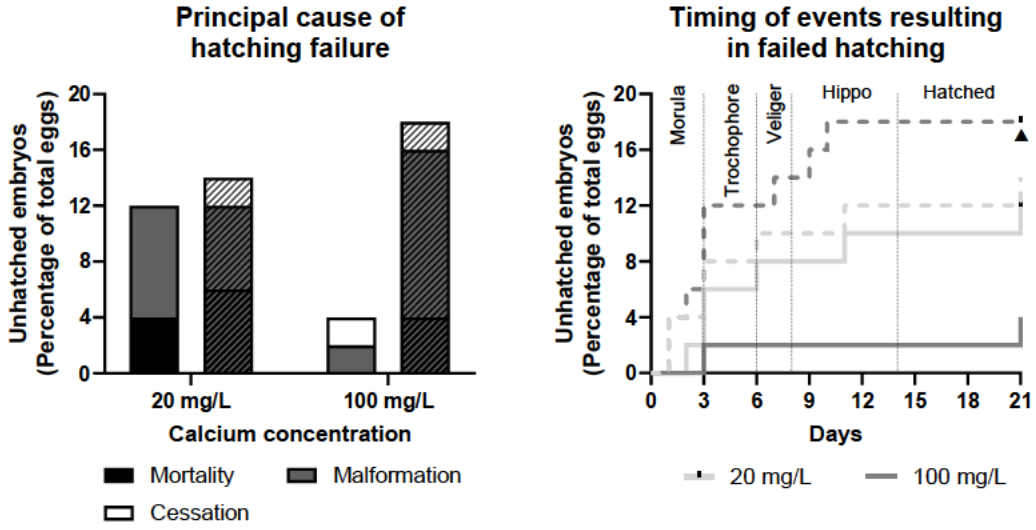

Figure 4.9: Summary of 21-day unsuccessful hatching. *Values represent percentages of total eggs pooled across egg clutches. Solid and hatched plots represent ambient and ferry sound treatments respectively. Left: Stacked bars show principal cause of hatching failure. Right: Timing at which mortality or terminal developmental abnormality was first evident. Vertical dotted lines denote median time boundaries of embryonic development with respect to oviposition. ▲significant pairwise difference (log rank Mantel-Cox, corrected p <0.05) between sound treatments with respects to calcium concentration.*

Table 4.9: Statistical summary onset of developmental abnormalities. *Log-rank Mantel-Cox comparisons. Uncorrected p values represent those from pairwise comparison. Corrected p values are modified values controlling for false discovery rate using Benjamini-Hochberg procedure (α = 0.05). Bold values signify statistical significance p < 0.05*

Contrast treatments		df	Z	Uncorrected p value	Corrected *p* value
Ambient – Low calcium	Ambient – High calcium	1	1.946	0.052	0.103
Ferry – Low calcium	Ferry – High calcium	1	-0.562	0.574	0.689
Ambient – Low calcium	Ferry – Low calcium	1	0.315	0.753	0.753
Ambient – High calcium	Ferry – High calcium	1	2.657	**0.008**	**0.047**

4.4.6 Duration of embryonic development

Duration of embryonic development was highly consistent across experimental treatments (Figure 4.10), with no statistically significant differences attributable to the fixed experimental factors. Significant differences in hatching time were present between eggs originating from different egg clutches (Table 4.10).

Table 4.10: Two-way PERMANOVA of duration of embryonic development. N=1000 permutations

	Df	Sums Sqs	Mean Sqs	F	R^2	p
Egg clutch	4	0.01370	0.00342	7.495	0.14993	**0.001**
Sound	1	0.00002	0.00002	0.047	0.00024	0.826
Calcium	1	0.00032	0.00032	0.693	0.00346	0.428
Sound:Calcium	1	0.00056	0.00056	1.230	0.00615	0.272
Residuals	168	0.07677	0.00046	0.840		

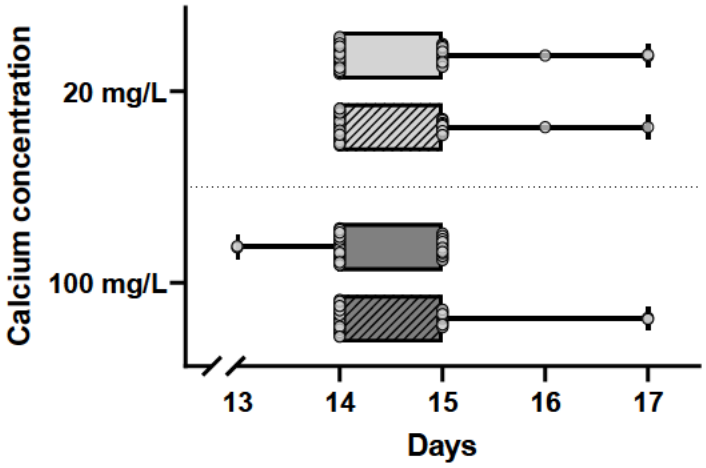

Figure 4.10: Duration of L. stagnalis embryonic development. *Box-and-whisker plot showing time to hatch as measured from oviposition; whiskers represent range, circles show individual data points. Solid and hatched plots represent ambient and ferry sound treatments respectively.*

4.4.7 Juvenile biometrics

Subtle, but significant (Table 4.11) differences were evident in both the shell length and heart rate of newly hatched snails with regards to calcium treatment, with increased calcium resulting in reduced hatching size, and increased heart rates (Figure 4.11).

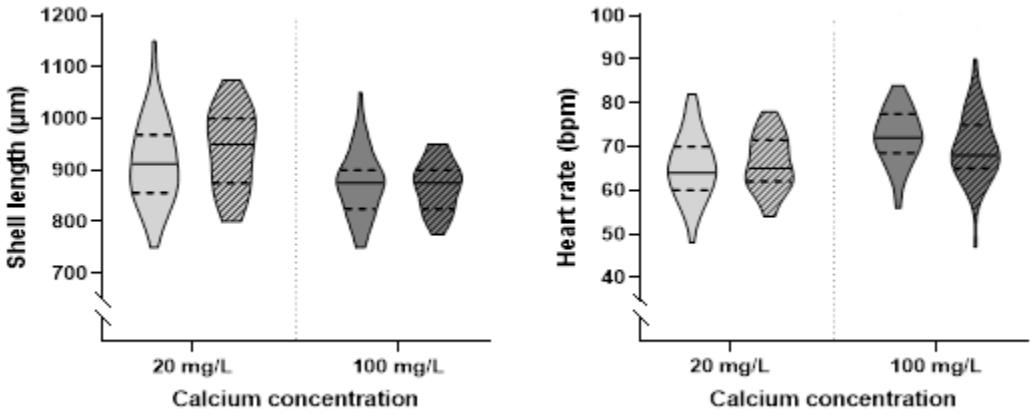

Figure 4.11:Biometric measurements of hatched L. stagnalis. *Violin plot. Solid and hatched plots represent ambient and piling sound treatments respectively. Solid and dashed horizontal bars within plots represent median and quartile values respectively..*

Table 4.11: Statistical summary of biometrics on newly hatched L. stagnalis. *Two-way PERMANOVA of shell length (top) and heart rate (bottom) on day of hatching. N=1000 permutations. Bold values signify statistical significance $p < 0.05$*

		Df	Sums Sqs	Mean Sqs	F	R^2	p
Shell length	Egg clutch	4	0.02500	0.00625	4.776	0.08620	**0.002**
	Sound	1	0.00193	0.00193	1.474	0.00665	0.214
	Calcium	1	0.04102	0.04102	31.354	0.14146	**0.001**
	Sound:Calcium	1	0.00223	0.00223	1.704	0.00769	0.215
	Residuals	168	0.21980	0.00131	0.758		
Heart rate	Egg clutch	4	0.00298	0.00074	0.22390	0.00487	0.939
	Sound	1	0.00018	0.00018	0.05390	0.00029	0.909
	Calcium	1	0.04749	0.04749	14.29000	0.07763	**0.001**
	Sound:Calcium	1	0.00275	0.00275	0.82730	0.00449	0.378
	Residuals	168	0.55834	0.00332	0.91271		

4.5 Discussion

This is the first study to investigate how the availability of an essential macronutrient impacts upon the susceptibility of a species to anthropogenic sound. As such, this study is the first to address how the risks posed by anthropogenic sound to a given species may vary spatially between environments with differing availability of a nutrient of documented importance to its vitality within environments susceptible to large temporal fluxes in nutrient availability. The study further addresses several separate aspects affecting species proliferation – namely adult fecundity, and the embryonic development of resultant offspring.

Calcium was a significant driver of fecundity. Contrary to the initial hypothesis, high calcium (100 mg L^{-1}) availability resulted in fewer eggs compared to the low calcium treatment (20 mg L^{-1}). Anthropogenic sound playbacks had no significant effect on fecundity in either calcium treatment, though there was an interaction between ferry playbacks and high calcium concentrations leading to a significant reduction in growth of adult snails during the fecundity study, suggesting that fecundity was being maintained through energetic plasticity at the expense of growth. A significant interaction between anthropogenic sound and calcium concentration was also seen with regards to embryonic development of snails, with ferry playbacks promoting failed hatching of snails in high calcium treatments relative to those observed in low calcium treatments. In both instances where interactions were evident, the mechanistic nature of the interactions could not be concluded as the study design precluded such determinations.

4.5.1 Exposures

Received sound pressure levels in the study were broadly consistent with expectations, however the corresponding RMS particle motion within ferry playbacks was lower than

expected – increasing only approximately 5 dB re: 1 µm s^{-2} relative to ambient playbacks, as compared to 20 dB re: 1 µPa increases in pressure. This likely reflects the comparably rapid attenuation of particle motion with increasing frequencies relative to pressure, and boundary effects related to the comparably small dimensions of the exposure system which are also known to influence particle motion (Gray et al., 2016). Resultantly, RMS values of particle motion calculated across broadband frequency ranges are likely constrained by the relative abundance of higher frequencies where particle motion is liable to be inherently lower. This supposition is supported by the more substantial separation of frequency-specific particle motion in the 5-3000 Hz range than reflected by the broadband RMS estimates (Figure 4.5).

Particle motion did not vary drastically between the two assays despite differences in placement of the exposure vessels within the exposure system, and the working volumes therein. Whilst received sound levels could be measured *in situ* within exposure vessels for the fecundity assay owing to the size of the beakers utilised, it was not possible to achieve the same for the embryo assays due the constraints of the well dimensions of the 96-well plates. Thus, whilst these sound levels are representative of those of the immediate environment of the well-plates, the *specific* received sound levels within each well within each plate is unknown. Realistically, sound within the well plates is likely further influenced by boundary effects; but given *L. stagnalis* routinely affix their egg clutches to solid substrates, it can be assumed that boundary effects are also likely characteristic of natural environments. Notably, additional sound pressure levels in the fecundity assay contributable to the necessary aeration did not translate to any noteworthy increase in sound particle motion within the replicates – to the benefit of comparability of sound exposures between the two assays.

Modulation of waterborne calcium via calcium sulphate enabled comparable pH and hardness levels between treatments, which are both known to otherwise affect both fecundity and growth of *Lymnaea spp.* (Cœurdassier et al., 2003; Hunter, 1975). Excepting nominal calcium content, composition and physicochemical properties of the experimental media were consistent between the experimental treatments, and assays. Dissolved oxygen and pH of solutions were within the limits stated by OECD guidelines (OECD, 2016), though measured hardness was below the OECD-advised value of 250 mg L^{-1} for the species, and the anticipated range of 160-180 mg L^{-1} stated for the unmodified water recipe (ASTM, 1975). These deviations from anticipated and recommended water-quality criteria are not considered detrimental to this study's validity as they were consistent between treatments.

4.5.2 Fecundity

The average number of eggs deposited within each clutch was consistent regardless of treatment. Correspondingly, cumulative egg clutches and cumulative eggs therein were strongly correlated, corroborating other studies (Gomot, 1998; OECD, 2016). As such, differences in fecundity primarily resulted from differences in the number of egg clutches being laid, as also observed by van Duivenboden et al. (1985). Nonetheless, in the present study, egg counts consistently proved an equally or more sensitive predictor of driver impacts than clutch counts alone, presumably due to the improved data resolution they provide. Thus, whilst assertions that counts of egg clutches alone are generally sufficient to assess fecundity effects (OECD, 2016), these data show fringe-case benefits to assessing both clutch and egg counts.

Fecundity in the low calcium treatments was similar to that observed in various other laboratory studies conducted under comparable conditions (Ducrot et al., 2014). However, contrary to expectations, fecundity was consistently lower in the high

calcium treatments compared to the low calcium treatments. Measures of cumulative fecundity, which better account for short-term variation in egg laying and the limited temporal resolution of the data, show that fecundity rates within each experimental treatment group remained consistent over the study duration. Whilst the study was not designed to directly assess how individuals respond to changes in environmental calcium conditions, it nevertheless suggests that acclimation to changes in calcium concentration happen rapidly. Indeed, increases in calcium uptake in response to shell damage in the ramshorn snail *Indoplanorbis exustus* were observed to happen within six hours (Vaidya & Nagabhushanam, 1980). Given the four-day experimental acclimation period in the present study covered the expected the oogenesis period of *Lymnaea stagnalis* (Vaughn, 1953), observed fecundity rates are presumed to relate directly to the prevailing environmental conditions during oogenesis, as opposed to latent factors such as body condition and/or energy reserves of the adults which are assumed to have been comparable upon allocation to experimental conditions.

Accounting for growth and food consumption of adult snails during the study suggests differences in life-history strategy between treatment groups, and that fecundity rates were maintained through a degree of physiological plasticity. Higher food consumption of adult snails in the low calcium treatment supports observations by Young (1975) that calcium needs, which could not be met through cutaneous absorption, may have been offset through dietary intake, although dietary calcium assimilation rates of snails may also be independently regulated (Beeby & Richmond, 2007).

Given the subtle differences in food consumption between calcium treatments, and the higher fecundity in the low calcium treatments, it is likely that egg production of *L. stagnalis* was not limited by calcium availability. Similarly, significant differences in accumulated shell growth between treatment groups did not correspond with

differences in food consumption, strongly suggesting differences in energy budgeting of adults between experimental treatments. Combining fecundity and growth into a single metric (eggs/snail-day/mm growth) enabled the detection of a significant interaction between calcium and sound exposures, suggesting that whilst exposure to ferry playbacks had no significant effect on any of the metrics in isolation, at given calcium concentrations, it is likely contributing to behavioural and/or physiological plasticity of the adults. Accepting the evidence that high calcium treatments were more physiologically stressful than low calcium treatments in this study, patterns in growth-dependent fecundity match known behaviours in some other aquatics gastropods whereby mild-to-moderate environmental stress reduces fecundity, but excessive environmental stress can promote reproduction (Boyle & Yoshino, 2000).

4.5.3 Embryo assay

Eggs isolated from their egg clutch, such as used in the present study, may exhibit higher sensitivity to environmental factors, particularly chemical pollutants, than those in retained in an egg clutch, although not to an extent that is significant in the context of environmental risk assessment (Stenton & Elphinstone-Davis, 2017). Otherwise, isolating eggs facilitates replication whilst better standardising against extraneous factors. *L. stagnalis* eggs can exhibit variability in developmental time dependent on their positioning within an egg clutch, with eggs located further from the periphery being slower to develop – likely resultant of competition for oxygen diffusing through the egg clutch matrix (Byrne et al., 2009). Individualised eggs are also not subject to declines in environmental conditions resultant of conspecific mortalities, which can lead to cascade failures of entire clutches (personal observation). Resultantly, excising individual eggs from the egg clutches is not considered to be detrimental to the study results despite reducing environmental realism. Rather, it enables better

standardisation, enabling a more robust ability to assess potential vulnerability to select drivers.

Whilst only sound was a statistically significant main-factor driver of failed hatching, the lack of statistical significance of calcium (and possibly the interaction of these factors) most likely indicates that the level of replication used in the present study was suboptimal, given the comparatively low incidence of failed development. Statistical significance notwithstanding, data suggest that under ambient sound playbacks high calcium conditions were beneficial to hatching success – somewhat contradictory to observations relating to adult fecundity. Ferry playbacks increased incidences of failed hatching in both low and high calcium treatments, although the effects were notably more detrimental in the high calcium treatment – both relatively to ambient playbacks and more generally – leading to a large increase in terminal malformations. In the combination of 100 mg L^{-1} calcium and ferry playbacks, the fact that most events resulting in hatching failure occurred during the morula phase also suggest that any stress imposed by sound exposure is independent of sensor perception, as the central nervous system and sensory organs are either be absent or underdeveloped at this development stage (Nagy & Elekes, 2000). Furthermore, the elevated incidence of non-hatching being resultant of a higher incidence of malformation suggests that sound was contributing to disruptions at a cellular level – possibly in the form of DNA damage as observed in blue mussels (*Mytilus edulis*) exposed to ship playbacks (Wale et al., 2019). Mechanistically, relative to hatching failure rates from the low calcium treatments, data indicate a synergistic interaction between ferry playbacks and high calcium concentrations – however in the absence of a true, definitive control for calcium, this assertion cannot be verified.

Developmental duration of successful embryos was broadly consistent across treatments groups, and not significantly affected by either sound or calcium. Newly hatched snails in the high calcium treatment were nonetheless both significantly smaller and had significantly higher heart rates than those in the low calcium treatment. Taking heart rate as a predictor of standard metabolic rate (SMR), as observed in the pulmonate gastropod *Cornu aspersum* (Bruning et al., 2013), data suggest that *L. stagnalis* in the 100 mg L^{-1} calcium treatments may have had intrinsically higher energetic demands. The smaller hatching size of snails in the high calcium treatment would corroborate this assertion, given that in the absence of external energy resources and assuming comparable nutrient provisioning of eggs allocated to each treatment, somatic growth would be constrained by energy availability. Unfortunately, the small size of newly hatched snails precluded taking accurate or precise measurements of tissue mass which would add surety to this hypothesis. Similarly, the limitations of the study design meant assessing SMR though more conclusive means such as respirometry was not practicable. Analysis highlighted that whilst heart rates of embryos originating from different egg clutches were consistent, significant differences in developmental time and shell length upon hatching were present. This variation is likely attributable to differences in parental nutrient provisioning given genetic factors are assumed to be minimal within the isogenic population used. In any case, potential variation between egg clutches was accounted for in the experimental allocation, and thus should have no bearing on the embryo assay results. Furthermore, comparisons and inferences between the two experimental assays are considered acceptable given that the isogenic experimental populations can be assumed to be genetically similar despite a generational gap.

4.5.4 Synthesis

Observations from both the fecundity and embryo assays showing the high calcium treatment to be on balance more detrimental to *L. stagnalis* compared to the low calcium treatment were unexpected given the species' acknowledged nature as a calciphile (Boycott, 1934). Cutaneous absorption is the predominant uptake pathway of calcium in *L. stagnalis* (van der Borght & van Puymbroeck, 1966). The 20 mg L^{-1} calcium content of the low treatment was conceived as the limit below which the electrochemical calcium gradient between the haemolymph and environment becomes insufficient to allow passive absorption. The chosen 20 mg l^{-1} is also the limit beyond which growth and survival of *L. stagnalis* would be highly detrimented (Greenaway, 1971). Although calcium uptake in the high calcium treatment would not be limited, it is possible that the concentration of calcium sulphate utilised in this study is approaching excessive levels for the population, and thus placing an energetic burden on snails to maintain a homeostatic calcium concentration below that of the environment. High energetic burdens during development have been a proposed cause of non-hatching of *L. stagnalis* embryos otherwise showing normal development (Vaughn, 1953), supporting evidence that otherwise unfavourable conditions can outweigh the direct benefits of calcium availability (Boycott, 1934). The importance of calcium provisioning to *Lymnaea spp.* eggs and its utilisation during embryonic development, including biological mechanisms, have been well documented (Ebanks et al., 2010b, 2010a; H. Piggott & Dussart, 1995b). More recently transgenerational effects evidenced that parental exposure to adverse chemical environmental conditions can negatively affect juvenile success (Reátegui-Zirena et al., 2017). Similarly, Leicht and Seppälä (2019) showed that adult *L. stagnalis* reared at higher temperatures produced smaller eggs which developed faster, had greater hatching

success, but lower long-term survival than those laid at lower temperatures. It is uncertain whether faster development in Leicht and Seppälä (2019) was purely caused by temperature, but observations of smaller hatching size under more challenging conditions are consistent with those of the high calcium treatment of the present study. It is possible that more rapid development at the expense of hatching size is a plasticity response to an adverse environment, where there is potential advantage in becoming freely motile, and thus being able to attempt seeking more favourable conditions earlier. It also highlights that higher hatching success may not necessarily translate into better long-term survival. Likewise, it is uncertain what impact higher hatching success is likely to have on long-term population stability. A multiple generation study of *Daphnia magna* exposed to ferry playbacks revealed that reduced brood sizes in F_1 generations did not persist across subsequent generations (Bolger, 2022), implying the potential of populations to acclimate to long-term sound exposures.

4.6 Conclusion

Anthropogenic sound exposure alone had no effect on *L. stagnalis* fecundity, and whilst it did result in a statistically significant reduction of hatching success, hatch success remained over 80%. In comparison, waterborne calcium content had a marked negative impact on fecundity rates which could translate into ecologically significant differences in population sizes – albeit results from this study directly contradict those of expected ecological understanding in this species – with higher calcium found to be more detrimental than low calcium. This highlights the complexities of laboratory studies, and in mimicking realistic environmental conditions even when adhering to accepted husbandry practices. In contrast, accepting evidence that the high calcium

treatments were the more stressful treatments to the experimental population in this study, the results conform with those of studies addressing known stressors. The observation from the present study that fecundity, in response to the combination of sound playbacks and calcium, is being maintained through life-history plasticity, indicates that hatching success may not equate to long-term health, and unknown trans- and/or multigenerational impacts complicate predictions of potential ecological impacts. More pertinently, the responses to the two calcium treatments used being entirely contradictory to those expected arguably reframe the experiment and warns of the perils of experimental designs featuring a limited number of treatments over a driver with a continuous range.

Chapter 5

Effects of Ferry and Pile Driving Playbacks on the Fertilisation and Immediate Development of the Pacific Oyster, *Magallana gigas*, at Different Salinities

5.1 Abstract

Variation in environmental factors is intrinsic of all ecosystems but is epitomised by intertidal zones and tidal estuaries. Though the organisms living in such environments are well-adapted to tolerate these changes, different environmental conditions at any given time may nonetheless represent substantially different challenges to these organisms – especially to events such as spawning and fertilisation which can occur rapidly. Similarly, these developmental stages represent potential bottlenecks to population proliferation, and thus may be more susceptible to acute, transient environmental drivers including anthropogenic sound. To address this potential, gametes of the Pacific oyster (*Magallana gigas*) were exposed to either ferry sound playback or piling playbacks in a variety of acute 1-hour exposure scenarios across a range of environmentally and ecologically relevant salinities to ascertain the potential effects on fertilisation and 48-hour developmental success. Salinity was a consistent significant driver of rates of fertilisation, normality of development, and failed development. Exposure to non-impulsive ferry sound playbacks led to a significant deviation in the predicted numerical trend of viable and failed embryos following 48-hour incubation compared to those predicted from salinity alone, though not to an extent likely to be of ecological relevance. No significant differences in any developmental outcomes resulted from fe/male gametes being exposed to sound in isolation or combination. Consequently, evidence suggests sound exposure during

spawning events presents minimal risk to the proliferation of *M. gigas*, especially in the context of other prevalent environmental factors.

5.2 Introduction

All aquatic ecosystems are subject to a degree of natural variation in environmental conditions, for example in temperature, salinity, or nutrient availability. Likewise, distributions and geographical limits of organisms are generally dictated by their varying tolerances to such environmental factors (Whalen, 2017). But whilst some ecosystems experience changes in environmental factors gradually (e.g. seasonal changes), or sporadically (e.g. weather events), others such as intertidal zones and tidal estuaries are defined by such changes – experiencing drastic changes both regularly and rapidly (Tomanek & Helmuth, 2002). Resultantly, organisms living in such environments have adapted exceptional biological tolerances for coping with such variations. These large environmental gradients nonetheless pose varying challenges to such organisms – be it on a temporal scale for those living under highly changeable conditions, or geographically between populations living under more stable conditions across these gradients (Sarkar et al., 2019; Lauchlan and Nagelkerken, 2020; Roy et al., 1998).

The Pacific oyster, *Magallana gigas* is typically considered a brackish species with an optimum salinity range between 20-30 ppt (Gosling, 2015; Nell & Holliday, 1988), though populations with tolerances to average salinities between 15 ppt and 50 ppt have been reported (Goulletquer & Héral, 1991). The species' wide environmental tolerance has seen it gain both commercial and food-security value (Willer & Aldridge, 2020), but also growing notoriety as an invasive species (Faust et al., 2017; King et al.,

2021). Resultantly, many studies have investigated propagation and survival of oyster larvae and spat with regards to salinity and other environmental factors, yet far fewer have looked directly at fertilisation rate.

Salinity is known to be capable of influencing the fertilisation and development of aquatic invertebrates. albeit with some uncertainties and inconsistencies in the predominating mechanisms between species, seemingly relating to their respective salinity tolerances. Some species adapt their reproductive strategies depending on salinity with *Gammarus lawrencianus* known to produce fewer but larger eggs in decreased salinities (Steele & Steele, 1991), and the hydrozoan *Eleutheria dichotoma* prioritising asexual reproduction in hypersaline conditions reducing planulae survival (Dańko et al., 2020). Fertilisation rates of the marine coral, *Acropora millepora* declined in a dose-dependent manner with decreasing salinity, with salinities < 30 ‰ leading to 100% occurrence of developmental abnormalities, and fertilisation completely inhibited at salinities < 28 ‰ (Humphrey et al., 2008). Pineda et al. (2012) showed hyposaline conditions led to significant reductions in both fertilisation and larval developmental success of two ascidian species, *Styela plicata* and *Microcosmus squamiger*, and determined that fertilisation and pre-settlement larvae were the developmental stages most sensitive to environmental drivers. In comparison, Caballes et al., (2017) found that early life stages of the crown of thorns startfish, *Acanthaster solaris*, demonstrate comparatively high tolerance to differences in salinity, though this tolerance also varies across different specific processes in their larval development.

The process of achieving gamete fusion and fertilisation is a complicated process (Siu et al., 2021) requiring multiple ordered events to occur successfully (Figure 5.1), making these stages particularly susceptible to so-called ‘bottlenecking’ events.

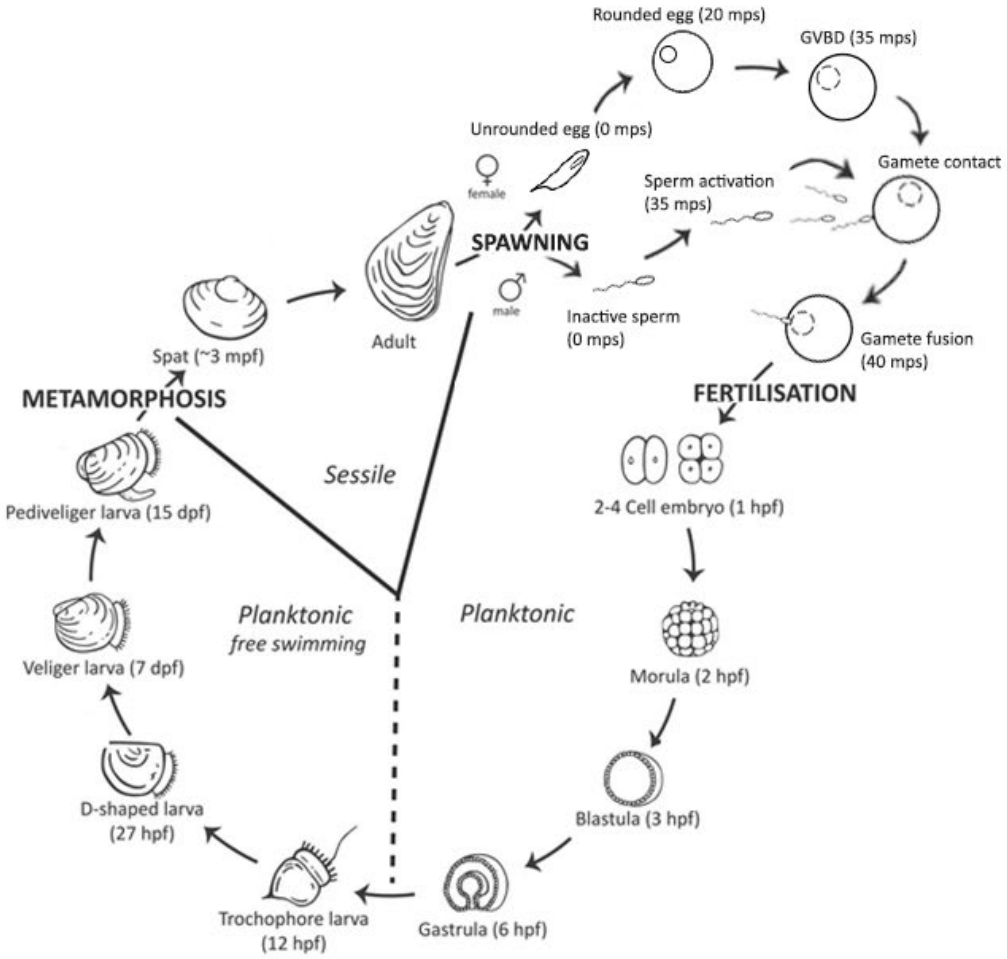

Figure 5.1: Life cycle of the Pacific oyster, Magallana gigas*. mps: minutes post spawning; hpf: hours post fertilisation; dpf: days post fertilisation; mpf: month post fertilisation. GVBD: germinal vesicle breakdown. Times are approximate and assume ideal environmental conditions. Modified from* Vogeler et al. (2016).

Bottlenecking events are occurrences that drastically reduce the effective size of a population, be it directly or indirectly (Armstrong, 2008). Bottlenecking can occur directly (such as a failure or reduction in reproductive success), but can also occur indirectly via a genetic component, where environmental conditions apply strong selection pressure for specific alleles, ultimately leading to reductions in genetic diversity which increase potential long-term susceptibility to other drivers (Bijlsma & Loeschcke, 2012).The fertilisation dynamics of species undergoing external fertilisation including broadcast spawners, are at particular risk in this regard given the lack of environmental control and/or stability in comparison to species that employ

reproductive strategies such as internal fertilisation and/or parental care (Wootton & Smith, 2014). Additionally, unlike larval development which can occur over several weeks and can be impacted upon by many variables during this time, fertilisation typically occurs within minutes of gamete contact (Babcock & Keesing, 1999; Levitan, 1991; Song et al., 2009). Thus, gametes of broadcast spawning species are particularly susceptible to selection pressures posed by transient environmental conditions which may contribute to more widely to population propagation.

Comparatively little is known about how sound exposure affects these initial early life stage developmental stages, with no known studies having yet addressed potential impacts on fertilisation rates. Whilst gamete fusion and development can be considered a predominantly biochemical process, and may therefore be susceptible to biochemical responses to sound exposure widely evidenced more generally in aquatic organisms (Stenton et al., 2022; Vazzana et al., 2020; Wale et al., 2019), there is also the biomechanical component of gametes successfully colliding. Sound exposure is known to be capable of causing physical damage to structures such as sensory hair cells (André et al., 2011; Day et al., 2016; Solé et al., 2016, 2013; Wagner and Shin, 2019) which share comparable size to sperm flagella. Thus, the biomechanical aspect of fertilisation may be susceptible to sound via a reduction in sperm motility relating to damage to the sperm cell flagella given. Similarly, it could be that additional particle motion resultant of anthropogenic sound otherwise disrupts the gamete binding following collision prior to successful fusion. Correspondingly, the impacts of sound on fertilisation and immediate developmental success are also completely unknown, yet worthy of consideration given drivers including temperature, ocean acidification, and waterborne metal concentration have been shown to interact with salinity in these contexts (Falkenberg et al., 2019; Pineda et al., 2012; Thiyagarajan et al., 2003).

To address knowledge gaps pertaining to sound exposure and its impacts on gamete fusion and investigate any additional variation contributable to different salinities, *M. gigas* gametes were exposed to acute one-hour exposures to one of two different anthropogenic sound (pile driving and passenger ferry) playbacks across a range of environmentally realistic salinities. These sound sources were chosen to reflect those which may occur in environments of different salinities, and to more broadly investigate potential differences in effects between impulsive (piling) and non-impulsive (ferry) sounds using a mechanistic approach to address the following key research questions:

- Does the combination of acute 1-hour anthropogenic sound impact upon the fertilisation and 48-hour developmental success of *M. gigas*, and how does this vary across an environmentally and ecologically realistic salinity gradient?
- How does the timing of anthropogenic sound exposure factor into fertilisation and developmental success rates, and how does this vary across an environmentally and ecologically realistic salinity gradient?
- How does fertilisation and developmental success vary between fe/male gametes separately exposed to anthropogenic sound and exposed to sound in combination with one another, and how do any such differences vary across an environmentally and ecologically realistic salinity gradient?

These research questions were selected not only to aid mechanistic understanding as to whether fe/male gametes are differently susceptible to anthropogenic sound, but also represent plausible ecological exposures which may arise from a desynchronised spawning of oysters. The chosen approach does not address related ecologically relevant concerns whether exposure of adult oysters impacts upon spawning

behaviour or gamete quality upon spawning, but provides additional experimental control and robustness benefitting the chosen scope.

5.3 Methods

5.3.1 Animal Husbandry

Ten mature *M. gigas* (five male, five female) were imported from Guernsey Sea Farms Ltd, Guernsey, UK, in full compliance with legislation and guidelines outlined by Marine Scotland's Fish Health Inspectorate. Supplied oysters were pre-assessed to ensure health (notifiable disease free), sexual maturity and reproductively ripe condition.

Upon arrival, oysters were individually wrapped in tissue dampened in 35 ppt artificial sea water (ASW) (Instant Ocean® Sea Salt reconstituted in UV-sterilised reverse osmosis (RO) water, aerated and aged for 24 hours), placed in separate 1-litre borosilicate beakers, and held 'dry' overnight in a Binder KBW Growth Chamber at 8 ± 1 °C under dark conditions to discourage spawning (Environment Agency, 2007).

Following initial overnight holding, all husbandry occurred at a water temperature of 20 ± 1 °C and under a 16:8 hours day:night cycle (IKS AquaStar Simmod 0-10 volt: colour temperature 6500 K; intensity 250-500 Lux). Prior to gamete stripping, mature oysters were revitalised individually via submersion in approximately 1 litre of 35 ppt ASW at 20 °C for one hour. Stripped gametes were activated for 45 minutes in 1 litre ASW at the intended experimental salinity (detailed in Section 5.3.4) at a density of 200 eggs ml^{-1} and 20,000 sperm ml^{-1} respectively. During exposures and subsequent observations, mixed gametes were held in sterile Nunc™ non-treated six-well plates, each well containing 5 ml of ASW at a desired experimental salinity containing 100

eggs ml^{-1} and 10,000 sperm ml^{-1}. All glassware and equipment utilised for the extraction and culturing of gametes was sterilised via autoclaving prior to use.

5.3.2 Experimental system

Two identical exposure systems were created each facilitating either the ferry or piling sound treatment. Each exposure system comprised a Crowson Technology Controlled Vibration™ EDP-2424 24 Inch Platform (frequency response: 5-2,000 Hz) coupled to a pair of Crown DSi 1000 amplifiers with signal input via an M-Audio M-Track QUAD Audio Interface. Each Vibration platform was placed on a separate rigid steel-framed table isolated from the external vibration using 25mm rubber sheeting. Each platform held 24 six-well plates acting as the exposure vessels. A third system, omitting the vibration platform, was used as an ambient sound control, enabling all sound exposure treatments to be run concurrently under otherwise comparable environmental conditions.

5.3.3 Sound exposures

Exposure to pile-driving and ferry sounds were conducted using playbacks of field-derived sound recordings. The ambient sound treatment involved no sound playback and was otherwise being conducted at the base sound level of the laboratory. All sound files for experimental playback were compiled in Audacity 2.2.2 and output as 24-bit WAV files.

Pile-driving recordings were made available by Rick Bruintjes (Defra, UK), Sophie Nedelec (University of Exeter, UK) and Irene Voellmy (University of Berne, CH). Piling playback tracks were compiled from multiple recorded strikes of a 1.2 m diameter monopole being driven approximately 25 m into the seabed in a water depth of 6.5 m. This piling had been recorded at distances between 87 and 200 meters from the sound source using a Hi Tech Inc. HTI-99HF hydrophone with inbuilt preamplifier

(manufacturer calibrated sensitivity -204 dB re: 1 V mPa^{-1}, 20– 125 000 Hz frequency range) and a RTsys EASDA data logger using a 44.1 kHz sampling rate. The piling playback consisted of a single track of 1:00 minute duration with a 'strike-interval' of 1.5 seconds looped continuously for the 1-hour duration of each sound exposure. Ferry recordings were compiled from 10-minute recordings capturing the arrival and departure of the Bowness ferry at Lake Windermere, UK, recorded in November 2014 by Bolgan et al. (2016). Recordings were made using an Aquarian H2a hydrophone (sensitivity -180 dB re 1V/µPa; frequency response 10 Hz-100 KHz) calibrated using a sine wave of known voltage (100 mV rms @1 kHz; Welleman Instruments HPG1) connected to a Zoom H1 16-bit recorder using a 44.1 kHz sampling rate. The ferry playback consisted of a single track of 1:00 minute duration capturing the arrival and departure of a ferry looped continuously for the 1-hour duration of each sound exposure. Received sound pressure levels (SPL) of both piling and ferry playbacks were targeted at and equivalent root mean square SPL (SPL_{RMS}) of 140 dB re: 1 µPa. These sound levels are consistent with environmentally and ecologically realistic potential sound exposures of both impact pile driving (Han & Choi, 2022; Martin & Barclay, 2019) and watercraft (Barlett & Wilson, 2002; Parsons et al., 2021) respectively. The ambient treatment occurred at the approximate noise-floor of the laboratory, 120 dB_{RMS} re: 1 µPa.

Dimensions of the wells within each exposure vessel prohibited direct measurement of received sound exposure levels *in situ.* Thus, measurements were conducted in a 100 ml 'proxy vessel' large enough to enable unimpeded suspension and full submergence of the sensors. Sound levels were then measured at the centre-point of each multi-well plate location in each experimental system using a manufacturer-calibrated HiTech HTI-94-MIN hydrophone (sensitivity: -165 dB re 1V mPa^{-1}) coupled with a calibrated

Roland R-26 2-channel Portable Recorder. Sound particle motion (PM) was measured using a calibrated custom-built triaxial accelerometer (STMicroelectronics LIS344ALH) potted within epoxy resin and suspended within the exposure vessels using 1.0 mm elastic cord (Wale, 2017).

5.3.4 Salinity exposures

All equipment and exposure vessels were thoroughly cleaned and sterilised via autoclaving prior to use. For experimental test media, nominal salinities of 15 ppt, 19 ppt, 23 ppt, 27 ppt, 31 ppt, 35 ppt were targeted, representing environmentally and ecologically relevant salinities for *M. gigas* (Gosling, 2015; Goulletquer & Héral, 1991; Nell & Holliday, 1988)

Separate one litre stock solutions of each salinity were made for both egg and sperm solutions. Each salinity was achieved by fully dissolving Instant Ocean™ Sea Salt into RO water at the concentrations stated in Table 5.1. Solutions were vigorously aerated for 24 hours prior to use to promote equilibrium of the solutions and consistent oxygenation between treatments. Prior to inoculation with gametes, salinity, temperature, and dissolved oxygen of each solution was measured using a calibrated WTW Multi 3430 portable multi-parameter probe.

Table 5.1: Concentration of Instant Ocean Sea Salt dissolved in RO water to form experimental salinities

Nominal salinity (ppt)	Instant Ocean Sea Salt (g/L)
15	18.125
19	23.675
23	28.725
27	34.275
31	39.075
35	43.375

5.3.5 Experimental exposure scenarios

Five experimental exposure scenarios (ES) were devised to address different exposure dynamics as detailed below. In all cases, upon completion of the prescribed exposure phases, replicates were histologically fixed by addition of 100 µl of 20% formalin buffered as described by Leverett and Thain (2013):

ES1) Simultaneous exposure of mixed eggs and sperm to experimental sound treatments for 1 hour, followed by 47-hour incubation under ambient sound conditions.

ES2) Exposure of eggs to experimental sound treatments for 1 hour, followed by inoculation with naïve sperm, and subsequent 47-hour incubation under ambient sound conditions.

ES3) Exposure of sperm to experimental sound treatments for 1 hour, followed by inoculation with naïve eggs, and subsequent 47-hour incubation under ambient sound conditions.

ES4) Simultaneous exposure of mixed eggs and sperm to experimental sound treatments for 1 hour followed by immediate fixation.

ES5) Simultaneous exposure of mixed eggs and sperm under ambient sound conditions followed by 47-hour incubation under ambient sound conditions.

A total of 90 six-well plates were set up, equally split between the five exposure scenarios and three sound treatments. Each plate contained one replicate of each of the six salinity treatment levels (n = 6 total replicates per treatment group), with locations of salinity treatments within each plate being randomised to control for random variation in sound and environmental factors. Whilst the experimental system theoretically allowed for concurrent running of all exposure scenarios, the study design

and allocation times prohibited this possibility. Rather, exposure scenarios were set up sequentially to minimise any potential time-related variation in gamete quality between replicates within any given scenario. Multi-well plates belonging to each of ES1-4 were then allocated to one quadrant of vibration platform for the duration of their sound exposure (Figure 5.2). This facilitated consistent sound exposure between exposure scenarios, whilst allowing multiple exposure scenarios to run in parallel where permittable.

Each well within multi-well plates was allocated 2.5 ml of both egg and sperm solutions of a desired salinity. When combined, this equated to a final working volume for gamete fertilisation of 5 ml, with an egg density of 100 cells ml^{-1} and an egg:sperm ratio of 1:100.

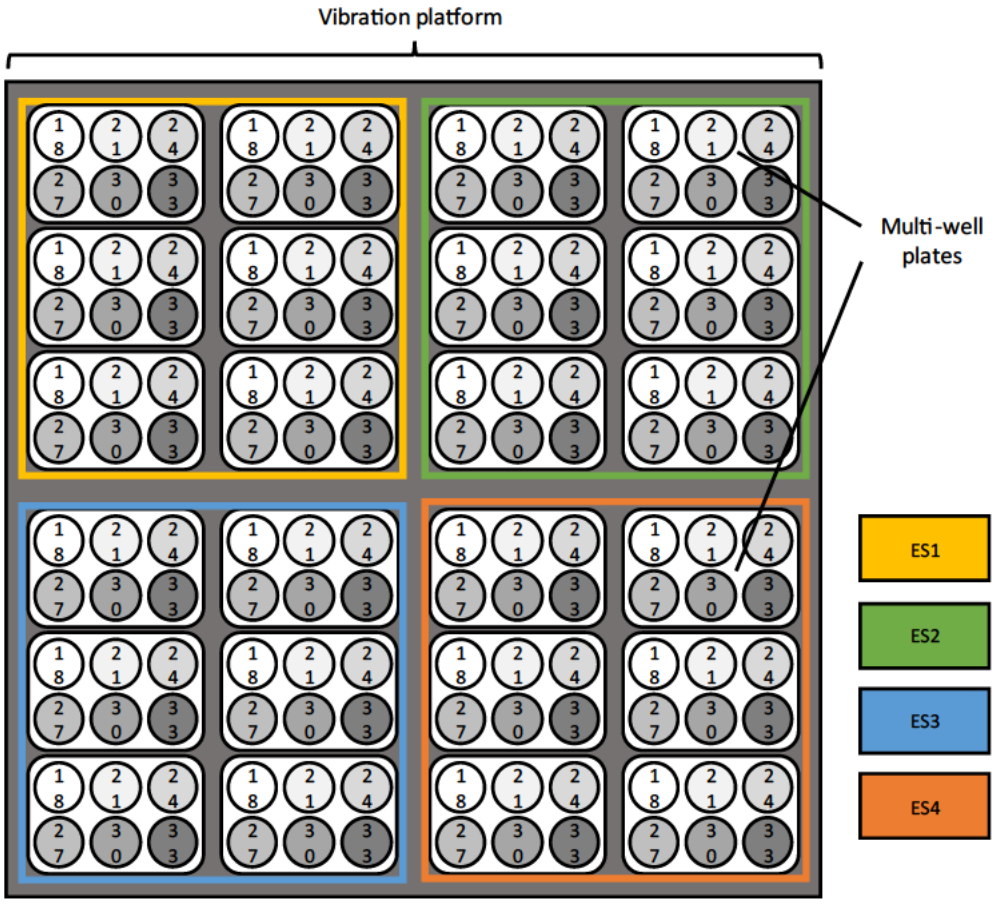

Figure 5.2: Schematic demonstrating distribution of multi-well plates on vibration platforms. *Coloured bounding boxes show quadrants of the vibration platform devoted to each exposure scenario. Values within each well of multi-well plates represent salinity treatments (salinity in ppt) within plates, but are*

not representative of actual wells to which each treatment was allocated, which was randomised within plates.

For ES1 and ES4 which required simultaneous exposure of mixed eggs and sperm to experimental sound treatments, empty multi-well plates were placed on the active vibration platforms and each well of each plate in sequence was simultaneously allocated with both eggs and sperm, ensuring that any fertilisation occurred whilst being exposed to the experimental sound, but minimising any pre-exposure of either fe/male gametes. Upon completion of the designated sound exposures, ES1 plates assigned to the piling and ferry sound treatments were removed from their respective vibration platforms and transferred to the location of ambient sound treatment. Plates for ES4 meanwhile were fixed *in-situ*.

For ES2 and ES3 where single-sex gametes were pre-exposed to sound, plates were fully allocated solely with eggs or sperm respectively under ambient conditions, and then placed on the vibration platform. Immediately following the designated sound exposures duration, plates were removed from their respective vibration platform, and the opposing gametes added to their respective wells. Plates were then transferred to the location of ambient sound treatment.

Upon completion of experimental sound exposures (ES1-4), the vibration platforms were switched off. Plates for ES5 were when filled with appropriate egg suspensions, followed by the corresponding sperm under ambient conditions. These plates were then evenly distributed between the locations of the ferry, piling, and ambient platforms respectively to capture and control for any environmental variation. Correspondingly, plates from ES1-4 were also randomly reallocated between the now inactive platforms for better account for potential environmental variation during incubation.

5.3.6 Observations of gametes and larvae

Fixed samples were examined with aid of an inverted stereoscopic microscope. For each replicate 100 randomly selected cells/larvae were visually observed, with prevalence counts recorded according to four criteria: 1) Unfertilised, 2) Normal, 3) Abnormal, 4) Failed. Unfertilised cells and ab/normal development were determined using criteria and examples outlined by Leverett and Thain (2013), Gamain et al. (2016) and Qin et al. (2018). Failed cells/larvae were defined as those having seemingly lost all cellular integrity (Figure 5.3).

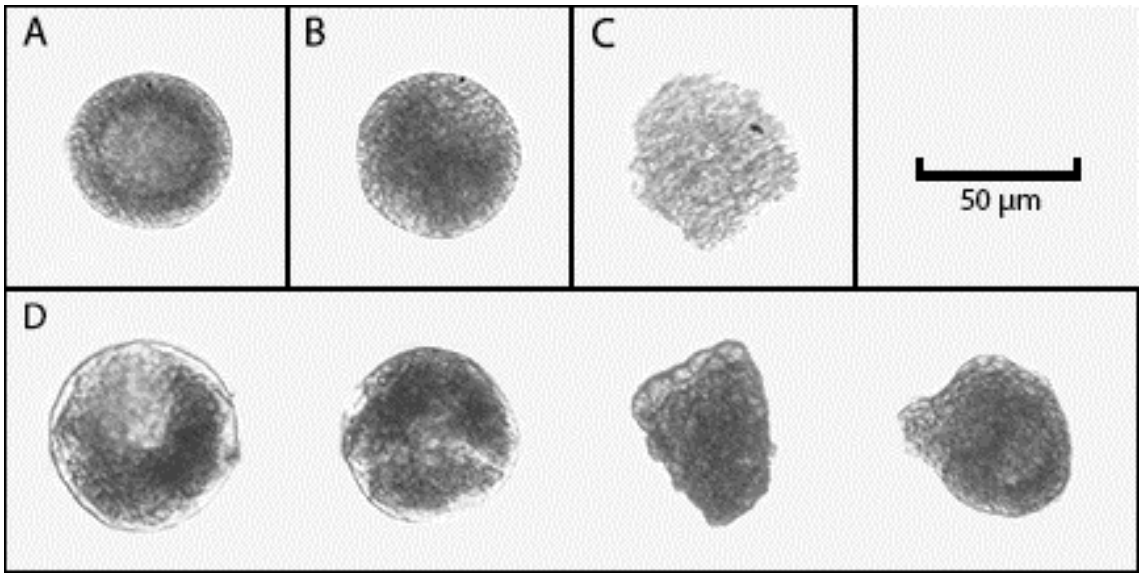

Figure 5.3: Magallana gigas larvae following 48h post-fertilisation. *A) Unfertilised egg. B) Normally developing larva. C) Failed larva. D) Larvae showing various structural abnormalities.*

For analytical purposes, additional data values for each replicate were calculated using raw cell/larva counts:

- Fertilisation rate – A measure of fertilisation based upon observed counts of Unfertilised cells:

$$Fertilisation\ rate\ (\%) = 100 - Unfertilsed\ \text{cells}$$

- Viable cells – A count of fertilised cells showing active development and not having evidentially failed:

$$Viable\ cells = Normal\ cells + Abnormal\ cells$$
$$= 100 - Unfertilised\ cells - Failed\ cells$$

- Normal:Abnormal – The proportion of Viable cells showing no visual abnormalities:

$$Normal{:}\,Abnormal = Normal\ cells / Viable\ cells$$

In all instances, observations were effectively conducted blind. Observations were conducted according to well-defined parameters by a single, experienced observer, and thus the impacts of any subjectivity or observational bias are considered negligible.

5.3.7 Statistical analyses

Statistical analyses were performed using R Statistical Software (version 4.1.0). Analyses were selected on the basis of data trends and distributions. Fertilisation rate, Viable larvae, Failed cells/larvae and Normal:Abnormal proportion were statistically assessed. All data were analysed using generalised additive models (GAM) assuming gaussian data distribution, fit using restricted maximum likelihood (REML). Effects of salinity were modelled using smooths limited to four knots to avoid over-fitting. Model residuals were checked visually and found to conform to normal distributions, but model assumptions pertaining to smooth fittings were violated. These violations seemingly resulted from the limited data availability and constrainment of the model fit parameters, but were considered to be a statistical artefact given the aforementioned residual distributions and corroboration between raw data and modelled outcomes.

Variation in gamete/larva count data contributable to environmental variation within the laboratory was modelled using data from ES5. Location of the exposure systems

within the room (Room location), and the location of plates on each the vibration platforms (Plate location) and interaction between these factors were contrasted parametrically against the salinity smooth.

Variation contributable to the age of the gamete suspensions was modelled using data from the ambient sound treatments from ES1 and ES5. Gamete age was contrasted parametrically, with separate smooths calculated for gametes from each exposure.

Comparison between developmental duration was conducted using data from ES1 and ES4. Separate smooths were calculated for each sound treatment, and sound and developmental duration also contrasted parametrically.

Data from ES1, ES2 and ES3 were combined into a single model comparing the effects of gamete sound exposure scenario. Separate smooths were calculated for each sound treatment and accounting for gamete age. Effects of sound and exposure scenarios were additionally contrasted parametrically.

5.4 Results

5.4.1 Sound exposures

Despite efforts to match sound pressure exposures between the ferry and piling sound treatments, there was a discrepancy of approximately 2.5 dB_{RMS} in the SPL between received playbacks of experimental recordings, leading to an approximate 6 dB re: 1 μPa^2 s discrepancy in SEL_{cum} between these treatments. In contrast, peak pressure levels in these two sound treatments were highly comparable. A discrepancy in the PM_{RMS} was also evident, being persistent with regards to peak particle acceleration. (Table 5.2).

Table 5.2: Received experimental playback sound levels in vicinity of 6-well plates.

Sound Treatment	Mean (± SD) received sound					
	Pressure			Particle motion		
	RMS (dB re: 1 µPa)	Peak (dB re: 1 µPa)	SEL_{cum} (dB re: 1 uPa^2 s)	RMS (dB re: 1 µm s^{-2})	Peak (dB re: 1 µm s^{-2})	SEL_{cum} (dB re: 1 (um s^{-2})^2 s)
Ambient	119.6 ± 0.2	-	195.0 ± 0.2	56.7 ± 0.1	-	132.1 ± 0.1
Ferry	140.1 ± 2.7	157.3 ± 3.2	215.5 ± 2.7	70.7 + 1.8	86.8 ± 2.1	146.1 ± 1.8
Piling	137.6 ± 3.2	157.1 + 3.4	221.0 ± 3.2	65.1 ± 1.5	83.7 ± 2.0	140.5 ± 1.5

Power spectral density (PSD) plots show that in both active sound treatments, SPLs were indistinguishable from the ambient sound of the laboratory above 4000 Hz – the harmonic frequency of the uppermost response range of the vibration platforms. Piling playbacks resulted in greater levels sub-150 Hz sound than ferry playbacks, but ferry playbacks displayed higher prevalence of sound frequencies exceeding 400 Hz.

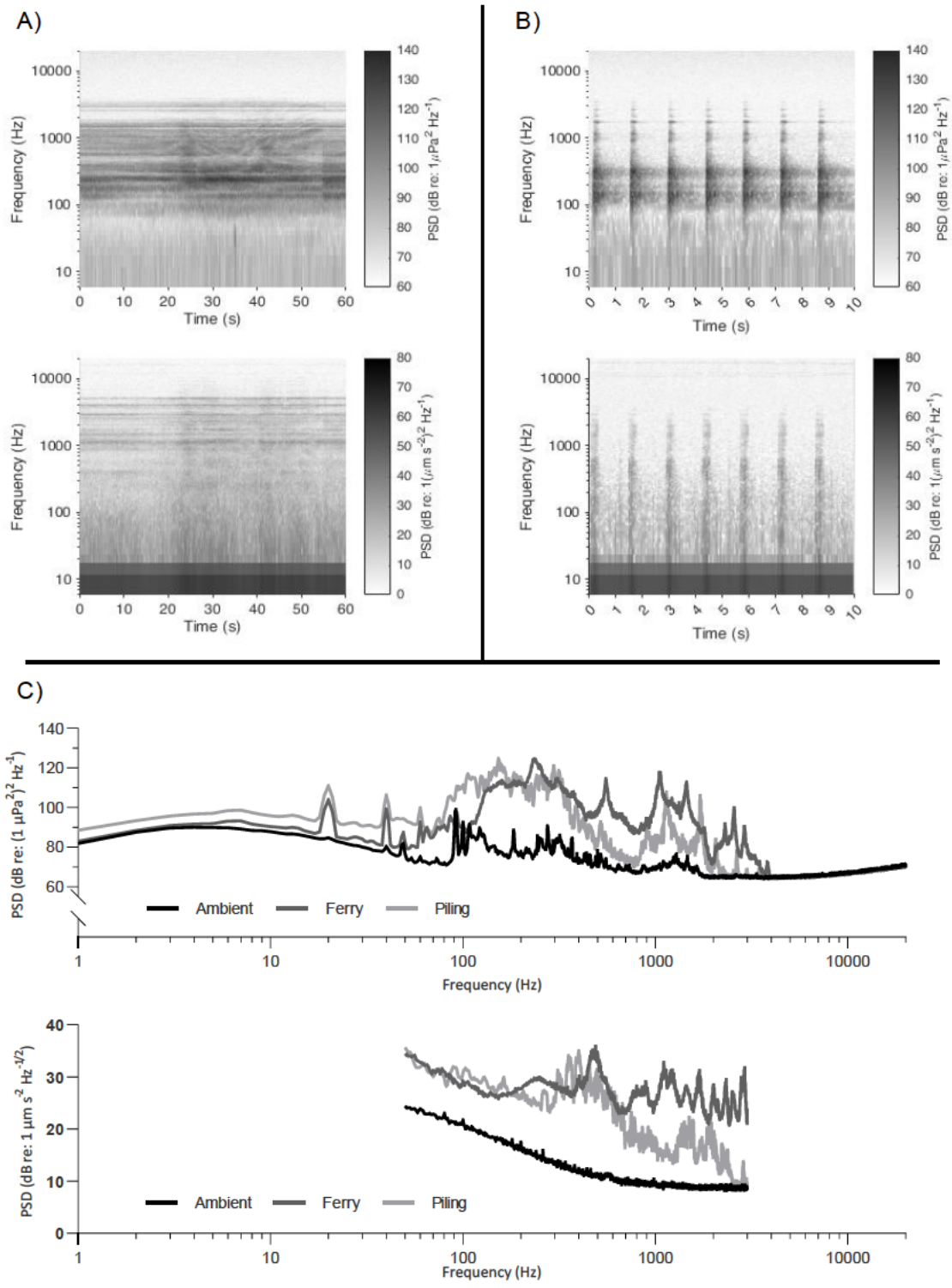

Figure 5.4: Spectrogram of received sound playback. *A) ferry (Top: pressure. Bottom: particle motion); B) Piling (Top: pressure. Bottom: particle motion). C) Comparison of root-mean-square PSD of received sound levels in each experimental treatment (Top: pressure. Bottom: particle motion). Spectrograms and PSD calculated using 0.1 second windows, 50 percent overlap. Hanning and Hamming windows utilised for pressure and particle motion calculations respectively.*

Particle motion in ferry and piling playbacks treatments was broadly comparable at frequencies below 700 Hz, although ferry playbacks contained more particle motion above 700 Hz (Figure 5.4), contributing towards the 6 dB re: 1 $(\mu m\ s^{-2})^2$ s difference in SEL_{cum} between these sound treatments.

5.4.2 Salinity exposures

Measured salinities were consistent with desired nominal concentrations, and dissolved oxygen, pH and temperature highly consistent across treatments (Table 5.3Table 4.3) and with those recommended for *M. gigas* larval bioassays (Leverett & Thain, 2013; Song et al., 2009).

Table 5.3: Water quality traits of experimental media.

Nominal salinity (ppt)	Measured Salinity (ppt)	Dissolved oxygen (%)	pH	Temperature (°C)
15 ppt	15.1 ppt	97.6 %	8.19	20.0 °C
19 ppt	19.0 ppt	97.8 %	8.20	20.0 °C
23 ppt	23.0 ppt	98.1 %	8.20	20.0 °C
27 ppt	27.1 ppt	97.7 %	8.19	20.0 °C
31 ppt	31.1 ppt	97.8 %	8.20	20.0 °C
35 ppt	35.0 ppt	98.1 %	8.21	20.0 °C

Salinity was a highly significant driver of every observed metric across all exposures and comparisons. To avoid repetition, further discussion of statistical significance shall be restricted to that of other drivers, and statistical significance of salinity otherwise accepted.

5.4.3 Spatial distribution control (ES5)

Spatial distribution with regards to both positioning of replicate plates upon each vibration platform and the locations of the vibration platforms within the laboratory had no significant effect on any of the assessed developmental criteria in the absence of experimentally introduced sound (Table 5.4). Fertilisation data were excluded from

consideration as the metric was deemed unreliuable in this context as detailed in Section 5.4.5.

Table 5.4: GAM results of effect of spatial variation within the laboratory on M. gigas larval developmetnal status following 48-hour incubation in the absence of experimentlly introduced sound. *Fertilisation data were excluded from consideration as the metric was deemed unreliuable in this context as detailed in Section 5.4.5 Bold values signify statistical significance $p < 0.05$*

	Parametric terms			Smooth terms			
	df	F	p	edf	Ref.df	F	p
Viable							
Plate location	5	0.389	0.855				
Room location	2	1.090	0.341				
Plate:Room locations	10	0.661	0.757				
s(Salinity)				2.924	2.995	307.1	**<0.001**
Failed							
Plate location	5	0.290	0.917				
Room location	2	0.635	0.532				
Plate:Room locations	10	0.550	0.850				
s(Salinity)				2.815	2.973	127.5	**<0.001**
Normal:Abnormal							
Plate location	5	0.274	0.926				
Room location	2	1.613	0.205				
Plate:Room locations	10	0.902	0.535				
s(Salinity)				2.777	2.962	12.64	**<0.001**

5.4.4 Temporal effects (ES1 and ES5)

The age of the gamete solution used for the experimental series resulted in a significant (Table 5.5) departure in the spline of Normal:Abnormal ratio of larvae following 48-hour incubation in the absence of experimentally introduced sound from that predicted by salinity alone. Older gametes displayed improved rates of normal development at salinities below 21 ppt relative to fresher gametes, but notably poorer rates at salinities exceeding this (Figure 5.5) – though these differences were not significant when considered parametrically (Table 5.5). Fertilisation data were excluded from consideration as the metric was deemed unreliable in this context as detailed in Section 5.4.5. Consequently, gamete age was included as a secondary modifier of splines for further analyses.

Table 5.5: GAM results showing temporal effects of gamete solution age on M. gigas larval developmetnal status following 48-hour incubation in the absence of experimentlly introduced sound. *Fertilisation data were excluded from consideration as the metric was deemed unreliuable in this context as detailed in Section 5.4.5. Bold values signify statistical significance $p < 0.05$*

	Parametric terms			Smooth terms			
	df	F	p	edf	Ref.df	F	p
Viable							
Gamete Age	1	3.467	0.067				
s(Salinity)				2.918	2.995	118.7	**<0.001**
s(Salinity):1 hr				1.001	1.002	3.281	0.074
s(Salinity):4 hr				0.000	0.000	0.005	0.999
Failed							
Gamete Age	1	2.028	0.159				
s(Salinity)				2.866	2.986	57.92	**<0.001**
s(Salinity):1 hr				1.000	1.001	0.174	0.678
s(Salinity):4 hr				0.000	0.000	0.022	0.999
Normal:Abnormal							
Gamete Age	1	0.978	0.326				
s(Salinity)				2.416	2.759	4.972	**0.004**
s(Salinity):1 hr				0.000	0.000	0.050	0.998
s(Salinity):4 hr				1.000	1.000	4.763	**0.033**

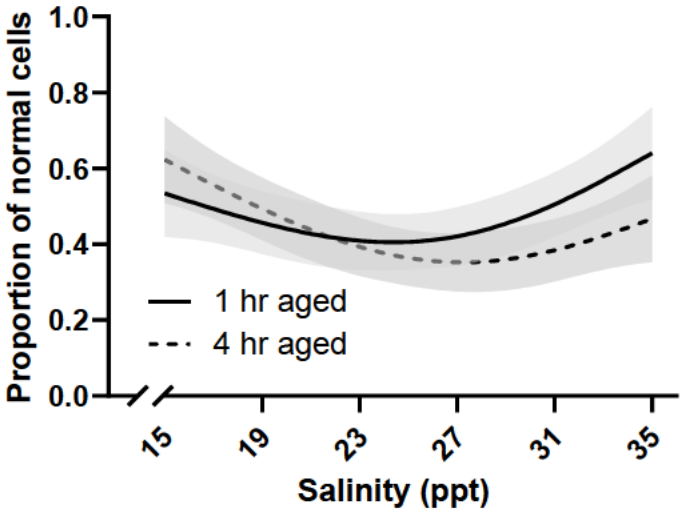

Figure 5.5: Comparison of gamete age on Normal:Abnormal ratio of larvae originating from gametes mixed and incubated for 48-hours in the absence of experimentally introduced sounds. *Lines represent mean values ± 95% CI (shaded regions).*

5.4.5 Fertilisation rate (ES4)

Sound had no significant effect on the ability of mixed gametes to fuse and successfully fertilise during the first hour of exposure (Table 5.6). An assessment of whether

maximal fertilisation potential was achieved across each salinity during the 1-hour sound exposure was not possible due to a significant increase in occurrence of Failed cells in replicates allowed to incubate to 48 hours (Table 5.7). Comparison of Viable larva counts following 1-hour incubation (directly equivalent to fertilisation rate given zero occurrence of failed cells) and 48-hour incubation (Figure 5.6) suggest that previously Unfertilised cells account for a substantial fraction of Failed cells after 48-hours. Consequently, for remaining data comparisons which were all incubated to 48-hours, fertilisation rate was deemed an unreliable metric and thus discounted from further consideration.

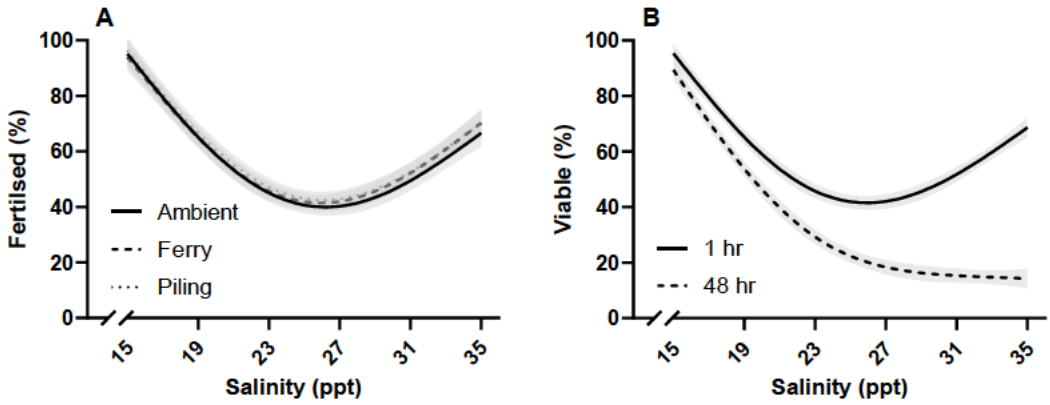

Figure 5.6: Fertilised cells vs Viable larvae. *A) fertilisation rates of mixed gametes in each sound treatment fixed immediately after 1-hour exposures. B) Comparison of the percentage of Viable larvae pooled across sound treatments after 1-hour incubation and 48-hour incubation. Lines represent mean values ± 95% CI (shaded regions).*

Table 5.6: GAM results comparing fertilisation rates of mixed gametes immediately following 1-hour exposures to experimental sound treatments. *Bold values signify statistical significance $p < 0.05$*

	Parametric terms			Smooth terms			
	df	F	p	edf	Ref.df	F	p
Fertilised							
Sound	2	0.966	0.384				

s(Salinity)	2.966	2.999	190.1	**<0.001**
s(Salinity):SoundAmbient	1.000	1.000	0.262	0.610
s(Salinity):SoundFerry	1.000	1.000	0.225	0.637
s(Salinity):SoundPiling	0.000	0.000	0.038	0.997

Table 5.7: GAM results comparing effect of incubation duration on developmetnal status of mixed M. gigas larvae following 1-hour exposure to experimentlly introduced sounds and 48-hour incubation.*Bold values signify statistical significance $p < 0.05$*

	Parametric terms			Smooth terms			
	df	F	p	edf	Ref.df	F	p
Viable							
Sound	2	0.382	0.683				
Duration	1	87.07	**<0.001**				
Sound:Duration	2	0.458	0.633				
s(Salinity)				2.948	2.998	154.7	**<0.001**
s(Salinity):SoundAmbient				1.000	1.001	0.179	0.673
s(Salinity):SoundFerry				1.000	1.001	0.775	0.380
s(Salinity):SoundPiling				0.000	0.000	0.002	0.999
Failed							
Sound	2	0.000	1.000				
Duration	1	87.37	**<0.001**				
Sound:Duration	2	0.337	0.714				
s(Salinity)				2.507	2.823	21.18	**<0.001**
s(Salinity):SoundAmbient				1.000	1.000	0.136	0.713
s(Salinity):SoundFerry				1.000	1.000	0.938	0.334
s(Salinity):SoundPiling				0.001	0.001	0.006	0.998
Normal:Abnormal							
Sound	2	0.630	0.533				
Duration	1	136.4	**<0.001**				
Sound:Duration	2	0.042	0.959				
s(Salinity)				2.504	2.821	6.495	**<0.001**
s(Salinity):SoundAmbient				1.002	1.004	0.029	0.872
s(Salinity):SoundFerry				1.000	1.000	1.134	0.288
s(Salinity):SoundPiling				0.000	0.000	0.005	0.999

5.4.6 Gamete sound exposure scenario (ES1, ES2, ES3)

No significant differences were present for any assessed metric dependent upon whether either eggs or sperm were exposed to sound in isolation, or whether they

were concurrently exposed. Likewise, no significant differences between sound treatments were present when considered parametrically. (Table 5.8)

Table 5.8: GAM results comparing exposure scenarios on developmetnal status of M. gigas larvae following 1-hour exposure to experimentlly introduced sounds and 48-hour incubation.*Bold values signify statistical significance p < 0.05*

	Parametric terms			Smooth terms			
	df	F	p	edf	Ref.df	F	p
Viable							
Sound	2	0.536	0.586				
Exposure	1	2.427	0.120				
Sound:Exposure	2	0.718	0.488				
s(Salinity)				2.979	3.000	560.2	**<0.001**
s(Salinity):SoundAmbient				1.000	1.000	0.014	0.906
s(Salinity):SoundFerry				2.001	2.002	3.178	**0.043**
s(Salinity):SoundPiling				2.000	2.000	1.103	0.333
Failed							
Sound	2	0.047	0.954				
Exposure	1	0.167	0.683				
Sound:Exposure	2	1.603	0.203				
s(Salinity)				2.885	2.983	100.7	**<0.001**
s(Salinity):SoundAmbient				1.764	1.938	1.644	0.178
s(Salinity):SoundFerry				2.000	2.001	3.974	**0.020**
s(Salinity):SoundPiling				2.000	2.000	0.444	0.642
Normal:Abnormal							
Sound	2	1.162	0.314				
Exposure	1	2.442	0.119				
Sound:Exposure	2	0.927	0.397				
s(Salinity)				2.825	2.965	30.66	**<0.001**
s(Salinity):SoundAmbient				2.267	2.451	1.612	0.151
s(Salinity):SoundFerry				1.000	1.000	2.041	0.154
s(Salinity):SoundPiling				2.000	2.000	2.482	0.085

Ferry playbacks led to subtle but statistically significant differences in splines for counts of Viable (GAM; edf =2.001, Red.df = 2.002, F = 3.178, p = 0.043) and Failed cells

(GAM; edf =2.000, Red.df = 2.001, F = 3.974, p = 0.020) (Figure 5.7).

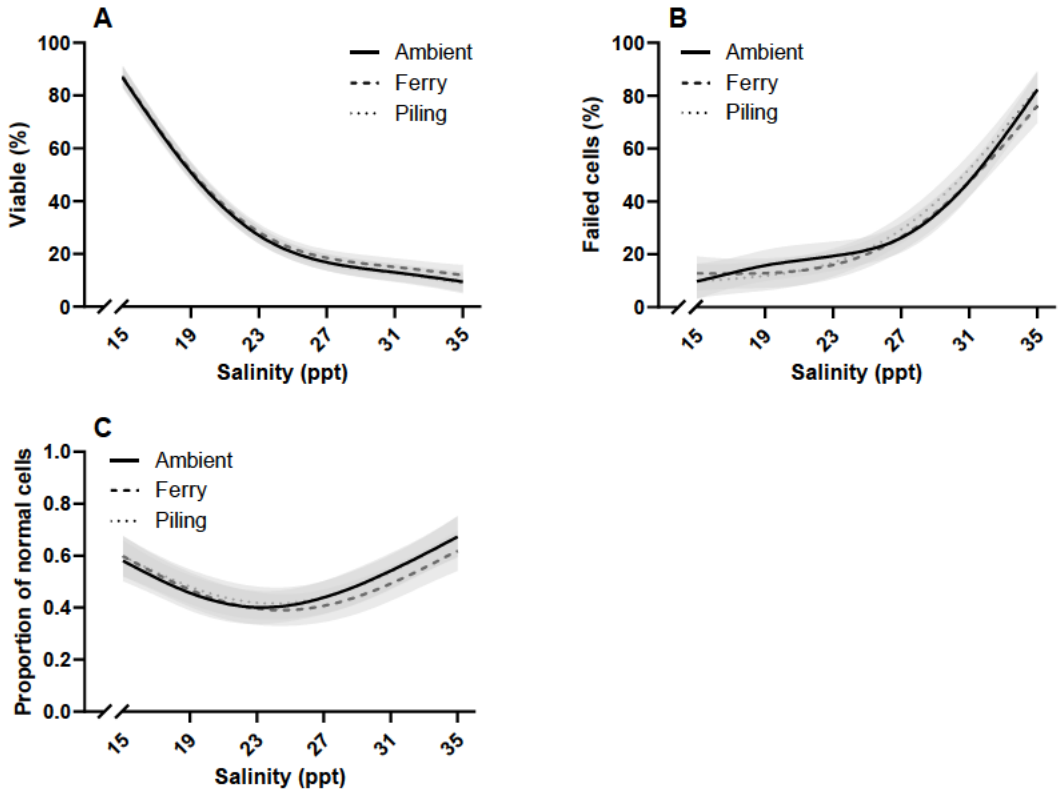

Figure 5.7: GAM modelled state of M. gigas larvae 48hr post-fertilisation. *A) Viable; B) Failed; C) Normal:Abnormal ratio. Lines represent mean values. Shaded areas represent 95% confidence intervals. Values are those pooled across exposure scenarios ES1, ES2 and ES3 for clarity.*

There was an observable salinity dependent decrease in the percentage of viable cells with increasing salinity mirrored by a salinity dependent increase in the number of failed cells (Figure 5.7). At salinities below 25 ppt, where cell failure rates are relatively consistent, the proportion of viable cells appears to be driven primarily by fertilisation rates, whereas above this salinity it is seemingly driven by failure rates. The Normal:Abnormal ratio of viable cells exhibited similar trends to that of fertilisation rates from ES4, with a decrease in the proportion larvae displaying visually normal development at intermediate salinities compared to those at the limits of the selected salinity range.

5.5 Discussion

This is the first study to investigate how exposure of spawned, waterborne gametes to acute anthropogenic sound affects the potential for fertilisation and immediate, short-term development of resultant embryos, and assess how this varies across a range of biologically and ecologically range of salinities. The study design, contrasting acute 1-hour sound exposures to impulsive and non-impulsive anthropogenic sounds prior to, and during gamete contact, provides a unique mechanistic assessment of how the timings of these sound exposures differently affect the potential proliferation of the Pacific oyster, *M. gigas*, by assessing fertilisation rate, developmental failures, and rates of ab/normal development over a 48-hour period inclusive of the sound exposure.

Salinity was a consistent significant driver of all investigated developmental outcomes across exposure scenarios. Exposure to non-impulsive ferry sound playbacks led to a significant deviation in the predicted numerical trend of viable and failed embryos following 48-hour incubation compared to those predicted of salinity alone, though these deviations were statistically insignificant when considered in terms of comparative magnitudinal differences. No significant differences in any developmental outcomes resulted from fe/male gametes being exposed to sound in isolation or combination. Consequently, evidence suggests sound exposure of during spawning events presents minimal risk to the proliferation of *M. gigas* minimal, especially in the context of other prevalent environmental factors.

5.5.1 Exposures

All reported sound exposure levels in this study must be considered with the caveat that quantification was conducted in a proxy vessel; therefore, actual received sound levels within the well plates may vary from those presented. Additional sound transmission to wells was however evident, both as visible displacement of the water within the wells corresponding to the sound playbacks, and preliminary measurements using a partially submerged hydrophone placed within a well (data not recorded). Preliminary spot-checks of received SPLs between the well measurement and the proxy vessel were also comparable. Use of the proxy was nonetheless deemed the more robust approach to quantitative assessment given the limitations of each approach. Therefore, whilst absolute specifics of received sound levels are unknown, results of the study can at the very least be considered in terms of 'additional' sound exposure, with an indication as to the likely characteristics of said sound.

Another caveat to the sound exposures is that of direct contact with the surfaces of the well plates. Eggs of *M. gigas* are more dense than seawater and therefore liable to settle in the well plates given the absence of water currents which would otherwise promote their suspension. Resultantly, experimental gametes will have received vibration transferred via direct contact with the well-plate surface in addition to that of particle motion within the water alone – an additional exposure mechanism not expected of natural environments.

Whilst the SPL_{RMS} and SEL_{cum} of each anthropogenic sound playback differed, the received SPL_{peak} of these treatments was consistent (though this did not translate to equivalent consistency in PM_{peak} values). Regardless, the two anthropogenic sound playbacks retained at least one common exposure metric as desired, allowing some

additional context for comparison between a non-impulsive and impulsive sound source.

Measured salinities of gamete solutions were consistent with desired nominal concentrations. Additionally measured water quality parameters were consistent across all salinity treatments, and within threshold criteria stated for accredited oyster larval bioassays (Leverett & Thain, 2013).

5.5.2 Subjectivity of visual assessment

Whilst Unfertilised and Failed cells were visually distinct and clearly identifiable, determination of ab/normally developing larvae (which lack clearly defined visual criteria) is more subjective. Many oyster developmental studies address this by assessing presence and counts of visually distinct D-stage veliger larvae as a developmental endpoint (Labbé et al., 2018; Ng et al., 2016; Suquet et al., 2016; Tervit et al., 2005). No larvae in the present study reached D-stage development during the 48-hour incubation period despite expectations – demonstrating slower than anticipated development likely resultant of a combination of the culture temperature and experimental salinities, both of which are known to influence growth and developmental rate (Magaña Carrasco et al., 2018; Ng et al., 2016; Y. Qin et al., 2018). Resultantly, fixed samples contained predominantly trochophore-stage larvae which are visually and morphologically more diverse than D-stage veligers, and therefore more susceptible to subjective assessment – particularly regarding ab/normal development. Subjectivity was managed by employing a single assessor tested for consistency of approach by repeated blind counts of multiple validation samples, and further blind assessment employed for all experimental samples. Consequently, subjectivity is considered to have had negligible contribution to the results.

5.5.3 Fertilisation & Development

Many factors are known to affect the successful fertilisation and development of oysters. These can vary between species and populations, but also potentially between oysters induced into spawning 'naturally' and those artificially stripped. Spawning in *M. gigas* can be induced via temperature ramping of holding water (Breese & Malouf, 1975), which retains better environmental realism than stripping as the gametes are being conditioned and released (or not) via natural means. But such induction methods come at the cost of unpredictability in the timing and success of spawning. These trade-offs were evidenced in a comparison of spawning methods of the black-lip rock oyster (*Saccostrea echinate*), where spawning induced via temperature and salinity ramping resulted in significantly higher fecundity and sperm motility than those gained via stripping, but could only be induced with a 22% success rate (Nowland et al., 2021). Stripping was used for the present study to facilitate experimental timing and dilution of gametes into the experimental salinities. This decision is considered unlikely to have had any negative bearing on the outcome of the study, and was conducted in accordance with accepted standard methods (Leverett & Thain, 2013). Indeed, evidenced differences in proportions of ab/normal and failed cells attributable to the duration for which gametes had been suspended in water support the choice to better control the experimental timings through use of gamete stripping.

Salinity profoundly impacted fertilisation rates with fertilisation rates being highest at the extremes of the experimental salinity range and decreasing towards the intermediate salinities. This is counterintuitive with what is otherwise widely be considered 'optimal' (C. Xu et al., 2020; Zhao et al., 2012), and directly contradictory to observations by Wang and Qi (2018), albeit in the Chinese pearl oyster *Pinctada*

martensii. Indeed, consensus within the literature is that fertilisation and developmental success is greatest under optimal conditions, and decreases with increasing deviation from these optima (Caballes et al., 2017; Falkenberg et al., 2019; Gamain et al., 2016; Moreira et al., 2018). This would suggest that in the present study, the selected median experimental salinity, recommended by Leverett and Thain (2013), was sub-optimal to the experimental oyster population. Similarly, differences in the observed trends for fertilisation and failure rates suggest that the optimal salinity may vary between different aspects of development, and/or that there are multiple mechanisms contributing to the final developmental outcomes.

Differences in the gamete densities between salinity solutions could also contribute to observed differences in quantities of Fertilised and Viable cells across salinity treatments, and cannot be entirely discounted given cell densities in individual salinity-dependent gamete solutions were not verified. Therefore, it cannot be discounted that the gametes existed in slightly different proportions between salinity treatments. However, all stocks were made using the same procedure, thus any such discrepancies would be expected to be random across treatments and highly unlikely to conform to a clear trend. Gamete stocking densities and ratios were additionally chosen to fall within range where any such discrepancies were unlikely to significantly affect outcomes (Song et al., 2009). The trend observed in fertilisation rates was also mirrored somewhat in the Normal:Abnormal ratio of viable cells which would be expected to be independent of gamete density, except in instances of polyspermy which were likely insignificant given the gamete concentrations and activation periods used in the study (Luis Stephano & Gould, 1988). Consequently, the results of the present study, whilst somewhat contradictory to expectations based upon the wider literature, are nonetheless considered to be experimentally robust.

Despite the prevalence of oyster larva bioassay studies, comparatively few data sets for fertilisation rates exist. This reflects the seeming prevalence of studies focusing on development which benefit in statistical power from pre-screening and selection for only fertilised larvae prior to exposures. A consequence of this is a relatively poor understanding of how disruption to fertilisation and gamete fusion manifests across various stages of this process, which is a crucial given several factors are known to impact upon oyster fertilisation. The age of gametes – or rather the length of time with which they have had contact with water – is also a factor. Sperm of *M. gigas* become activated rapidly upon suspension in seawater thought to be mediated by changes across sodium-proton exchanger (Boulais et al., 2018). Despite the importance of Na^+ in sperm motility, *M. gigas* sperm motility rates were consistent between 14 ppt - 33.8 ppt, and velocities consistent between 14 ppt - 29.7 ppt (Boulais et al., 2018). Conversely Falkenberg et al. (2019) found a significant decrease in *M. gigas* sperm motility with decreasing salinity between 33 ppt and 13 ppt. The Eastern oyster (*Crassostrea virginica*) sperm displays similar stability in motility rates at salinities between 12 ppt - 24 ppt, but markedly decreased velocities outwith this range – although motility at each salinity was consistent across post-activation times ranging between 1-180 minutes (Nichols et al., 2021). *C. virginica* eggs are also activated upon contact with seawater, undergoing a 'rounding' process over approximately 30 minutes (Leverett and Thain, 2013; Thanormjit et al., 2020). Germinal vesicle breakdown (GVBD) rate was positively correlated with increasing salinity in both stripped and spawned oyster oocytes, with significantly reduced GVDB ratios evident at salinities below 24 ppt (Li et al., 2021; Y. Qin et al., 2018). Neither sperm motility nor GVDB were assessed in the current study, with experimental procedures instead being selected to minimise the potential impact of both, and to robustly favour fertilisation

potential. Resultantly, the study not only lacks any ability to distinguish any potential mechanistic role sperm motility and GVDB may play, the study design also arguably lacks sensitivity to such impacts. Future studies assessing sound and salinity impacts on fertilisation may benefit from assessing impacts across a range of lower gamete densities. Likewise, pooling gametes from multiple lineages as in this study results in a lack of understanding as to intraspecific variation in gamete quality and the relative contribution of each lineage to observed results – although this is true of natural mass-spawning events and resultant competitive reproductive fitness. A basic understanding of response variation within a population would however add substantially to the scientific knowledge base.

Occurrence of Failed cells was time-dependent, with zero occurrence in all samples fixed immediately after the 1-hour exposure period, but significant presence after 48 hours regardless of treatment. Contrasting counts of Fertilised and Viable larvae between 1-hour and 48-hour exposures strongly suggest that both fertilised and unfertilised eggs are prone to failure, although in the absence of a negative control comprising deliberately non-fertilised eggs this cannot be definitively verified. If true, it suggests that the mechanism driving larval Failure in this study is not exclusively a result of developmental failure. Given the definition Failed eggs/larvae in this study, and the correlations of such occurrences with increasing salinity, osmotic shock seems the likely mechanism (Aldarmahi, 2007; J. Weiss & Devoto, 2016).

The low prevalence of Failed cells at the lowest investigated salinity and modelled relationship with salinity also demonstrate a baseline rate of larva/egg failure, and the presence of contributary drivers additional to salinity. Although a clear visual differentiation could be made between Failed and Abnormal larvae (justifying inclusion of both criteria), cell lysis is the likely eventuality of any non-functional larvae. Thus,

delineation of different causal mechanisms of larval failure will become less robust over longer time periods. In any case, the underlying mechanisms promoting non-Normal development are somewhat irrelevant in the context of environment-specific outcomes and ecological significance.

There were statistically significant variations in the Normal:Abnormal proportion of larvae across the studied salinity range, but no effect of anthropogenic sound. Observed differences were however likely ecologically irrelevant in comparison to the scale-change in the proportions of Viable larvae these proportions correspond to across the same salinity range. Observed trends mirroring those of Fertilisation rates nonetheless support assertions that the intermediate salinities used were sub-optimal to the studied oysters. Gamete age also had a statistically significant effect on the Normal:Abnormal proportion, with gametes aged for 4 hours displaying higher rates of normal development at salinities below approximately 22 ppt, but lower rates of normality exceeding this. Again, the ecological significance of this is likely minimal as in the event of asynchronous spawning, reduction of gamete concentrations by water currents and predation leading to lower fertilisation rates are likely to have a larger impact on potential propagation numbers. Indeed, hydrodynamic factors are considered the dominant factor in oyster distribution across an estuary (Kim et al., 2010). Furthermore, synchronisation of oyster spawning is known to occur via various pathways (Galtsoff, 1938, 1940; Nowland et al., 2021; Ubertini et al., 2017), and gamete contact and fertilisation to occur rapidly in such instances.

Accounts of the effects of salinity on oyster development vary within the literature, both within and between species. Gamain et al. (2016) observed lower incidence of abnormality in *M. gigas* D-larvae with increasing salinity, whereas Moreira et al. (2018) found minimal difference in malformation rates of 48-hour old larvae. It is possible

that these differences reflect the age of the larvae and differential survival between larval stages. Reduced survival at Intermediate salinities has been observed in umbonate and eyed larvae of the black-lip rock oyster *Saccostrea echinate* (Nowland et al., 2021). Growth rates of juvenile Iwagaki oyster *Crassostrea nippona* were also greater at intermediate 'optimal' salinity ranges and reduced towards the upper and lower limits of the species – although survival was greater at lower salinities with reduced growth rates (T. Wang & Li, 2018). Variation in the literature reflects assertions that 'optimal' conditions will vary by species and plasticity of broodstock related to parental lineage and environmental conditions during gametogenesis (Meng et al., 2021; Scharping et al., 2019).

The results of the present study suggest that acute exposure to the SPL_{peak} levels received have no effect on the development of *M. gigas*, be this in the form of a non-impulsive or impulsive sound signal. Sound exposure to sub-200 Hz impulsive sound (SEL 165 dB re: 1 μPa^2 s) reduced developmental rate and increased occurrence of abnormalities in New Zealand scallops, *Pecten novaezelandiae* (de Soto et al., 2013). This pulse equated to approximately 20 dB re: 1 μPa^2 s greater power at all frequencies below 2000 Hz compared to the present study, which may contribute to differences in observations. Increased mortalities following seismic air gun sound exposure have also been observed in adult scallops (*Pecten fumatus*), although again peak sound levels were in excess of 30 dB re: 1 µPa greater than the present study (Day et al., 2017). It therefore seems likely that sound would be capable of disrupting *M. gigas* development and survival, but only at notably higher peak sound levels than used in the current study. Equally, compared to the aforementioned studies, the lower sound levels of the present study are arguably more reflective of exposure levels likely to be

experienced by organisms, be that consequential sound propagation and attenuation, or simply lower energy sound sources.

There was no observable driver interaction between anthropogenic sound and salinity. It is possible that this is genuinely reflective of a non-interactive relationship between the drivers, but equally may reflect a lack of sensitivity of the experimental design to any present interactions. There are no directly comparable studies against which to measure these two assertions, and attempting to derive this context from studies using different combinations of multiple drivers is fraught with difficulties. A meta-analysis of various biological responses of multiple early life stage invertebrate phyla found changes in salinity typically results in synergistic driver interactions (Przeslawski et al., 2015). However, most studies included in the metanalysis paired salinity with physicochemical drivers such as temperature, pH, oxygen, or pollutants, all of which are well-established and substantial drivers of early life stage development in their own rights. Despite this, sub-optimal salinities provoke stronger negative impacts in aquatic organisms than many other co-occurring drivers (Velasco et al., 2019). In any case, in the context of the present study, the evident impacts of salinity were clearly the predominant driver of all observed developmental outcomes, and whether or not the study design lacked sensitivity to potential effects of anthropogenic sound and any related interaction, results of this study suggest any such effects would likely remain ecologically insignificant in comparison to the impacts of salinity.

An interesting and warranted continuation of this research would be to incorporate the use of molecular techniques into the experimental design to ascertain whether there are any correlations between expression and/or quantity of cellular biomarkers and developmental state of the embryos. It is also possible that molecular techniques would uncover non-visible differences between treatment groups, providing additional

sensitivity to the experiment, whilst also aiding in mechanistic understanding. Indeed, such approaches have been used to such effect in studies addressing chemical toxicants (Akcha et al., 2012; Mai et al., 2020; Ringwood et al., 2009), but were considered incompatible with the current study due to experimental logistics imposed and required to ensure the robust delivery of the primary objectives. Similarly, expansion of the experiment to monitor the developmental success of the embryos throughout their continued development would be interesting and could potentially reveal latent impacts of the anthropogenic sound exposures, however this too was beyond the scope and objectives of this experiment.

5.6 Conclusion

Salinity had a profound impact on *M. gigas* gametes/larvae, being a statistically significant driver of all endpoints in each exposure scenario. There were no significant differences between gamete exposure scenarios concerning the ferry pile driving sound playbacks. Only ferry playbacks elicited a significant deviation from values predicted of salinity effects alone, marginally increasing the proportion of Viable cells at salinities at salinities above 23 ppt and decreasing Failed cells below 27ppt – although not to extents that were significant when considered parametrically. Fertilisation rates and cell failure rates are considered the primary driving mechanisms of the results, with the former of greater importance at salinities below 25 ppt, and the latter at salinities exceeding this. Collectively these two aspects led to a salinity dependent decrease in the proportion of Viable larvae with increasing salinity, with the lowest salinity (15 ppt) the most conducive to proliferation. The results of the present study indicate that acute anthropogenic sound exposures < 120 dB re: 1 μPa^2 Hz^{-1} had little-to-no impact on gamete fusion and fertilisation, or the development of resultant

larvae in the immediate short-term at sound levels. Hence, such acute sound exposures would likely not be significant risk factor for proliferation of *M. gigas* in a wider ecological context.

Chapter 6
Discussion and Synthesis

A succinct summary of this thesis could simply state, *"multiple driver studies are complicated"*, yet this somehow fails to fully capture the realities of the undertaking. It is not so much that any one aspect of multi-driver studies is particularly difficult, more that the interplays between *each and every* aspect of the experimental process lead to profound complexity. Whilst this is true to an extent of most (if not all) experimental undertakings, the incorporation of additional drivers/levels to experimental designs adds to these challenges. In this way, the design and conduction of multiple driver studies almost create a synergy of their own.

The experimental design philosophies for this thesis and justification for these choices are addressed in Chapter 2, however choices inevitably have ramifications, some of which are not always immediately evident. Thus, despite steadfastly backing the methodological approaches taken in this thesis, it is prudent to reflect on some of the different choices taken between the studies of this thesis before addressing their findings, such that their differing limitations and benefits can be properly appreciated. Some of discussed limitations relate specifically to the comparability studies employing different methods and equipment. It was never the focus or design of this thesis to enable direct comparisons between the studies undertaken. Rather, any such limitations are discussed as a means of exploring and exampling similar difficulties in comparing findings of the wider literature.

6.1 Anthropogenic sound impacts in wider contexts

This thesis sought not only to build on the limited understanding of the impacts of anthropogenic sound on early life stage aquatic invertebrates, but also to expand this understanding to include how these impacts change in the context of different environmental drivers. Principally, the aims were to ascertain (i) whether anthropogenic sound playbacks and/or different environmental chemistries affect early life stages of invertebrates, (ii) whether and how these drivers combine and interact, and (iii) the potential mechanisms contributing to any such interactions.

Rather than placing emphasis upon any one aquatic environment or species, it was decided to focus on key early-life developmental stages – fertilisation, embryonic development, and larval development – adding context regarding fecundity and juvenile behavioural fitness where practicable. Three model species were selected, each suited for assessing each of these developmental stages – namely the Pacific oyster (*Magallana gigas*), the great pond snail (*Lymnaea stagnalis*), and the Norway lobster (*Nephrops norvegicus*) respectively. Additional post-exposure assessment of fecundity and biometric assessments were undertaken in adult and juvenile *L. stagnalis* respectively, and biometric and behavioural assessments of post-exposure juvenile *N. norvegicus* also undertaken. The three species, *N. norvegicus*, *M. gigas*, and *L. stagnalis* likewise represent marine, brackish, and freshwater habitats, thus three corresponding environmental drivers – cadmium, salinity and calcium were chosen as additional environmentally relevant drivers. Detailed justification, results and discussion for each study are contained within their respective chapters, and thus are not reiterated to any great extent here. Rather, this synthesis seeks to briefly expand on the collective understanding gained from these studies and reflect upon the wider implications of anthropogenic sound in a multiple driver context.

Different taxa and developmental stages can display very different responses and susceptibility to environmental factors (Moreira et al., 2018; Pineda et al., 2012). Understanding the potential impacts of driver combinations on any one developmental stage is informative, but ultimately limited if more concerning impacts were to occur at the preceding or following developmental stage. This reasoning led to the decision to address impacts of anthropogenic sound on fecundity, gamete fertilisation, embryonic development, larval development, and juvenile behavioural fitness. Whilst an assessment of these life stages using a single model species would have been comprehensive and robust, this was not experimentally practical and would have limited the scope of the thesis; likewise, for addressing only a single environmental chemistry driver across all studies and species. Rather, the various combinations of drivers, species, and endpoints were carefully selected to demonstrate the propensity for interaction between anthropogenic sound across a range of environmental factors in an attempt to ascertain any commonality in responses which could be generalised beyond specific species, taxa, or developmental stage.

6.1.1 Disambiguation of embryos and larvae

This thesis makes common reference to gametes, embryos, larvae, and juveniles (defined in Chapter 2), yet the ecological difference of these developmental stages – particularly embryos and larvae – is sometimes unclear. The distinction between larval and embryonic developmental stages in the context of this thesis is arguably semantic – the terminology demarking developmental differences between the species, without necessarily having any experimental consequence. This is because different taxa and species have differing reproductive and developmental strategies, which may or may not feature both embryonic and larval developmental phases. For example, *N.*

norvegicus would be considered as undergoing embryonic development whilst encapsulated within the egg, larval development upon hatching, before transitioning to juvenility following metamorphosis. In comparison*, L. stagnalis* transitions directly from embryonic development to juvenility upon hatching, whilst *M. gigas* transitions from larval development to juvenility. Thus, in the context of this thesis (and molluscan development generally), the developmental progression of *M. gigas* larvae and *L. stagnalis* embryos are very similar. Indeed, the development of other closely related molluscan species share practically identical developmental progression despite some undergoing planktonic larval development and others encapsulated embryonic development (Collin, 2003; Pechenik, 1979). Likewise, *L. stagnalis* embryos, *M. gigas* larvae, and *N. norvegicus* larvae are broadly analogous in that they all represent the developmental stage immediately preceding juvenility; albeit their ecological-, taxa-, and species-dependent differences again preclude direct comparison.

6.1.2 Gametes to juveniles

A comprehensive assessment of acute sound exposure of *M. gigas* gametes provided no evidence of either ferry- or piling sound playbacks impacting upon fusion and fertilisation of gametes, nor on the subsequent development of resultant embryos/larvae in the context of variable salinity. For b

roadcast-spawning taxa including fish, corals, sponges, it therefore seems unlikely anthropogenic sound would notably impact upon this initial stage of proliferation in a wider ecological context (Monteiro et al., 2015; Vendrami et al., 2021) – though this may depend upon the magnitude of the sound exposure.

Results from this thesis evidenced that chronic exposure to an environmentally realistic regime of ferry sound playbacks significantly decreased successful embryonic developmental and hatching of *L. stagnalis* (attributable to a greater occurrence of

developmental abnormalities), but only under seemingly more challenging environmental conditions. Similarly, piling sound playbacks led to an increase in *N. norvegicus* larval mortality at more challenging cadmium concentrations, but promoted survival in the absence of additional cadmium burdening. In the case of *N. norvegicus*, mortalities often coincided with moulting events, with oxidative stress and increased energetic demands and resultant identified as potential contributing factors. Along with potential mechanosensory disruption, differences in energetic demands and energy partitioning of post-exposure juvenile *N. norvegicus* were a postulated contributary factor to differences in the likelihood of eliciting escape responses, and the magnitude of any such responses. Notably, ferry sound playbacks also resulted in a consistent trend for reduced hatching size and increased heart rate in juvenile *L. stagnalis*, though neither to a statistically significant extent. The combination of reduced growth and elevated heart rate are nonetheless consistent with increased metabolic requirement in a resource-limited system (Bruning et al., 2013; Ward et al., 2017). Similarly, whilst gross fecundity of adult *Lymnaea* was unimpacted by sound exposures, it became apparent that this was likely a consequence of different life-history strategies and physiological plasticity when accounting for adult growth during the exposure period. This observation again likely indicates that sound exposure increases energetic burden – which becomes more apparent in organisms additionally burdened by other environmental drivers.

It is notable that in both the *Nephrops* and *Lymnaea* studies, where sound was observed to promote mortality and developmental abnormalities leading to non-hatching events, many of the observed effects occurred within the first days of exposures. This clearly demonstrates that comparatively short sound exposures can have deleterious impacts. More importantly, evidence of sound exposure impacts on

embryonic stages of *L. stagnalis* preceding development of the species' principal mechanosensory structures and associated nervous pathways shows that sound can cause deleterious effects in organisms aside of their sensory and perceptual capability.

Whether the lack of any observable effect of sound exposure in *M. gigas* gametes is therefore demonstrative of differences in species sensitivity, exposure duration, or the sound characteristics remains uncertain. Assuming the energetic consequences raised above and existing evidence of DNA damage resultant of sound exposure (Wale et al., 2019), developmental morbidity events resultant of sound exposure may be more likely when coinciding with periods of rapid cellular proliferation, or specific periods of cellular differentiation. Any such DNA damage and/or energetic impacts are also likely to be accumulative. Unfortunately, comparatively little is currently known about dose-response dynamics of aquatic anthropogenic sound (Popper et al., 2020; Wale et al., 2021).

In summary, the lack of any observable impact of anthropogenic sound on *M. gigas* fertilisation rates and early larval development suggests acute exposure of broadcast-released gametes to anthropogenic sound is not a concern, and presents no form of proliferation bottleneck. In comparison, larvae and embryos of *N. norvegicus* and *L. stagnalis* did show susceptibility to chronic exposure to anthropogenic sound resulting in a reduction of recruitment to juvenile developmental stages under more detrimental environmental conditions. Exposure of early life stage *N. norvegicus* and *L. stagnalis* to anthropogenic sound also translated to measurable differences in behavioural physiology of juvenile-stage specimens, although the long-term impacts and likely persistence of these effects are uncertain.

6.1.3 Environmental and ecological gradients

Interactions and interplays between sound exposure and other environmental chemistry drivers evidenced in this thesis are mutually inclusive; meaning the statements, '*sound exposures influence the chemical exposure impacts*' and '*chemical exposures influence the sound exposure impacts*' are equally valid. Consequently, rather than discussing both mutualisms, here the discussion is framed primarily with regards to anthropogenic sound exposure – discussing its potential impacts across various ecological contexts. This reflects anthropogenic sound's status as an evolutionary irrelevant driver, as opposed to the accompanying environmental drivers which are ecologically and biologically intrinsic. More specifically, in addition to respectively representing freshwater, brackish and marine environments, calcium, salinity, and cadmium further reflect chemical drivers considered to be mostly detrimental, beneficial, and neutral from a biological perspective, respectively.

As with comparisons between developmental stages, direct contrasts between the three studies of this thesis are difficult given this was never a primary objective, yet across the studies, a pattern in which exposure conditions resulted in significant biological effects was apparent. Anthropogenic sound exposures in isolation were never a significant driver of biological effects, and where pairwise interactions between anthropogenic sound and the accompanying environmental chemistry drivers were apparent, these exclusively occurred when environmental chemistry in isolation were either sub-optimal or demonstrably detrimental to the species in question. This was most evident in the *Nephrops* study given that the accompanying cadmium driver can readily be accepted as being a net stressor to larval development at the experimental concentrations. Correspondingly, cadmium led to concentration dependent increases in larval mortality, with anthropogenic sound increasing the

sensitivity of larvae to increasing cadmium concentrations. In the *Lymnaea* study, calcium's role as an essential macronutrient negated the use of a calcium control treatment, and the limitation in treatment levels across the calcium driver precluded any similar mechanistic assessment. Despite this, the study's results clearly showed that of the two calcium levels used, the high calcium treatment was less conducive to growth and development, reducing both reproduction rate of adults and hatching size of embryos. Likewise, it was in the high calcium treatment that anthropogenic sound exposure led to a significant reduction in hatching success of embryos and in growth-dependant fecundity of adults. However, in the *Magallana* study, anthropogenic sound had no impacts upon larvae even when coupled with salinities which were clearly detrimental to fertilisation and development. Thus, whilst patterns exist which may aid in predicting situations in which anthropogenic sound exposure might pose an elevated risk, these patterns are not a steadfast rule.

In addition to patterns of occurrence of interaction between anthropogenic sound and accompanying physicochemical drivers, results indicate a potential commonality in the underlying biological mechanisms underlying these interactions. In both the *Lymnaea* and *Nephrops* studies, significant sub-lethal effects all indirectly related to changes in energy burdening and energy partitioning. In *N. norvegicus,* differences in both the developmental rate of the larvae and escape-response dynamics of the juveniles can be explained by differences in energy utilisation. Differences in energy partitioning in *L. stagnalis* are likewise directly evident through differences in adult growth, and growth-dependant fecundity; the latter of which also indicated that sub-lethal impacts of sound exposure might have been masked by behavioural and physiological plasticity.

6.1.4 Implications

Results presented throughout this thesis indicate that the effect of anthropogenic sound on early life stage aquatic invertebrates is contextual, rather than sound being a consistent and predictable driver of concern. The impact of anthropogenic sound exposures not only varied between species, developmental stages, exposure durations, and the sound characteristics, but more widely according to the environmental conditions. The interplay and interaction between anthropogenic sound and environmental chemistry drivers seemingly relate to the underlying environmental stress experienced by an organism, with anthropogenic sound exposure only provoking significant effects in sub-optimal environmental conditions.

Accepting earlier conjecture that sub-lethal impacts relate to differences in energy budgeting, this suggests that the risks posed by anthropogenic sound exist along a continuum, rather than being discrete. Results presented in this thesis suggest that anthropogenic sound exposures likely add to the cumulative stress burden of aquatic invertebrates, albeit to a proportionately small extent compared to environmental chemical drivers. As such, organisms exposed to comparatively favourable conditions and low stress burdens may have the energetic latitude to effectively mitigate against impacts of anthropogenic sound and absorb the energetic cost via plasticity. However, when already burdened by other drivers, such energetic latitudes would be diminished and eventually surpassed, beyond which point additional sound exposure would be a cumulative burden leading to observable negative impacts. Correspondingly, any seeming resistance to the effects of anthropogenic sound under more favourable conditions is likely an inaccurate interpretation/conclusion, with it more probably reflecting better tolerance to the additional stress burden rather than outright immunity. The ramifications of tolerance to additional sound exposure are difficult to

quantify. Assuming that impacts of sound exposure are cumulative, it is possible that tolerances could be eroded over chronic exposures. Conversely, it is possible that organisms would acclimate. In any case, any biological impact of sound exposure may alter the susceptibility to, and ability to cope with, other environmental drivers. In this context, the variable risks of anthropogenic sound across environmental gradients are constrained less by the environmental conditions themselves than the tolerance breadth of any given species or individual in said environment. This is turn might explain a degree of intraspecific variably within any given experimental treatment group.

Regarding lethal effects of sound exposure on early life stage organism, the implications are obvious, yet the risks harder to quantify and variable according to the route cause or mortality. If energetic consequences are the likely cause (as suspected in *N. norvegicus*), then the risks of mortality would scale in the same manner as non-lethal effects. However, if DNA damage or disruption to other biomolecular and biomechanical pathways affecting somatic growth is a possible mechanism (as in *L. stagnalis*), then the inherent uncertainties in the propensity of such effects makes associated risks far more difficult to judge. This is compounded given such effects may occur independently of an organism's ability to perceive sound. Resultantly, it is reasonable to suggest that anthropogenic sound exposures may have multifaceted modes of action, which may or may not occur in parallel.

6.1.5 Summary and future directions

This thesis provides a framework for conducting multiple driver research in the context of sound, including reflection on the many challenges and limitations involved, and advice on approaches towards addressing these. Also explored are the impacts of anthropogenic sound on key early life developmental stages across a range of taxa and

environmental conditions, and the propensity for interaction between anthropogenic sound and environmental chemistry. The framework and experimental design approaches featured in this thesis can be readily expanded upon and/or adapted for assessment of a wide range of environmental sounds (anthropogenic or otherwise), and an equally variable range of waterborne chemical drivers. Likewise, the approach is not limited in applicability to early life stage organisms, and could be expanded to include other life history stages, species, and taxa. Such expansion in scope would greatly improve current understanding of the scope of driver interactions, and likely reveal more intrinsic mechanistic pathways of sound exposures.

The benefits of assessing chemical drivers across multiple concentrations have been remarked upon frequently in the literature, yet little is known about the dose-dependency of sound exposure. Expanding sound studies to explore how sound amplitude and duration of exposure influence biological impacts would add greatly to mechanistic understanding in both single and multiple driver contexts, which in turn would improve the ability to dynamically assess risk.

Overarching themes of energetic consequences discussed in this chapter highlight the added value that would be gained from understanding how anthropogenic sound exposure influences energy budgeting under different environmental conditions. Approaches including lipid analysis and controlled respirometry could help to address these uncertainties but may be prohibitive in multiple driver studies concerning minute larvae due to tissue limitations and the sensitivity/resolution of analytical techniques. Likewise, a better understanding of uptake kinetics, bioaccumulation, and homeostatic fluxes of chemical drivers may add much needed context.

Evidence of multi-driver impacts of anthropogenic sound in various developmental stages has been discussed. However, understanding carry-over effects and the long-

term ramifications of these impacts is important, particularly any propensity for long-term acclimation. Furthermore, such studies should be expanded to consider trans- and multigenerational effects which may further compound impacts and lead to interactions of their own.

This thesis barely scratches the surface of multiple driver research, yet still represents a formidable undertaking. It is hoped that rather than dissuading from any continuation, it serves to educate and encourage others to expand and improve upon this crucially important research field, provide better understanding, and hopefully help mitigate the effects of multiple drivers on aquatic invertebrates.

6.2 Limitations and lessons learned

6.2.1 Pseudoreplication of sound

Pseudoreplication is sometimes raised as a limitation of sound-based studies, though the validity of this statement is contentious. Those purporting pseudoreplication (especially of laboratory-based studies) often do so on the grounds that playbacks of sounds compiled from a limited number of *in situ* recordings do not capture the full acoustic variation of such sounds (McGregor et al., 1992). This is undoubtedly true, but strictly speaking attests to a lack of acoustic replication rather than pseudoreplication (Kroodsma et al., 2001) – the implications of which vary dependent on study aims, interpretations, and assertions. For example, it would be inappropriate to make unilateral blanket statements over implications of ferry- and pile-driving sounds based upon the results of this thesis, as playbacks represent only a single example of each sound source and could therefore be an atypical statistical outlier (Kroodsma et al., 2001). Such limitations also apply to field-based studies and are not an inherent

limitation of laboratory-based studies. On the contrary, studies using sound playbacks potentially enable a greater variation of sound sources to be included – although the benefits and best approach for doing so again depend upon the experimental question.

An approach for accounting for limited variation in acoustic profiles is to include examples originating from various different sources, for example several different types of boat, or from several different pile installations – though the limitation may then become access to ecologically relevant sound source recordings. Additionally, whilst inclusion of multiple sound sources within a single compiled sound treatment will capture greater acoustic variation, it makes disentanglement of any effects more difficult as it is impossible to determine which of the included sounds were responsible for any observed effects. Furthermore, such an approach, whilst capturing more variation, would still constitute pseudoreplication as only a single *amalgamation* of sounds is used (Kroodsma et al., 2001). Thus, inclusion of more variation in this manner might benefit effect-screening studies, but is less informative than separate exposures to multiple individual sound sources – although conducting multiple independent sound exposure may prove logistically prohibitive. Likewise, although the inclusion of sound source variation has worth, it's value may be overstated given that any such inclusions still represent only a minute degree of true variation in potential sound exposures. Thus, whilst the piling-playbacks used in this thesis benefit from inclusion of some degree of variation, and the ferry playbacks are arguably limited in this regard, the validity of these studies' results is not impacted – only the extrapolations and conclusions which can be drawn from them.

6.2.2 Speakers vs vibration platforms

Decisions over whether to introduce sound playbacks by speaker or vibration platform were predominantly motivated by experimental logistics – namely temperature control, replication, and space. The *Nephrops* study (Chapter 3) was not possible to conduct in a controlled temperature room due to space requirements. Thus, it was decided to employ an exposure system which could double as a water bath to facilitate temperature regulation. Whilst in principle it would be possible to place a tank acting as a water bath on a vibration platform and create a similar set-up within that tank, such a setup would be limited in size and volume, making temperature regulation more difficult and limiting space availability and replication potential. Underwater speaker-based playback systems have an undoubted advantage with regards to the area of sound exposure they can generate, with the sound front being proportional to the distance from the speaker resultant of hemispherical propagation (Rienstra & Hirschberg, 1952). This allows great flexibility in experimental design compared to vibration platforms which are more limited by their physical dimensions (size of tank they can support). The ability to create a larger sound field with speakers was essential to facilitate the scope and scale of replication of the *Nephrops* study. Being able to achieve separation from the speaker and the subject also helps to manage and limit near-field sound tank-acoustic effects in laboratory studies (depending on tank size), though 'tank-acoustic' effects are realistically still a likely concern compared to open bodies of water (Gray, Rogers, Popper, et al., 2016). However, speakers can and have been deployed in open water bodies to facilitate sound playbacks not subject to tank-acoustic artefacts (Nedelec, Radford, et al., 2017; Simpson et al., 2016), though such an approach was not practicable for the studies presented in this thesis.

Whilst vibration platforms are constrained in their size, they provided more consistent and predictable sound fields across their surface than speakers – undoubtedly reflecting their smaller size and the reduced spreading and attenuation effects of sound from the source (Rienstra & Hirschberg, 1952). They proved beneficial in the *Magallana* study (Chapter 5) as the provisioned multi-well plates were buoyant and unstable in water-filled tanks. Given the need for regular handling of the plates during the study, the better stability provided to the plates by the vibration platforms was highly beneficial. The consistency in sound field across the vibration platforms was also essential to concurrently running multiple experiments whilst maintaining consistent sound exposures. The use of the vibration platforms for this experiment, however, was only feasible given the high replication potential facilitated by multi-well plates, which offset the limitations in the size of the available sound field.

Vibration platforms were not used for the *Lymnaea* study (Chapter 4) despite the use of multi-well plates, as the experimental system was originally envisioned and created to allow concurrent running of both the fecundity and embryo assays, and thus space was a premium. Secondly, *Lymnaea* routinely occupy the surfaces and lay and adhere their eggs onto such surfaces (Willows, 2003), thus it was decided that vibration transferred directly from the platform into the beaker/plates walls would constitute an unrealistic exposure dynamic (Rogers, Hawkins, Popper, Fay, & Gray, 2016). Whilst this is also true of the *Magallana* study, the *Lymnaea* were considered more susceptible to these effects given the differing mechanosensory abilities of the developmental stages involved in each study (Croll & Dickinson, 2004; Nagy & Elekes, 2000; Yurchenko et al., 2018).

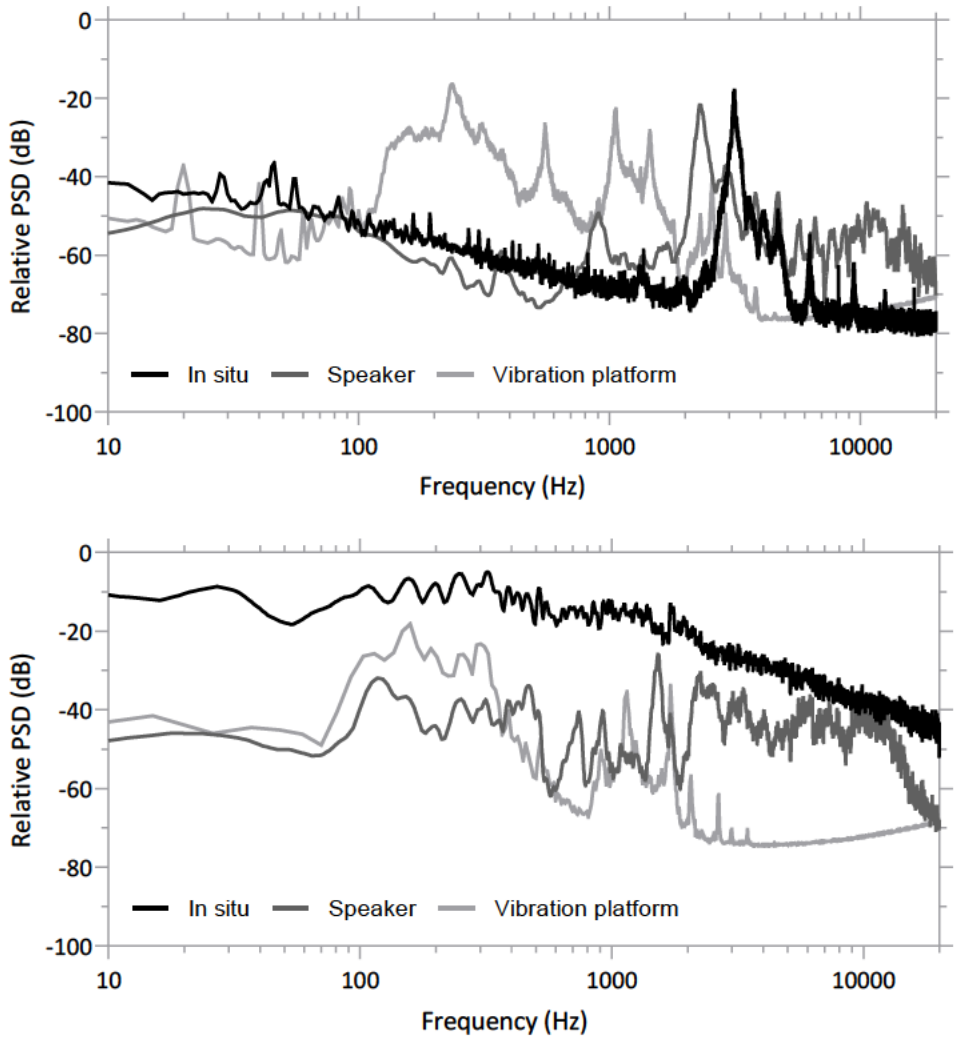

Figure 6.1: Relative RMS power spectral density comparing sounds as recorded in situ and as reproduced experimentally. *Top: Ferry sound (1 second Hann window, 50-percent overlap). Bottom: Piling sound (0.1 second Hann window, 50-percent overlap). Speaker refers to Clark Synthesis Diluvio AQ339 Aquasonic and DNH Aqua-30 for piling and ferry respectfully. Vibration platform refers to Crowson Technology Controlled Vibration™ EDP-2424 24 Inch Platform Shaker.*

The speakers and vibration platforms used throughout the research of this thesis also differ in the frequency range of sound they produce, and thus in fidelity with which they can reproduce various sounds. Figure 6.1 shows how total sound power of both the ferry and pile driving sounds used were distributed across sound frequencies as recreated by both speakers and vibration platforms. Both the dis/similarities between *in situ* sound exposures are demonstrated, and those achieved via playbacks, but also those between equipment with different specifications. Evident is the difference in upper frequency response between the speakers (18-20 kHz) and the vibration

platform (2 kHz). Such differences in the frequency composition of sounds also have ramifications on the sound analyses themselves, as does the frequency range across which such metrics are calculated (Foreman, 1990). Although the latter is controlled for in this thesis by a consistent approach towards sound measurements between studies, broadband sound metrics may not appropriately capture variation across comparatively narrow bandwidths or transient sound signals (Madsen, 2005). This was the justification for limiting the frequency bandwidth for particle motion analyses, as the comparatively modest increases in particle acceleration coupled with high attenuation rates at higher frequencies otherwise resulted in spurious root mean square (RMS) and sound exposure level (SEL) metrics if considered over a 24 kHz range as in the case of pressure. Therefore, whilst such metrics remain meaningful for comparisons within studies, their value when contrasting between sounds generated by different sources or conducted in environments with different acoustic properties is diminished. Resultantly, comparisons between PSD estimates at specific frequencies is arguably more appropriate in such instances.

6.2.3 Exposure vessels, aeration, and sound quantification

The size and materials of the exposure vessels varied notably between studies. Selection of exposure vessels was primarily driven by the husbandry and welfare requirements of each study species, and experimental logistics.

Differences in the volume and material of the exposure vessels between the two *Nephrops* experiments had little impact on the received sound profile of experimental playbacks. This was verifiable as the received sound levels could be measured within the exposure vessels, fully accounting for any transmission losses and/or acoustic aberrations caused by the exposure vessels. This was not possible for the embryo and gamete experiments of the *Lymnaea* and *Magallana* studies. The use of multi-well

plates for these experiments precluded the measurement of sound within the exposure vessels themselves, instead requiring measurements to be taken from within a proxy vessel. A proxy vessel was used for sound measurement in an attempt to capture any sound discrepancies attributable to the material properties of the plates, though the exact nature of the received sound is nonetheless unknown. This approach was however considered a more appropriate compromise than measuring the sound without the benefits of a proxy vessel.

Aeration of the exposure vessels was also required for the *Lymnaea* fecundity assay given the volume of the exposure vessels, stocking densities, and the respiration rate of the adult specimens. Whilst aeration notably increased sound pressure levels, this did not translate to any notable increase in particle motion. Thus, for invertebrates and other taxa primarily responsive to sound particle motion, the inclusion of aeration may have little impact on experimental design – at least from an acoustic standpoint.

6.2.4 Controlling for spatial variability and mutual exclusivity

A cornerstone of experimental design is accounting for environmental variation which might otherwise influence the results of a study. For laboratory studies this effectively equates to controlling for spatial variation in experimental set-ups. When counteracting unknown or unexpected variation, randomising spatial distribution of experimental treatments is an acceptable methodological approach (Lazic, 2016). Where a known gradient exists, other approaches like block designs distributions are more suitable (Lazic, 2016). However, such distribution approaches do not work where treatment locations are mutually exclusive – such as when there is a requirement for separate tanks recreating different sound treatments. A solution to this could be to use multiple tanks in different locations, each providing replication of any given sound source, however this becomes unfeasible when resources and space are limited.

Each study presented in this thesis used a different approach towards control, each with different limitations:

1) Substantive monitoring of environmental variables between sound treatment locations

2) Periodic rotation of sound treatments (and all treatment replicates therein) between exposure systems facilitating sound playbacks

3) Secondary experimental exposures conducted under ambient sound conditions in all sound treatment locations

Approach 1 was used during the *Nephrops* study. The presence of a sound cone effect within the tank providing piling playbacks necessitated cadmium treatment replicates in each sound treatment be distributed according to a Latin-square block design. This distribution effectively controlled for spatial distribution within each sound treatment, but not between sound treatments. Approach 2 was not practicable due to the large number of replicates, the highly specific distribution of cadmium treatments, the added risks of excessive handling of chemical treatments, and time constraints required to maintain temperature control. The lack of more meaningful control means it is *technically* impossible to delineate between a tank/location effect and a sound effect between treatments. However, substantive monitoring of environmental factors liable to impact results (temperature, pH, salinity, light intensity, dissolved oxygen) was conducted at multiple locations within each tank throughout the study duration, with little or no measurable differences between tanks. Thus, sound exposure levels were the only environmental variable proven to be substantially different between the tank locations, and thus the assumed likely driver of experimental endpoint variation between locations.

Approach 2 was used for the *Lymnaea* study. The lower working replication, easier handling requirements, and better environmental temperature control compared to the *Nephrops* study diminished the risks and challenges of rotating sound treatment locations. The 21-day study duration enabled multiple opportunities for repositioning of sound treatments between locations, enabling variation throughout the developmental period of embryos. Whilst this is notably more robust than Approach 1, it does not fully preclude short-term temporal variation, which may not be adequately captured depending on rotation frequency.

Approach 3 was used for the *Magallana* study. The short sound exposure duration (1 hr) and concurrently running exposure scenarios precluded rotation of sound exposure locations. The sound exposure duration did however enable secondary experimental exposures to address the impacts of room location as a factor rather than sound. This data could be analysed separately to discount the impacts of location on experimental endpoints or incorporated into further analysis as a random variable. Moreover, following the sound exposure phase, treatment replicates were randomly assigned to different sound exposure locations to capture environmental variability during post-sound exposure. Thus, environmental uncertainly was reduced to a one-hour time period.

Of these methods, Approach 1 is objectively the least robust, with Approach 2 and 3 being increasingly controlled. However, Approach 2 is predicated on a suitably long experimental period to enable repeated rotation of sound locations, whereas Approach 3 requires a suitably short experimental period. Thus, these approaches may not be practicable, depending on the specific research questions. If resource limitation was not a factor, multiple sound source replicates would prove beneficial, enabling use of statistical approaches controlling for any random effects between sound-specific

locations. Failing this, Approach 1 may become less a choice than a lack of options. Likewise, as alluded to previously, there are situations where the risks of increasingly robust experimental control might outweigh the benefits, and an informed decision to compromise being made. In either case, whenever resorting to Approach 1, considerable thought should be given to monitoring practices, and the limitations fully understood and considered.

6.2.5 Scope and replication

Time-specific availability of larvae was a key driver in the selection of model organisms featured in this thesis. Females of species such as *Nephrops norvegicus* (Chapter 3) only produce a single brood per year, limiting the opportunities available to conduct research. In comparison species such as *Lymnaea stagnalis* (Chapter 4) can readily be laboratory cultured free in high numbers and have a comparatively short generational time, thus availability of embryos constituted less of a limitation subject to adequate planning. Very little limitation existed in availability of gametes for the *Magallana gigas* (Chapter 5) given the species' fecundity and existence of services providing reproductively conditioned adults to order. This combination of species and approach aided the ability to conduct research less encumbered by limitations of reproductive seasonality. However, they also influenced decisions regarding the diversity in genetic lineage across which experimental replication was derived. Seasonally reproductive *N. norvegicus* demonstrate a high degree of variation in the timing of larval release between females, and to a lesser a degree, in specific hatching date of larvae within a brood. It was also noted the berried females would occasionally 'drop' their broods or moult their exoskeletons (and attached broods) after which the eggs rapidly ceased to be viable. Based on these observations and the number of remaining berried females available upon the initial release of larvae from the broodstock, it was considered an

unacceptable risk to deliberately under-populate the experiments with readily available larvae of single maternity in the hope that desired replication could later be fulfilled using larvae of differing maternity. Therefore, each experiment in the *Nephrops* study utilised larvae originating from a single berried female. A potential limitation of only using larvae from a single female could be a lack of genetic diversity in the test population, and a related limitation in observed responses and/or resilience to any stressors (Benedetti-Cecchi, 2003). Such limitations of single maternity may be partially offset given the evidence that *N. norvegicus* larvae within a single brood can have multiple paternities (Streiff et al., 2004) and may therefore contribute more genetic variability than genetic recombination alone. Additionally, to properly address differences between larvae of different broods, larvae from several different females would be required. Given space limitations within the experimental systems, incorporating this diversity would have necessitated a reduction in brood-specific replication to an extent where statistical power would likely have been insufficient to assess such variation even if present. Ultimately, in the case of the *Nephrops* study, populating each experiment with larvae from a single brood was a pragmatic decision rather than a design choice. In the case of *L. stagnalis*, whilst the broodstock produced embryos of multiple lineages, the effective genetic diversity was expected to be minimal, in part due to the species ability to reproduce parthenogenetically, but predominantly due to the isogenic nature of the original seed culture. Thus, even though the species preferentially reproduces sexually (Koene et al., 2007; Koene & ter Maat, 2007), the genetic diversity this introduces is likely minimal. Similarly, even though the *Magallana* study used gametes from multiple *M. gigas* lineages, the genetic diversity of commercially cultured oysters may be lower than natural populations (Lind et al., 2009).

The implications of any potential lack of genetic diversity depend upon the specific research questions and aims of a study. It is conceded that to confidently extrapolate results to population-level ecology, larvae from a variety of parental lineages would be preferable to address potential variations in response to consequential genetic and life-history factors (Jansen et al., 2015), however this was not the principal aim of the research in this thesis. Rather, given the novelty of the approach and infancy of the research field, here the research priorities were towards ensuring data integrity and robustly establishing *potential* effects in key species – considered an essential initial platform of knowledge from which to expand and build research further (Griffen et al., 2016). In this context, limited genetic diversity can be beneficial as it controls for phenotypical variation to the benefit of statistical power (Festing, 2004).

6.2.6 Statistical analysis

As stated in Chapter 2, all statistical approaches used were selected on the basis of data distributions and underlying model assumptions, and without employing data transformation techniques. Though transformation of non-parametric data to meet data-normality assumptions in so-called 'surrogate models' (Schneider, 1992) is commonplace in scientific literature, the practice can lead to false conclusions. Whilst data transformation can help meet model assumptions, it can also impact the likelihood of Type I (false rejection of the null-hypothesis) errors. Using simulated non-normal data, McArdle & Anderson (2004) demonstrated a Type I error rate for ANOVA of 10.6% using raw data (greater than the 5% rate expected of normally-distributed data), increasing to 66.8% following log-transformation. These inflated error rates are a result of discrepancies between the arithmetic means and log-transformed means of data, and consequently errors will likely be present in any analyses comparing sample means. Similar issues were evidenced by Feng et al (2014) who also cautioned against

the false assumption that log-transformations inherently achieve normality and reduce variation in all skewed data. Whilst these cases example log-transformation, fundamental discrepancies between mean and median values in raw and surrogate data will persist regardless of the type of transformation, thus the null-hypothesis being modelled, the outcome, and the resultant inferences are prone to the same misinterpretations. By avoiding data transformations and selection of statistical analyses compatible with the raw data distributions, studies in this thesis minimise the opportunities for an such errors or misinterpretations.

Post-hoc analyses can likewise lead to complications in interpretation of statistical models – particularly when including multiple potential pairwise comparisons. As stated in Chapter 2, false-detection rate (FDR) was controlled for using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995), however any such method for controlling FDR, whilst controlling against Type I errors, runs the risk of introducing Type II errors (Craiu & Sun, 2008) – especially when correcting for a large number of extraneous pairwise combinations lacking ecological relevance. These issues of family-wise error corrections grow when contrasting over several factor levels using linear models such as ANOVA – especially when treatment combinations display treatment-dependent interactions, complicating the baseline average against which the model compares. This becomes fully apparent when subsetting a dataset to mimic a plausible reduced experimental design, which would otherwise result in a statistically significant result. For this reason, statistical probabilities with an without correction for FDR were presented, allowing for more informed interpretation of the statistical analysis.

Replication limitations, whatever their cause, can also substantially impact a study. As explained in Chapter 2, the scope of the studies was modulated in favour of providing better replication and statistical power – this does however impact on the selection of

analyses and the inferences that can be drawn for each study. For example, restricting a continuous driver variable to only two discrete treatment levels as in the case of calcium concentrations in the *Lymnaea* study necessitates a linear or comparative approach to modelling (J. Antony, 2014). Whilst appropriate for the given data, such an approach would fail to capture concentration-dependent effects and non-linear behaviours such as those evident in the *Magallana* study. Thus, whilst limited scope studies can provide specific comparative results, they lack the resolution and variability across driver levels required for more meaningful mechanistic or predictive assessment.

Theoretical statistical replication can also be eroded by experimental realities, for example left-censoring. Left-censoring is a term commonly used in survival analyses (such as Kaplan-Meyer used in Chapter 2) to describe the removal of a replicate from statistical consideration. Left-censoring may result from mortality (where mortality isn't the focal endpoint), or withdrawal of a subject from the experiment for other reasons, and can introduce bias in survival analyses as the models apply an equal, proportional weighting to each uncensored individual in the models (Cain et al., 2011). However, upon being censored, the weighting of the removed individual is equally distributed amongst the remaining participants. Resultantly, as effective replication numbers decrease, individuals have a proportionally larger impact on the survival curves. Therefore, if left censoring is disproportionate amongst treatment groups, the statistical comparisons can become less reliable, and therefore the use of the analysis, and/or the validity of any statistical outcome should be considered carefully.

Reduction in effective replication resultant of experimental mortalities is evident in the analyses of developmental endpoints of all three studies presenting in this thesis. It is most evident in the biometric and behavioural analyses of the *Nephrops* study where

mortality between treatment groups ranged from 15% to 100%. Conversely, effective replication for analysis of the principal cause of hatching failure in the *Lymnaea* study was considerably limited due to the low incidences of failed hatching. Whilst the respectively high and low rates of incidence in the aforementioned examples somewhat preclude the need for statistical analysis to understanding any likely ecological significance, or lack thereof, the data would nonetheless be valuable for understanding mechanistic effects. Data on incidence rates could also be used in conjunction with power analyses to inform replication of future research focusing on such studies (Cohen, 1992), but this is less helpful in retrospect. Thus, when conducting novel research, greater resource might need to be given to replication depending on the specific objectives – which may in turn require reductions in scope elsewhere to be achievable.

6.2.7 Suggestions and summary

Regarding suggestions for others intending to undertake multiple-driver studies, the advice is simply to fully understand what the primary research goals are, and to tailor the experimental design to achieving these. If this includes sound exposure, special consideration should be given to the sound frequency range of interest and the most appropriate way of achieving the sound exposures. Equally, thought should be given to how best to manage spatial distribution and mutually exclusivity of sound treatments.

It is worth acknowledging that whilst studies in this thesis employed full-factorial designs with the aim of understanding mechanistic effects, other experimental approaches can be employed. For example, approaches focussing on only certain axes of driver combinations can considerably reduce treatment groups and replication requirements – which may be suitable for establishing differences in effect, but limit context for mechanistic understanding. Such approaches may offer particular benefit

for preliminary range-finding studies, helping to refine the scope and replication needs of future studies, assuming this is a viable approach.

Regarding the choice of statistical approach, there is a strong argument for avoiding those requiring extensive use of *post-hoc* analyses where family-wise error may impact upon your statistical power and interpretations; though in reality this may not be possible if data availability, data distributions, and research questions most favourably suit such approaches. In these cases, it is advisable to include statistical probabilities both of direct, uncorrected statistical comparisons, and those correcting for FDR (by a suitably appropriate method) as employed throughout this thesis. These data can then be fully and transparently interpreted by future audiences, enabling them to draw their own conclusions on the validity of each approach and their requirements of statistical certainty.

In summary, the studies undertaken in this thesis were not without limitations – most of which were understood prior to their undertaking, and others which were more emergent in nature. Nonetheless, the comparative lack of understanding pertaining to both sound and multiple driver impacts on aquatic organisms arguably instils value of any such experimental undertaking, assuming limitations are understood and interpretations suitably caveated and constrained. To this end, such undertakings should not be dismissed out of hand. Rather, as with all research, such studies should be weighed and considered against the benefits they offer despite their shortcomings – particularly when concerning drivers or research questions that are otherwise prohibitively constrictive to address.

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Appendix A: Supplementary Materials to Chapter 3: Effects of Pile Driving Playbacks and Cadmium Co-exposure on the Early Life Stage Development of the Norway Lobster, *Nephrops norvegicus*

Methods

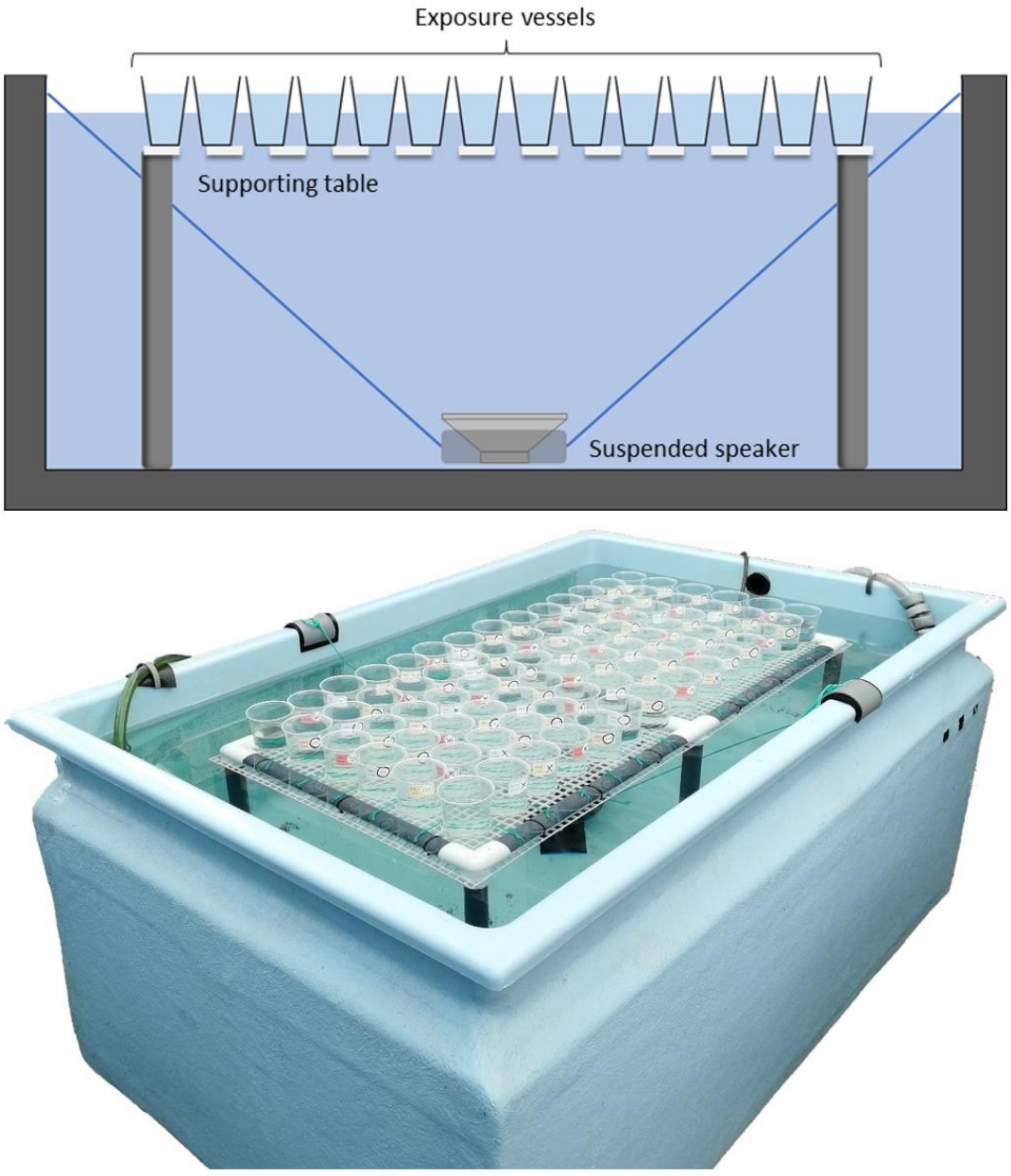

Figure A1 Top: Longitudinal cross-sectional schematic of the exposure systems. Bottom: Photograph showing exposure vessels within an exposure system.

Chemical exposures

6.2.7.1 Dosing solutions

Cadmium dosing solutions were made from a 1 $g_{[Cd]}$ L^{-1} primary stock solution of cadmium chloride (Sigma Aldrich, CAS# 654054-66-7, 99.995%) prepared in deionised (DI) water. Dosing solutions were subsequently created from the primary stock via serial dilution with DI water.

Concentrations of dosing solutions and dilution factors varied between experiments according to required working volumes. For Experiment 1, where working volumes were lower, dosing solutions of concentrations of 0 $\mu g_{[Cd]}$ L^{-1}, 250 $\mu g_{[Cd]}$ L^{-1}, 2500 $\mu g_{[Cd]}$ L^{-1}, 25000 $\mu g_{[Cd]}$ L^{-1} were created. For Experiment 2, dosing solutions of concentrations of 0 $\mu g_{[Cd]}$ L^{-1}, 800 $\mu g_{[Cd]}$ L^{-1}, 8000 $\mu g_{[Cd]}$ L^{-1}, 80000 $\mu g_{[Cd]}$ L^{-1} were utilised (Table A6.1).

Table A6.1: Details of the dilution series for creation of cadmium dosing solutions

	To Create:	Stock to Use	Quantity of Stock	Volume of deionised water (ml)	Dosing Stock Cd^{2+} concentration
Experiment 1					
	Primary stock	$CdCl_2$	0.082 g	50.00	1 g L^{-1}
	$High_{[Cd]}$ stock	Primary Stock	0.833 ml	32.50	25000 µg L^{-1}
	$Medium_{[Cd]}$ stock	$High_{[Cd]}$ stock	3.30 ml	29.70	2500 µg L^{-1}
	$Low_{[Cd]}$ stock	$Medium_{[Cd]}$ stock	3.00 ml	27.00	250 µg L^{-1}
Experiment 2					
	Primary stock	$CdCl_2$	0.082 g	50.00	1 g L^{-1}
	$High_{[Cd]}$ stock	Primary Stock	4.000 ml	46.00	80000 µg L^{-1}
	$Medium_{[Cd]}$ stock	$High_{[Cd]}$ stock	5.00 ml	45.00	8000 µg L^{-1}
	$Low_{[Cd]}$ stock	$Medium_{[Cd]}$ stock	5.00 ml	45.00	800 µg L^{-1}

6.2.7.2 Dosing dynamics

Chemical exposures were conducted under semi-static renewal conditions, with 95% water changes and full cadmium renewal each water change. Water changes occurred twice weekly during Experiment 1 and daily during Experiment 2.

At each dosing interval, one millilitre of dosing solution was added to 249ml (Experiment 1) and 799 ml (Experiment 2) of UV sterilised seawater, resulting in final working volumes of desired nominal concentrations. Where mortalities occurred in individual replicates, both dosing and dilution volumes were adjusted proportionately to maintain comparable stocking densities and exposure concentrations.

In all cases, dosing solutions were diluted into working solutions in the absence of larvae to prevent exposure to excessive cadmium concentrations. The large dilution factors from dosing stocks to working solutions were similarly chosen to increase likelihood of achieving near-nominal cadmium concentrations, as well as to limit the undesirable reduction of salinity in the exposure vessels.

Experiment 1: Phenomenological effects

A total of 160 ZI larvae were utilised. Larvae were evenly distributed between treatment groups resulting in n=20 independent replicates per treatment. Due to timing of hatching, larvae were allocated over a two-day period, with 80 larvae allocated on each of the two days.

Larvae were maintained individually in 330 ml BPA-free, food-grade virgin polypropylene plastic cups containing 250 ml of UV sterilised seawater arranged with the exposure system according to their assigned cadmium concentration in a 14 x 6 Latin-square array to account for environmental factors and sound gradient effects (Figure A2)

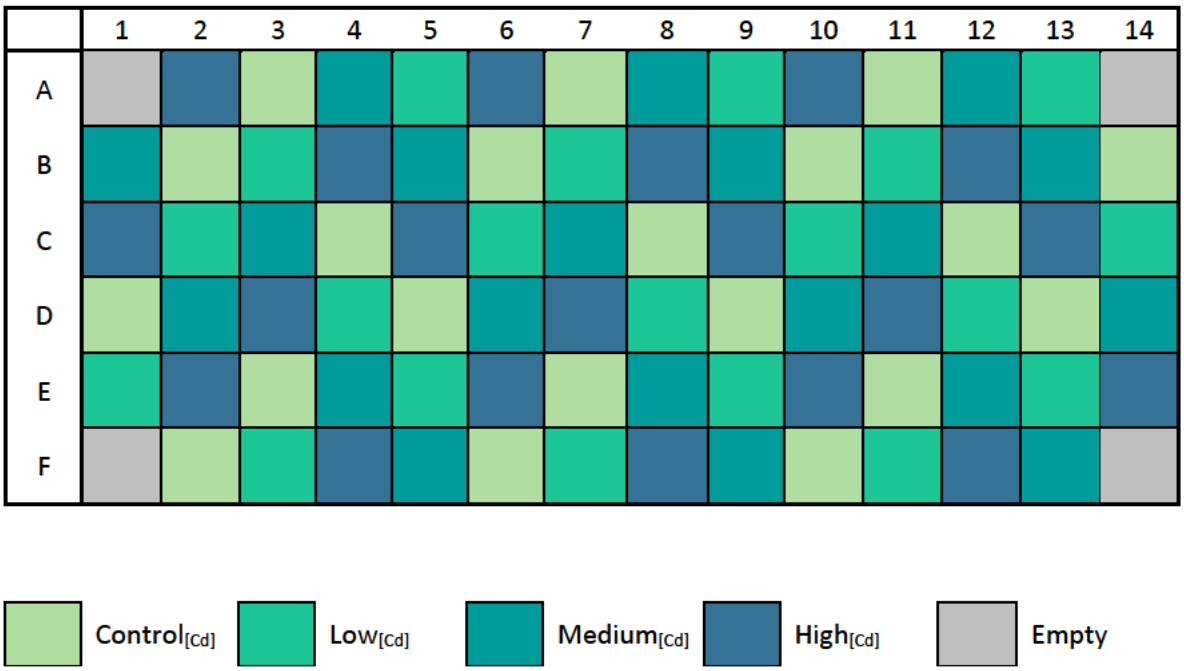

Figure A2: Schematic showing Latin-square arrangement of cadmium treatments within the exposure systems. *Alphanumeric indices refer to the equally spaced positions on the acoustically transparent table as shown in Figure A1*

Experiment 2: Mechanistic effects

A total of 672 larvae were utilised for this study. Exposures were undertaken in 1000 ml borosilicate glass beakers, containing a maximum of 800 ml of UV sterilised seawater of a stated nominal cadmium concentration. To provision sufficient tissue quantities to enable biomarker analyses, 12 larvae were allocated to each exposure vessel, with each exposure vessel being regarded as a single independent replicate. Over the course of 12 days, a total of seven replicates was set up for each treatment group.

Exposure vessels were randomly allocated to one of 16 positions within the central portion of the exposure system where sound levels were most consistent (Figure A3).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A					**1**		**5**		**9**		**13**			
B					**2**		**6**		**10**		**14**			
C					**3**		**7**		**11**		**15**			
D					**4**		**8**		**12**		**16**			
E														
F														

Figure A3: Schematic showing available positions of cadmium treatments within the exposure systems. *Alphanumeric indices refer to the equally spaced positions on the table as shown in Figure A1.*

Tissue homogenisation for oxidative stress assays

Replicate whole-organism samples were homogenised in 800 µl Tris-HCl (50 mM, 0.15 M KCl, pH 7.4) buffer solution using a motorised pestle, and spun at 10,000 RPM for three minutes in an Eppendorf Mini Spin centrifuge. The resulting supernatant was split into aliquots of sufficient volume for each of the oxidative stress assays, and these aliquots re-frozen at -80 °C until required. This splitting and aliquoting step minimised requirement for repeated freezing/thawing of the samples and assisted with standardisation of each tissue sample between assays. Quantitative assays were corrected for protein content as determined by Bradford assay).

Oxidative stress assays were conducted in 96 well plates using the colorimetric methods described below. In all instances, samples were plated and absorbance read in triplicate using a Spectramax M5 Multi-Mode Microplate Reader. Where required, standards, blanks, and positive controls were likewise conducted in triplicate within the same plate as samples for consistency and robustness.

Superoxide Dismutase (SOD) inhibition

Superoxide Dismutase inhibition was quantified using a Sigma-Aldrich SOD Determination Kit (19160). Each 20 µl of sample homogenate was combined with 200 µl of WST Working solution and 20 µl of Enzyme Working solution. In addition, three blanks were also created. Blank 1 replaced tissue homogenate with ultrapure (Milli-Q) water. Blank 2 replaced the Enzyme Working solution with Dilution buffer. Blank 3 replaced both the tissue homogenate and dilution buffer. Plates were incubated at 37 °C for 20 minutes prior to being read at 450 nm on the plate reader. SOD inhibition was then calculated using the equation:

$$\text{SOD inhibition rate } (\%) = \frac{(\text{A}_{\text{Blank 1}} - \text{A}_{\text{Blank 3}}) - (\text{A}_{\text{Sample}} - \text{A}_{\text{Blank 2}})}{(\text{A}_{\text{Blank 1}} - \text{A}_{\text{Blank 3}})} \times 100$$

Catalase (CAT)

Catalase activity was quantified using a Cayman Chemical Catalase Assay Kit (707002), utilising the peroxidatic conversion of methanol to formaldehyde. Firstly, 20 µl of sample homogenate was combined with 100 µl of diluted Assay Buffer, 30 µl of methanol, and the catalytic reaction initiated by addition of 20 µl of diluted hydrogen peroxide. Plates were then covered and incubated at room temperature on a plate shaker for 20 minutes before the reaction was terminated by addition of 30 µl of potassium hydroxide. Colour was developed by addition of 30 µl of Catalase Purpald, a further 10-minute room-temperature incubation on the plate shaker, addition of 10 µl of catalase potassium periodate, and a final five-minute incubation on the plate shaker at room temperature. Absorbance was then read at 540 nm, and samples compared to a range of formaldehyde standards and a catalase positive control developed using the same method.

Glutathione (GSH)

Glutathione concentration was determined according to methods outlined by Smith et al. (2007) adapted from (Owens & Belcher, 1965). Each 20 µl homogenate sample was combined with 20 µl of 10 mM 5,5-dithiobis-2-nitrobenzoic acid) (DNTB), 260 µl Tris-HCl (50 mM, 0.15 M KCl, pH 7.4) buffer, and 20 µl of 2U ml^{-1} glutathione reductase (GR). Reaction was initiated by addition of 20 µl NADPH, samples incubated at room temperature for six minutes, and absorbance read at 412 nm against a 125-1000 µM GSH standard range.

Glutathione Peroxidase (GPx)

Glutathione peroxidase was quantified using Cayman Chemical Glutathione Peroxidase Assay Kit (703102). The assay indirectly measures GPx activity through a coupled reaction with glutathione reductase (GR), whereby glutathione is oxidised and subsequently recycled to a reduced state by GPx and GR respectively. Each 20 µl homogenate sample was combined with 50 µl of Assay Buffer, 50 µl of Co-Substrate Mixture, and 50 µl NADPH. Redox reactions were initiated by addition of 20 µl of cumene hydroperoxidase, and the plate absorbance immediately read at 340 nm. Further absorbance readings at 340 nm were then taken at one-minute intervals for five minutes to produce activity curves. Sample absorbance readings were corrected for background non-enzyme related absorbance using background wells which replaced sample homogenate with additional Assay Buffer. GPx activity was then calculated using the following equations between two time-points along the linear proportion of the absorbance curves:

$$\Delta A_{340}/\text{min} = \frac{A_{340}(\text{Time 2}) - A_{340}(\text{Time 1})}{\text{Time 2(min)} - \text{Time 1(min)}} \qquad \text{GPx activity} = \frac{\Delta A_{340}/\text{min}}{0.00373\ \mu\text{M}^{-1}} \times \frac{0.19\ \text{ml}}{0.02\ \text{ml}}$$

Thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) were quantified using a method derived from those described by from those described by (Bouskill et al., 2006; Camejo et al., 1998; C. J. Smith et al., 2007). Here, 40 µl tissue homogenate, 10 µl of 1M butylated hydroxytoluene (BHT) in ethanol, 140 µl of 1mM ethylenediaminetetraacetic acid phosphate buffered saline (EDTA PBS) at pH 7.4, 50 µl of 50% (w/v) trichloroacetic acid (TCA), and 75 µl of 1.3% (w/v) thiobarbituric acid (TBA) in 0.3% (w/v) sodium hydroxide (NaOH) were combined. Plates were then incubated at 60 °C for one hour, and absorbance read at 530 nm and 630 nm wavelengths, and absorbance calculated as, $\Delta A_{TBARS} = A_{530} - A_{630}$. Samples were then compared against a 0.5-25 nM 1,1,3,3-tetraethoxypropane (TEP) in ethanol standard range.

Protein quantification (Bradford assay)

Sample protein was quantified using the method outlined by (Bradford, 1976). Sample homogenates were diluted 1:10 using Tris-HCl buffer, and in quadruplicate, 10 µl of diluted homogenate combined with 290 µl of Bradford reagent. Plates were incubated at room temperature for five minutes, and absorbance read at 595 nm. Samples were then compared against a 0-1000 µg L^{-1} bovine serum albumin (BSA) standard range.

Metallothionein (MT)

Metallothioenein was quantified in accordance with the methods derived from (Viarengo et al., 1997) and (Cenov et al., 2018), excepting the homogenisation buffer which was as previously described, but with the addition of 0.01% v/v 2-mercaptoethanol as a reducing agent. The tissue homogenate was further centrifuged at 20,000 x g for 20 minutes, and 200 µl of supernatant extracted. To the supernatant, 210 µl of cold (-20 °C) absolute ethanol and 16 µl of chloroform were added, and the

samples centrifuged cold (0-4 °C) at 6000 x g for 10 minutes. The supernatant was extracted, and three volumes of cold (-20 °C) absolute ethanol added, and the solution left to precipitate at -20 °C for one hour, before being centrifuged at 6000 x g for 10 minutes. The resulting pellets were washed using ethanol:chloroform homogenization buffer (87:1:12), centrifuged again at 6000 x g for 10 minutes, and the pellets dried under a nitrogen gas stream to complete evaporation. Dried pellets were resuspended in 300 µl of 5mM Tris-HCl, 1 mM EDTA, pH 7, and 20 µl of the resuspended metallothionein fraction combined with 280 µl of 0.43mM DNTB buffered to pH 8 using 0.2 M phosphate buffer. Samples were incubated at room temperature for 30 minutes, and absorbance read at 412 nm against a 0-1000 µM GSH standard range assuming 18 Cys residues per metallothionein residue (Cenov et al., 2018; Zhu et al., 1994).

Results

Sound exposures

A)

Ambient sound treatment: RMS SPL (dB re: 1 µPa)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	117.6	117.3	118.1	117.8	118.1	118.1	118.4	117.9	118.4	118.4	118.1	117.8	117.8	117.4
B	118.4	118.0	118.2	118.2	118.0	118.4	118.5	118.5	118.5	118.5	117.6	117.9	118.0	117.5
C	118.2	118.2	118.1	118.0	118.1	117.9	118.5	118.6	117.6	118.5	117.9	117.8	117.8	117.6
D	118.1	117.9	118.1	118.2	118.0	118.1	117.4	118.5	118.7	118.3	117.7	117.8	117.9	117.7
E	118.0	117.8	117.8	118.0	117.5	118.2	118.4	118.3	118.7	118.0	118.0	117.8	117.7	117.7
F	118.1	117.8	117.5	118.0	118.1	118.2	118.3	118.3	118.6	118.0	117.8	117.9	117.7	117.2

Piling sound treatment - ambient phase: RMS SPL (dB re: 1 µPa)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	117.8	117.9	118.0	118.1	118.8	118.1	118.1	117.7	117.8	118.0	118.3	118.0	118.4	118.0
B	118.0	118.1	118.0	118.2	118.4	118.3	117.8	117.7	118.0	118.1	118.1	118.1	118.4	118.0
C	117.9	118.2	118.0	118.0	118.3	118.6	117.6	117.7	118.2	117.9	118.1	118.4	118.4	118.1
D	117.8	118.1	118.1	118.1	118.2	118.3	117.7	118.0	118.2	117.4	118.2	118.5	118.2	118.2
E	117.8	118.3	118.1	118.2	118.4	118.2	117.7	118.1	118.1	118.0	118.0	118.3	118.2	118.0
F	117.8	118.1	118.0	118.5	118.1	118.1	117.8	118.1	117.8	118.0	118.0	118.4	118.1	117.9

B)

Piling sound treatment - piling phase: Peak-Peak SPL (dB re: 1 µPa)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	167.2	166.1	166.3	169.8	169.0	171.9	170.9	171.7	170.2	168.4	169.7	166.4	167.1	167.7
B	167.0	168.2	168.7	169.7	171.2	172.1	170.7	171.3	171.4	167.7	169.3	168.3	167.2	167.6
C	166.8	165.4	169.3	170.8	171.7	171.3	171.4	171.4	171.2	169.6	169.6	167.4	166.6	166.4
D	165.6	167.8	168.6	169.8	171.7	170.9	171.8	171.8	170.4	170.9	170.8	169.6	168.1	166.4
E	166.9	167.0	168.5	168.6	170.5	170.6	171.8	171.8	169.9	170.8	168.0	168.0	169.1	167.0
F	166.7	169.1	166.3	169.3	170.4	170.9	170.7	171.3	170.6	170.2	169.3	167.7	168.4	168.3

Piling sound treatment - piling phase: RMS SPL (dB re: 1 µPa)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	135.7	133.4	134.9	136.6	136.9	139.5	138.9	140.3	137.1	135.9	136.5	133.8	135.1	135.7
B	134.7	135.5	136.4	137.0	138.7	139.9	137.5	138.6	138.3	134.7	136.8	136.2	134.7	135.5
C	134.7	133.0	136.9	138.6	139.0	139.0	138.2	138.2	138.7	136.5	137.1	135.2	134.7	134.8
D	133.5	135.2	136.2	137.9	139.5	138.0	139.1	139.5	138.2	138.3	138.9	136.6	135.9	134.8
E	135.1	134.2	136.1	136.1	137.4	138.6	139.0	139.4	136.5	137.3	135.2	136.0	136.2	135.1
F	134.8	135.8	134.8	136.7	137.7	138.4	138.2	138.7	137.8	137.8	136.5	135.0	136.4	136.7

Figure A4: Experiment 1 sound pressure measurements. *Heatmap of received sound pressure levels within each exposure vessel as located in the exposure system during A) ambient-playback; B) piling-playback. All measurements are absolute values taken at each location. Alphanumeric indices refer to the equally spaced positions on the acoustically transparent table as shown Figure.*

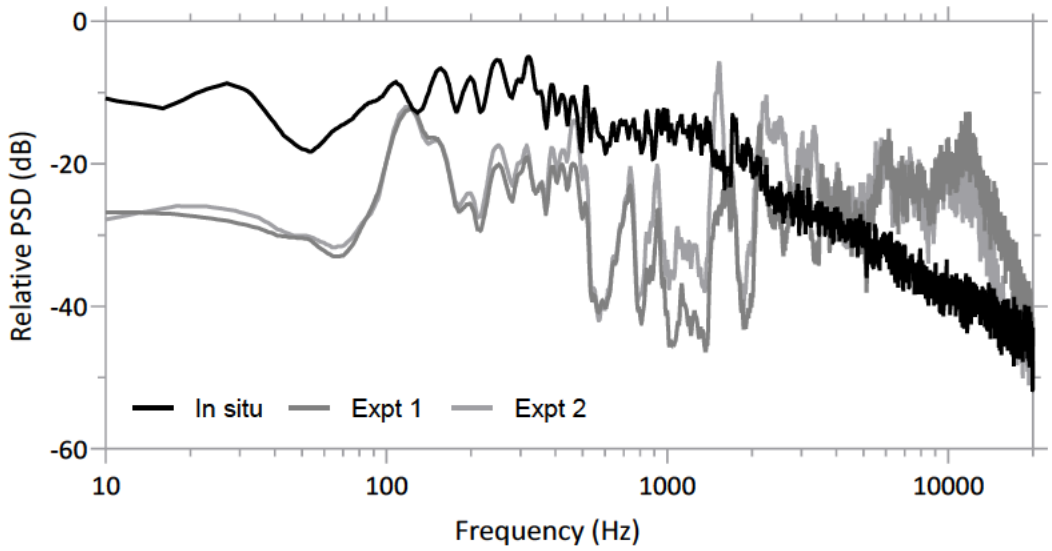

Figure A5: Relative RMS power spectral density of piling as recorded in situ and as received in exposure vessels via playbacks in each experiment. *(0.1 second Hann window, 50-percent overlap)*

Mortality

Total Mortality

Table A6.2: Statistical summary of logistic regression model of overall N. norvegicus mortality. *Bold values signify statistical significance $p < 0.05$*

	Estimate	SE	Z value	*p*
(Intercept)	-0.162	0.242	-0.668	0.504
Cadmium	0.474	0.218	2.173	**0.030**
Sound	-0.764	0.398	-1.923	0.055
Sound x cadmium	0.773	0.363	2.130	**0.033**

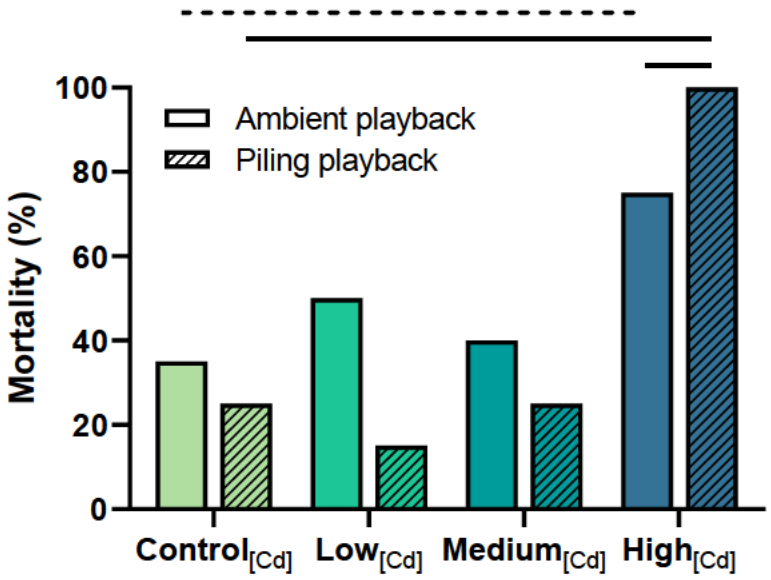

Figure A6: Total N. norvegicus larval mortality. Solid and hatched bars represent ambient- and piling-playback sound treatments respectively. *Control[Ca], Low[Ca], Medium[Ca], and High[Ca] represent Cd^{2+} ion concentrations of 0.08 µg L^{-1}, 0.71 µg L^{-1}, 6.48 µg L^{-1}, 63.52 µg L^{-1} respectively Horizontal markers above bars denote significant differences between groups (Fisher's exact test, dashed line uncorrected p <0.05, solid line corrected p <0.05).*

Temporal patterns of mortality

Table A6.3: Statistical summary of N. norvegicus larval mortality curves. *Post-hoc log-rank Mantel-Cox comparisons. Uncorrected p values represent those from pairwise comparison. Corrected p values are modified values controlling for false discovery rate using Benjamini-Hochberg procedure (α = 0.05). Bold values signify statistical significance p < 0.05*

Contrast treatments		df	Z	Uncorrected *p* value	Corrected *p* value
Ambient - Control[Cd]	Ambient - Low[Cd]	1	1.0057	0.314	0.454
	Ambient - Medium[Cd]	1	0.300	0.765	0.793
	Ambient - High[Cd]	1	2.017	**0.043**	0.100
Piling - Control[Cd]	Piling - Low[Cd]	1	-0.807	0.420	0.534
	Piling - Medium[Cd]	1	-0.036	0.971	0.971
	Piling - High[Cd]	1	4.464	**<0.001**	**<0.001**
Ambient - Control[Cd]	Piling - Control[Cd]	1	-0.643	0.521	0.561
Ambient - Low[Cd]	Piling - Low[Cd]	1	-2.310	**0.020**	0.051
Ambient - Medium[Cd]	Piling - Medium[Cd]	1	-1.039	0.300	0.454
Ambient - High[Cd]	Piling - High[Cd]	1	2.631	**0.005**	**0.015**

Developmental duration

Table A6.4: Statistical summary of N. norvegicus development.*Dunn's test post-hoc analysis of timing of transition to Zoea III (top) and Zoea IV (bottom). Uncorrected p values represent those from pairwise comparison. Corrected p values are modified values controlling for false discovery rate using Benjamini-Hochberg procedure (α = 0.05). Bold values signify statistical significance p < 0.05*

	Contrast treatments		n1, n2	Z	Uncorrected p value	Corrected p value
Transition to Zoea III	Ambient - Control[Cd]	Ambient - Low[Cd]	14, 12	-0.177	0.859	0.891
		Ambient - Medium[Cd]	14, 14	1.794	0.073	0.197
		Ambient - High[Cd]	14, 12	2.616	**0.009**	**0.042**
	Piling - Control[Cd]	Piling - Low[Cd]	16, 18	-1.568	0.117	0.209
		Piling - Medium[Cd]	16, 17	-2.630	**0.009**	**0.042**
		Piling - High[Cd]	16, 5	0.578	0.563	0.717
	Ambient - Control[Cd]	Piling - Control[Cd]	14, 16	1.558	0.119	0.209
	Ambient - Low[Cd]	Piling - Low[Cd]	12, 18	0.272	0.786	0.846
	Ambient - Medium[Cd]	Piling - Medium[Cd]	14, 17	-2.837	**0.005**	**0.042**
	Ambient - High[Cd]	Piling - High[Cd]	12, 5	-0.306	0.760	0.846
Transition to Zoea IV	Ambient - Control[Cd]	Ambient - Low[Cd]	14, 10	0.703	0.482	0.595
		Ambient - Medium[Cd]	14, 12	1.818	0.069	0.212
		Ambient - High[Cd]	14, 5	1.938	0.053	0.212
	Piling - Control[Cd]	Piling - Low[Cd]	15, 17	-1.649	0.099	0.212
		Piling - Medium[Cd]	15, 15	-2.744	**0.006**	0.050
		Piling - High[Cd]	-	-	-	-
	Ambient - Control[Cd]	Piling - Control[Cd]	14, 15	1.678	0.093	0.212
	Ambient - Low[Cd]	Piling - Low[Cd]	10, 17	-0.631	0.528	0.616
	Ambient - Medium[Cd]	Piling - Medium[Cd]	12, 15	-2.823	**0.005**	0.050
	Ambient - High[Cd]	Piling - High[Cd]	-	-	-	-

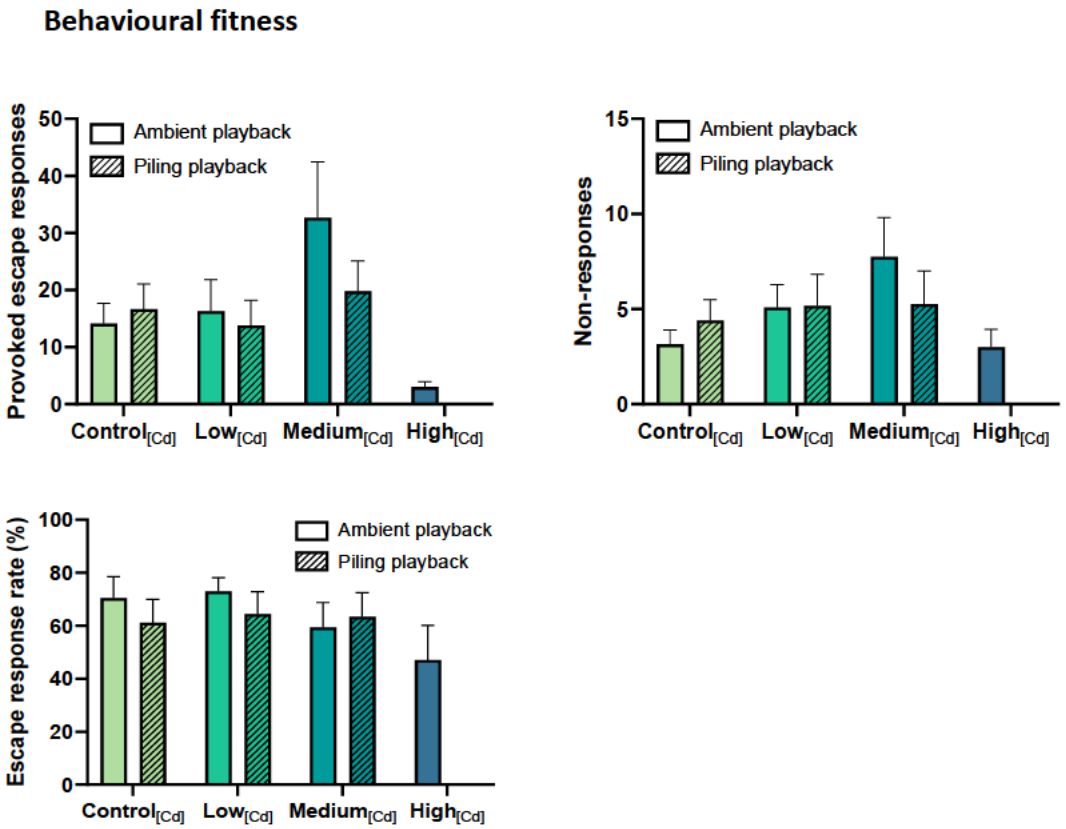

Figure A7: Responses of N. norvegicus juveniles to a simulated threat*. Top-left: number of escape responses provoked. Top-right: number of non-responses to simulated threat. Bottom-left: escape response rate. Solid and hatched bars represent ambient- and piling-playback sound treatments respectively. Control$_{[Cd]}$, Low$_{[Cd]}$, Medium$_{[Cd]}$, and High$_{[Cd]}$ represent Cd^{2+} ion concentrations of 0.08 µg L^{-1}, 0.71 µg L^{-1}, 6.48 µg L^{-1}, 63.52 µg L^{-1} respectively. All bars represent mean values. Error bars represent SE. Absent violins in piling – 100 µg$_{[Cd]}$ L^{-1}treatment consequent of no larvae surviving to metamorphosis)*

Table A6.5: Statistical summary of N. norvegicus responses to simulated threat.*Comparison by experimental treatment; Kruskal-Wallis test.*

Response	X^2	df	*p*
Total induced escape responses	5.37	6	0.498
Total non-responses	4.55	6	0.602
Response rate (%)	3.65	6	0.725

Table A6.6: Statistical summary of juvenile N. norvegicus escape responses.*Dunn's test post-hoc analysis of principal component scores of escape behaviour dynamics. Uncorrected p values represent those from pairwise comparison. Corrected p values are modified values controlling for false discovery rate using Benjamini-Hochberg procedure (α = 0.05). Bold values signify statistical significance p < 0.05*

	Contrast treatments		n1, n2	Z	Uncorrected *p* value	Corrected *p* value
Principle Component 1	Ambient - Control$_{[Cd]}$	Ambient - Low$_{[Cd]}$	679, 623	-0.206	0.837	0.878
		Ambient - Medium$_{[Cd]}$	679, 1076	8.633	**<0.001**	**<0.000**
		Ambient - High$_{[Cd]}$	679, 41	1.470	0.142	0.212
	Piling - Control$_{[Cd]}$	Piling - Low$_{[Cd]}$	782, 768	-1.167	0.243	0.301
		Piling - Medium$_{[Cd]}$	782, 849	-2.385	**0.017**	**0.030**
		Piling - High$_{[Cd]}$	-	-	-	-
	Ambient - Control$_{[Cd]}$	Piling - Control$_{[Cd]}$	679, 782	4.661	**<0.001**	**<0.001**
	Ambient - Low$_{[Cd]}$	Piling - Low$_{[Cd]}$	623, 768	3.648	**<0.001**	**0.001**
	Ambient - Medium$_{[Cd]}$	Piling - Medium$_{[Cd]}$	1076, 849	-6.466	**<0.001**	**<0.001**
	Ambient - High$_{[Cd]}$	Piling - High$_{[Cd]}$	-	-	**-**	**-**
Principle Component 2	Ambient - Control$_{[Cd]}$	Ambient - Low$_{[Cd]}$	679, 623	-1.765	0.078	0.116
		Ambient - Medium$_{[Cd]}$	679, 1076	-15.860	**<0.001**	**<0.001**
		Ambient - High$_{[Cd]}$	679, 41	-2.417	**0.016**	**0.027**
	Piling - Control$_{[Cd]}$	Piling - Low$_{[Cd]}$	782, 768	-1.664	0.096	0.135
		Piling - Medium$_{[Cd]}$	782, 849	-1.317	0.188	0.219
		Piling - High$_{[Cd]}$	-	-	-	-
	Ambient - Control$_{[Cd]}$	Piling - Control$_{[Cd]}$	679, 782	-3.264	**0.001**	**0.003**
	Ambient - Low$_{[Cd]}$	Piling - Low$_{[Cd]}$	623, 768	-2.927	**0.003**	**0.008**
	Ambient - Medium$_{[Cd]}$	Piling - Medium$_{[Cd]}$	1076, 849	11.781	**<0.001**	**<0.001**
	Ambient - High$_{[Cd]}$	Piling - High$_{[Cd]}$	-	-	-	-

Biometrrics

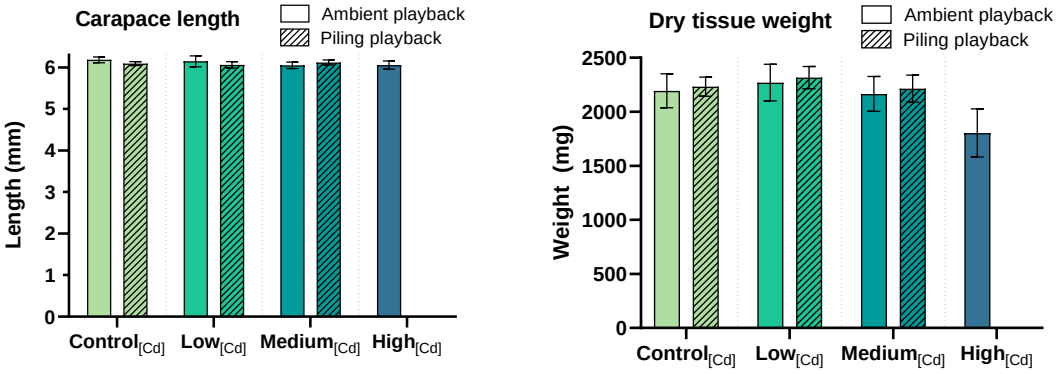

Figure A8: Biometric measurements of N. norvegicus. *Solid and hatched bars represent ambient- and piling playback sound treatments respectively. $Control_{[Cd]}$, $Low_{[Cd]}$, $Medium_{[Cd]}$, and $High_{[Cd]}$ represent Cd^{2+} ion concentrations of 0.08 µg L^{-1}, 0.71 µg L^{-1}, 6.48 µg L^{-1}, 63.52 µg L^{-1} respectively. All bars represent mean values. Error bars represent SE. Absent violin in piling – 100 $µg_{[Cd]}$ L^{-1}treatment consequent of no larvae surviving to metamorphosis.*

Table A6.7: Statistical summary of N. norvegicus oxidative stress biomarker measurements.*Top: Results of two-way analysis of variance contrasting the effects of sound and cadmium. Bottom: Results of Kruskal-Wallis Rank Sums by experimental treatment groups.*

ANOVA

Biomarker	Factor	df	Sum Sq	Mean Sq	F	*p* value
GPx	Sound	1	0.07	0.068	0.011	0.917
	Cadmium	3	40.54	13.513	2.181	0.103
	Sound x cadmium	3	36.59	12.197	1.968	0.132
	Residuals	46	285.05	6.197		
GSH	Sound	1	4808	4808	3.790	0.058
	Cadmium	3	2812	937	0.739	0.534
	Sound x cadmium	3	5777	1926	1.518	0.222
	Residuals	46	58358	1269		
SOD	Sound	1	0.30	0.282	0.019	0.890
	Cadmium	3	56.30	18.755	1.294	0.288
	Sound x cadmium	3	21.00	6.996	0.483	0.696
	Residuals	46	666.70	14.494		

Kruskal-Wallis Rank Sums

Biomarker	χ^2	df	*p* value
CAT	2.8044	7	0.902
TBARS	7.3269	7	0.396
MT	14.565	7	**0.032**

Table A6.8: Statistical summary of N.norvegicus metallothionein measurements.*Dunn's test post-hoc analysis. Uncorrected p values represent those from pairwise comparison. Corrected p values are modified values controlling for false discovery rate using Benjamini-Hochberg procedure (α = 0.05). Bold values signify statistical significance p < 0.05*

Contrast treatments		n1, n2	Z	Uncorrected *p* value	Corrected *p* value
Ambient - Control$_{[Cd]}$	Ambient - Low$_{[Cd]}$	7, 6	0.000	1.000	1.000
	Ambient - Medium$_{[Cd]}$	7, 7	1.580	0.114	0.355
	Ambient - High$_{[Cd]}$	7, 7	-0.323	0.747	0.863
Piling - Control$_{[Cd]}$	Piling - Low$_{[Cd]}$	7, 6	-1.137	0.256	0.550
	Piling - Medium$_{[Cd]}$	7, 7	-2.718	**0.007**	0.092
	Piling - High$_{[Cd]}$	7, 7	-0.408	0.683	0.863
Ambient - Control$_{[Cd]}$	Piling - Control$_{[Cd]}$	7, 7	0.629	0.530	0.810
Ambient - Low$_{[Cd]}$	Piling - Low$_{[Cd]}$	6, 6	-0.514	0.607	0.810
Ambient - Medium$_{[Cd]}$	Piling - Medium$_{[Cd]}$	7, 7	-3.669	**<0.001**	**0.007**
Ambient - High$_{[Cd]}$	Piling - High$_{[Cd]}$	7, 7	0.544	0.587	0.810

Table A6.9: Statistical summary of N. norvegicus oxidative stress biomarker Principal Component Analysis.*Results of two-way analysis of variance contrasting the effects of sound and cadmium.*

	Factor	df	Sum Sq	Mean Sq	F	*p* value
Principle Component 1	Sound	1	4.02	1.341	0.583	0.629
	Cadmium	3	1.62	1.618	0.704	0.406
	Sound x cadmium	3	6.12	2.041	0.887	0.455
	Residuals	46	105.8	2.3		
Principle Component 2	Sound	1	2.48	0.826	0.889	0.454
	Cadmium	3	5.67	5.672	6.104	**0.017**
	Sound x cadmium	3	16.25	5.417	5.83	**0.002**
	Residuals	46	42.75	0.929		

Table A6.10: Statistical summary of N. norvegicus oxidative stress biomarker PC2 post hoc analysis.*Dunn's test. Uncorrected p values represent those from pairwise comparison. Corrected p values are modified values controlling for false discovery rate using Benjamini-Hochberg procedure (α = 0.05). Bold values signify statistical significance p < 0.05*

Contrast treatments		n1, n2	Z	Uncorrected *p* value	Corrected *p* value
Ambient - Control$_{[Cd]}$	Ambient - Low$_{[Cd]}$	7, 6	0.188	0.851	0.953
	Ambient - Medium$_{[Cd]}$	7, 7	-1.767	0.077	0.240
	Ambient - High$_{[Cd]}$	7, 7	0.781	0.435	0.692
Piling - Control$_{[Cd]}$	Piling - Low$_{[Cd]}$	7, 7	0.892	0.372	0.651
	Piling - Medium$_{[Cd]}$	7, 7	2.361	**0.018**	0.102
	Piling - High$_{[Cd]}$	7, 7	1.002	0.316	0.590
Ambient - Control$_{[Cd]}$	Piling - Control$_{[Cd]}$	7, 7	-0.238	0.812	0.947
Ambient - Low$_{[Cd]}$	Piling - Low$_{[Cd]}$	6, 7	0.459	0.646	0.823
Ambient - Medium$_{[Cd]}$	Piling - Medium$_{[Cd]}$	7, 7	3.890	**<0.001**	**0.003**
Ambient - High$_{[Cd]}$	Piling - High$_{[Cd]}$	7, 7	-0.017	0.986	0.986

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Appendix B: Supplementary Materials to Chapter 4: Effects of Ferry Noise on the Embryonic Development of the Pond Snail, Lymnaea stagnalis, at Different Waterborne Calcium Concentrations

Sound exposures

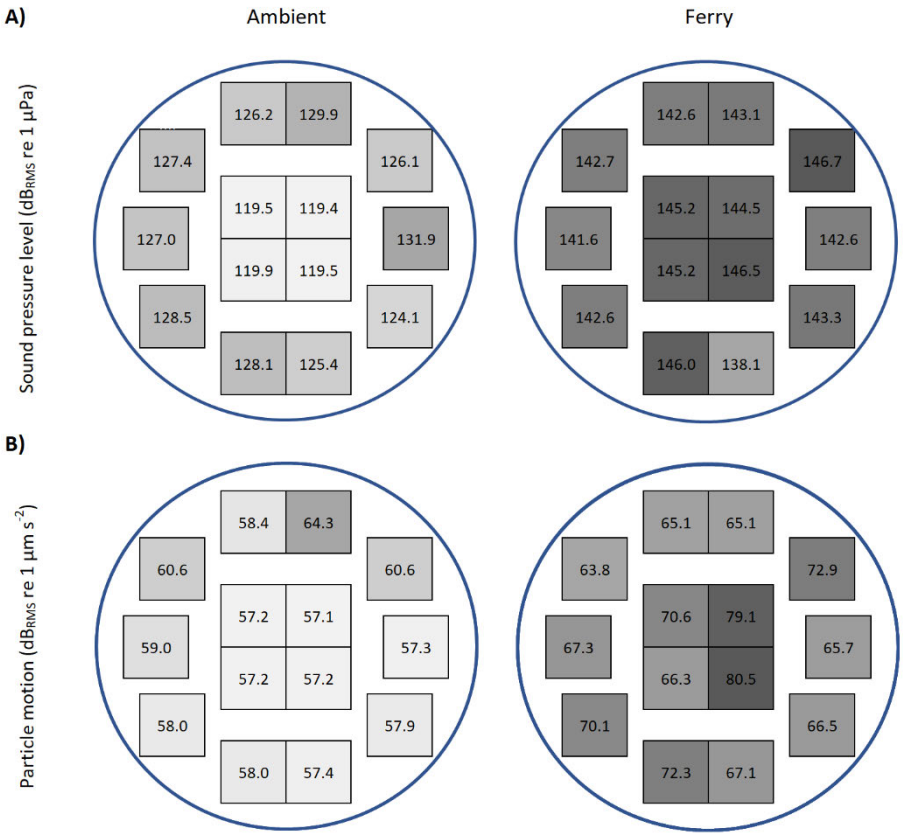


Figure B1: Sound pressure measurements. *Heatmap of received sound pressure levels at each exposure vessel location withing in an exposure system during A) ambient-playback; higher received sound levels of perimeter locations vessels compared to central regions reflect additional sound introduced by aeration in perimeter exposure vessels. B) ferry-playback; higher received sound levels of central locations compared to perimeter reflect the sound cone produced by the speaker.*

Fecundity assay biometrics

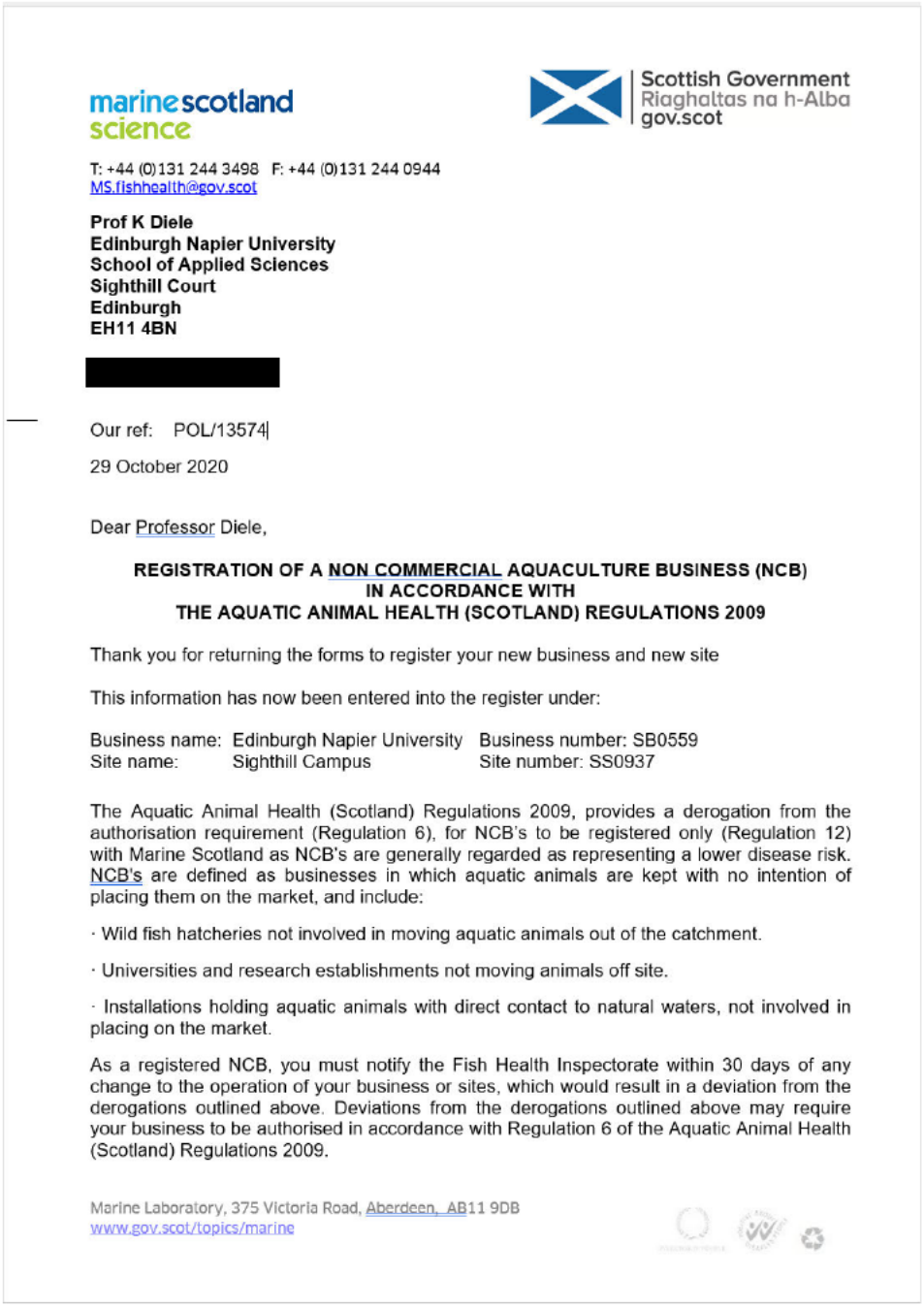
Table B6.11: Biometric measurements of mature L. stagnalis at time of allocation, and completion of the 28-day fecundity assay. *All values represent mean ± SD. Day -4 represents time of allocation. Day 28 represents study completion.*

Sound	Calcium	Exposure vessel	Length (mm) ± SD		Weight (g) ± SD	
			Day -4	Day 28	Day -4	Day 28
Ferry	Low	1	25.18 ± 1.31	25.81 ± 1.53	0.85 ± 0.13	0.96 ± 0.13
Ferry	Low	2	25.08 ± 1.24	26.38 ± 1.13	0.85 ± 0.15	1.03 ± 0.15
Ferry	Low	3	25.36 ± 1.60	26.63 ± 1.47	0.84 ± 0.20	1.01 ± 0.21
Ferry	Low	4	25.44 ± 1.42	26.65 ± 0.97	0.90 ± 0.08	1.09 ± 0.16
Ferry	Low	5	25.14 ± 1.04	26.13 ± 1.34	0.90 ± 0.09	1.06 ± 0.16
Ferry	High	6	25.12 ± 1.19	25.87 ± 1.53	0.87 ± 0.09	0.96 ± 0.13
Ferry	High	7	25.22 ± 1.43	25.72 ± 0.56	0.84 ± 0.19	0.96 ± 0.07
Ferry	High	8	25.30 ± 1.38	26.19 ± 1.41	0.87 ± 0.19	1.00 ± 0.19
Ferry	High	9	25.46 ± 1.45	25.80 ± 0.67	0.84 ± 0.11	0.89 ± 0.08
Ferry	High	10	25.17 ± 1.14	25.95 ± 1.05	0.89 ± 0.14	0.97 ± 0.19
Ambient	Low	11	25.14 ± 1.29	26.11 ± 1.17	0.90 ± 0.16	0.97 ± 0.16
Ambient	Low	12	25.14 ± 1.28	26.46 ± 0.71	0.93 ± 0.12	1.12 ± 0.15
Ambient	Low	13	24.83 ± 1.54	25.34 ± 1.14	0.86 ± 0.17	0.91 ± 0.12
Ambient	Low	14	25.43 ± 1.43	26.21 ± 0.91	0.93 ± 0.08	1.02 ± 0.05
Ambient	Low	15	25.15 ± 1.06	26.51 ± 0.62	0.88 ± 0.07	1.05 ± 0.09
Ambient	High	16	25.13 ± 1.26	26.83 ± 1.15	0.77 ± 0.12	1.03 ± 0.03
Ambient	High	17	25.15 ± 1.33	26.12 ± 1.28	0.89 ± 0.18	1.01 ± 0.16
Ambient	High	18	25.37 ± 1.51	26.63 ± 1.39	0.92 ± 0.21	1.06 ± 0.23
Ambient	High	19	25.45 ± 1.42	26.37 ± 1.08	0.87 ± 0.13	0.97 ± 0.14
Ambient	High	20	25.17 ± 1.09	26.06 ± 1.02	0.86 ± 0.16	0.96 ± 0.17

Appendix C: Supplementary Material to Chapter 5: Effects of Ferry and Pile Driving Playbacks on the Fertilisation and Immediate Development of the Pacific Oyster, *Magallana gigas*, at Different Salinities

Import of Magallana gigas

Non-commercial aquaculture business status



marinescotland science

Scottish Government
Riaghaltas na h-Alba
gov.scot

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MS.fishhealth@gov.scot

Prof K Diele
Edinburgh Napier University
School of Applied Sciences
Sighthill Court
Edinburgh
EH11 4BN

[redacted]

Our ref: POL/13574

29 October 2020

Dear Professor Diele,

REGISTRATION OF A NON COMMERCIAL AQUACULTURE BUSINESS (NCB) IN ACCORDANCE WITH THE AQUATIC ANIMAL HEALTH (SCOTLAND) REGULATIONS 2009

Thank you for returning the forms to register your new business and new site

This information has now been entered into the register under:

Business name: Edinburgh Napier University Business number: SB0559
Site name: Sighthill Campus Site number: SS0937

The Aquatic Animal Health (Scotland) Regulations 2009, provides a derogation from the authorisation requirement (Regulation 6), for NCB's to be registered only (Regulation 12) with Marine Scotland as NCB's are generally regarded as representing a lower disease risk. NCB's are defined as businesses in which aquatic animals are kept with no intention of placing them on the market, and include:

· Wild fish hatcheries not involved in moving aquatic animals out of the catchment.

· Universities and research establishments not moving animals off site.

· Installations holding aquatic animals with direct contact to natural waters, not involved in placing on the market.

As a registered NCB, you must notify the Fish Health Inspectorate within 30 days of any change to the operation of your business or sites, which would result in a deviation from the derogations outlined above. Deviations from the derogations outlined above may require your business to be authorised in accordance with Regulation 6 of the Aquatic Animal Health (Scotland) Regulations 2009.

Marine Laboratory, 375 Victoria Road, Aberdeen, AB11 9DB
www.gov.scot/topics/marine

In accordance with Regulation 5 of the 2009 Regulations, the fish health inspectorate may require a NCB to be authorised, where it is considered that authorisation is necessary in order to prevent or limit the spread of disease. In this event, a written notice will be served to the operator to this effect.

NOTIFICATION OF INCREASED MORTALITY OR SUSPICION / PRESENCE OF A LISTED DISEASE

In accordance with Article 23 of The Aquatic Animal Health Regulations 2009, it is a legal requirement that you notify[1] the Fish Health Inspectorate immediately where you:

i. know or suspect that a listed disease[2] is present in aquatic animals;
ii. know or suspect that increased mortality has occurred or is occurring in aquatic animals.

AMENDMENTS TO BUSINESSES OR SITES

Any change to your business or site details, including the status of the business or site, the species held, contact details and changes to the facilities must be notified in writing to the Fish Health Inspectorate at Marine Scotland Marine Laboratory, Aberdeen within 30 days of the change taking place.

The status of a business may be either **active** or **deregistered** and the status of a site may be **active, inactive or deregistered. Active** refers to the status of a business or site that is stocked or fallow with the intention of stocking in the foreseeable future. **Inactive** refers to the status of a site that is fallow and is unlikely to be stocked in the foreseeable future and no longer requires authorisation. **Deregistered** refers to the status applied to a business or site that is fallow and no longer used for fish or shellfish production, where the lease has been surrendered.

Please complete the **'Application to Amend an Authorisation** or **Notify of a Change to Registration'** form should you require to make any changes to the registration of your business or site. Please contact the Fish Health Inspectorate, at Marine Scotland Marine Laboratory, Aberdeen at the address below, should you require this form.

Yours sincerely

[redacted]

Joe Triscott

Senior Fish Health Inspectorate

---

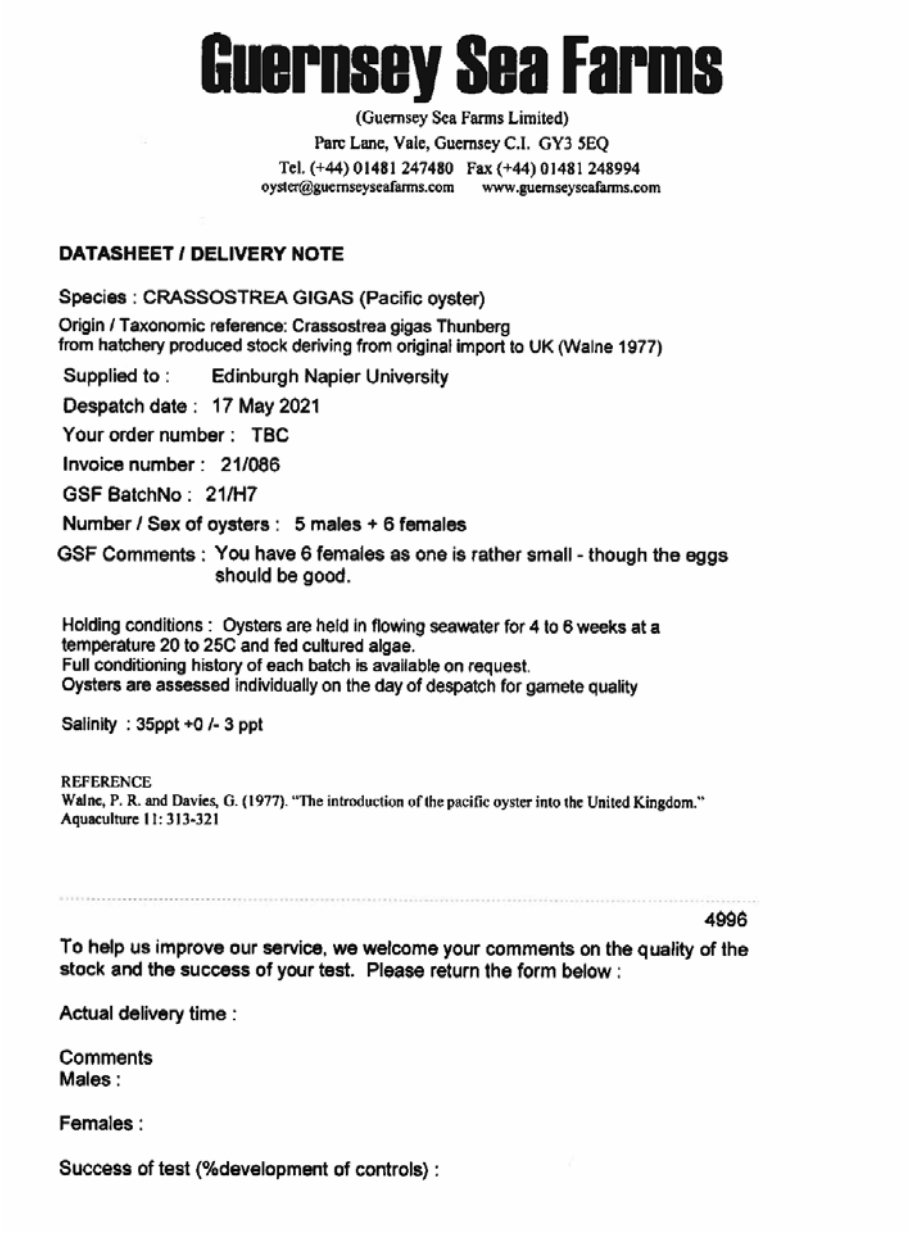
[1] Notification should be made in writing to the Fish Health Inspectorate, Marine Scotland Science, Marine Laboratory, 375 Victoria Road, Aberdeen AB11 9DB; by Fax to 0131 244 0944; or by email to MS.fishhealth@gov.scot

[2] "Listed disease" means–

(a) a disease listed in the second column of Part II of Annex IV of Council Directive 2006/88/EC [O.J. No. L 328, 24.11.2006, p. 14 as amended by Commission Directive 2008/53/EC (OJ No L. 117, 1.5.2008, p. 27). Please note that the most current version of Council Directive 2006/88/EC can be obtained from the European Union website (www.eur-lex.europa.eu) or by contacting Marine Scotland.]; or
(b) a disease listed in the first column of Schedule 1 to the Aquatic Animal Health (Scotland) Regulations 2009.

Figure C1: Confirmation of non-commercial aquaculture business status

Delivery note



Guernsey Sea Farms

(Guernsey Sea Farms Limited)
Parc Lane, Vale, Guernsey C.I. GY3 5EQ
Tel. (+44) 01481 247480 Fax (+44) 01481 248994
oyster@guernseyseafarms.com www.guernseyseafarms.com

DATASHEET / DELIVERY NOTE

Species : CRASSOSTREA GIGAS (Pacific oyster)
Origin / Taxonomic reference: Crassostrea gigas Thunberg
from hatchery produced stock deriving from original import to UK (Walne 1977)

Supplied to : Edinburgh Napier University
Despatch date : 17 May 2021
Your order number : TBC
Invoice number : 21/086
GSF BatchNo : 21/H7
Number / Sex of oysters : 5 males + 6 females
GSF Comments : You have 6 females as one is rather small - though the eggs should be good.

Holding conditions : Oysters are held in flowing seawater for 4 to 6 weeks at a temperature 20 to 25C and fed cultured algae.
Full conditioning history of each batch is available on request.
Oysters are assessed individually on the day of despatch for gamete quality

Salinity : 35ppt +0 /- 3 ppt

REFERENCE
Walne, P. R. and Davies, G. (1977). "The introduction of the pacific oyster into the United Kingdom." Aquaculture 11: 313-321

4996

To help us improve our service, we welcome your comments on the quality of the stock and the success of your test. Please return the form below :

Actual delivery time :

Comments
Males :

Females :

Success of test (%development of controls) :

Figure C2: Delivery note from Guernsey Sea Farms

Health certificate

Placing on the market of aquaculture animals for farming, relaying, put and take fisheries, open ornamental facilities and restocking. GBHC158

COUNTRY: BAILIWICK OF GUERNSEY

Health certificate to Great Britain from Northern Ireland and the Crown Dependencies

Part I: Details of dispatched consignment

I.1. Consignor
Name **Mark Dravers**
Address **Guernsey Sea Farms**
Le Parcq Lane
Vale
Guernsey
GY3 5EQ
Tel. **+44 (0)1481 247480**

I.2. Certificate reference number: **WK/202101223**

I.2.a. Local reference number: **GB06101**

I.3. Central Competent Authority: **DEFRA (GB)**

I.4. Local Competent Authority: **STATES OF GUERNSEY (GG)**

I.5. Consignee
Name **Edinburgh Napier University**
Address **Sighthill Campus, 9 Sighthill court, Edinburgh, Scotland**

Postal Code **EH11 4BN**
~~Tel.~~

I.7. Country of origin	ISO code	I.8 Region of origin	I.9. Country of destination	ISO code
Guernsey	**GG**	**GGYLBM004**	**Great Britain**	**GB**

I.11. Place of origin

Approved aquaculture holding ☒ Other ☐
Name **Mark Dravers**
Address **Guernsey Sea Farms**
Le Parcq Lane
Vale
Postal Code **GY3 5EQ**
Approval number: **GX117**

I.12. Place of destination

Approved aquaculture holding ☒ Other ☐
Name **Edinburgh Napier University**
Address **Sighthill Campus, 9 Sighthill court, Edinburgh, Scotland**
Site number: **SS0937**

Postal Code **EH11 4BN**

I.13. Place of loading: **Guernsey**

I.14. Date and time of departure: **Monday 17th May 2021**

I.15. Means of transport

Aeroplane ☒ Ship ☐ Railway wagon ☐
Road vehicle ☐ Other ☐

Identification: **DHL**
Documentary references: **N/A**

I.16. Transporter

Name **DHL International (UK) Ltd**

Address **Unit 8-9 Dunbenth Road, Elgin Ind., Swindon**

Postal Code **SN2 8EA**

I.17.

I.18. Description of commodity: **Oysters for research**

I.19. Commodity code (HS code): **0307**

I.21.

I.20. Quantity: **2kg**

I.22. Number of packages: **1**

I.23. Seal/Container No.

I.24. Type of packaging: **QR**

I.25. Commodity certified for:

Breeding ☒ Game restocking ☐ Relaying ☐ Pets ☐ Quarantine ☐ Other ☐

I.28. Identification of the commodities

Species (Scientific Name)	Quantity
Crassostrea gigas	**10**

States of Guernsey GG011 Authorised Veterinarian

Version 1.0 Nov 2020

[redacted]/6

COUNTRY: BAILIWICK OF GUERNSEY

Placing on the market of aquaculture animals for farming, relaying, put and take fisheries, open ornamental facilities and restocking

Part II: Certification

II. Health information

II.a. Certificate reference number	II.b
WX/202101223	

II.1 **General requirements**

I, the undersigned official inspector, hereby certify that the aquaculture animals referred to in Part 1 of this certificate:

either

II.1.1 (1) [have been inspected within ~~(1) (2) [72]~~ (2) [24] hours of loading, and showed no clinical signs of disease]

~~*or*~~

~~(1) in the case of eggs and molluscs, come from a farm or molluse farming area where, according to the records of the farm or molluse farming area, there is no indication of disease problems]~~

~~*or*~~

~~(1) (2) [in the case of wild aquatic animals, according to the best of my knowledge and belief are clinically healthy];~~

II.1.2 are not subject to any prohibitions due to unresolved increased mortality;

II.1.3 are not intended for destruction or slaughter for the eradication of diseases;

~~II.1.4 comply with the requirements for placing on the market laid down in Regulation (EC) 1251/2008;~~

II.1.5 (1) [in the case of molluscs, were subject to an individual visual check of each part of the consignment, and no molluscs species other than those specified in Part 1 of the certificate were detected.]

~~II.2 (1) (4) (5) [Requirements for species susceptible to Viral haemorrhagic septicaemia (VHS), Infectious haematopoietic necrosis (IHN), Infectious salmon anaemia (ISA), Koi herpes virus (KHV), Marteilia refringens, Bonamia ostreae, and/or White spot disease~~

~~I, the undersigned official inspector, hereby certify that: the aquaculture animals referred to above:~~

~~*either*~~

~~(1) (4) [originate from a territory, zone or compartment declared free from (1) [VHS] (1) [IHN] (1) [ISA] (1) [KHV] (1) [Marteilia refringens] (1) [Bonamia ostreae] (1) [White spot disease] in accordance with a process equivalent to Decision 2009/177.]~~

~~*or*~~

~~(1) (3) (4) [in the case of wild aquatic animals, have been subject to quarantine in accordance with Decision 2008/946/EC.]]~~

~~II.3 (1) (2) [Requirements for vector species to Viral haemorrhagic septicaemia (VHS), Infectious haematopoietic necrosis (IHN), Infectious salmon anaemia (ISA), Koi herpes virus (KHV), Marteilia refringens, Bonamia ostreae, and/or White spot disease~~

~~I, the undersigned official inspector, hereby certify that the aquaculture animals referred to above which are to be regarded as possible vectors to (1) [VHS] (1) [IHN] (1) [ISA] (1) [KHV] (1) [Marteilia refringens] (1) [Bonamia ostreae] (1) [White spot disease] as they are of species listed in Column 2 and fulfil the conditions set out in Column 3 of the table in Annex I to Regulation (EC) No 1251/2008:~~

~~*either*~~

~~(1) (4) [originate from a country/territory, zone or compartment declared free from (1) [VHS] (1) [IHN] (1) [ISA] (1) [KHV] (1) [Marteilia refringens] (1) [Bonamia ostreae] (1) [White spot disease] in accordance with a process equivalent to Decision 2009/177.]~~

~~*or*~~

~~(1) (4) (7) [have been subject to quarantine in accordance with Decision 2008/946/EC.]]~~

[redacted]

COUNTRY: BAILIWICK OF GUERNSEY

Placing on the market of aquaculture animals for farming, relaying, put and take fisheries, open ornamental facilities and restocking

II. Health information	II.a. Certificate reference number WK/202101223	II.b

II.4 **Transport and labelling conditions**

I, the undersigned official inspector, hereby certify that:

II.4.1 the aquaculture animals referred to above,

(i) are placed under conditions, including with a water quality, that do not alter their health status,

(ii) as appropriate, comply with the general conditions for the transport of animals laid down in Article 3 of Regulation (EC) No 1/2005;

II.4.2 the transport container or well boat prior to loading is clean and disinfected or previously unused; and

II.4.3 the consignment is identified by a legible label on the exterior of the container, or when transported by well boat, in the ship's manifest, with the relevant information referred to in boxes I.7 to I.13 of Part 1 of this certificate, and the following statement:

either (1) ['(1) ~~[Wild]~~ (1) ~~[Fish]~~ (1) [Molluscs] (1) ~~[Crustaceans]~~ intended for farming in Great Britain'],

~~or~~ (1) ['(1) ~~[Wild]~~ (1) ~~[Molluscs] intended for relaying in Great Britain'~~],

~~or~~ (1) ['(1) ~~[Wild]~~ (1) ~~[Fish]~~ (1) ~~[Molluscs]~~ (1) ~~[Crustaceans] intended for put and take fisheries in Great Britain'~~],

~~or~~ (1) ['(1) ~~[Wild]~~ (1) ~~[Ornamental fish]~~ (1) ~~[Ornamental molluscs]~~ (1) ~~[Ornamental crustaceans] intended for open ornamental facilities in Great Britain'~~],

~~or~~ (1) ['(1) ~~[Fish]~~ (1) ~~[Molluscs]~~ (1) ~~[Crustaceans] intended for restocking in Great Britain'~~],

~~or~~ (1) [']/[1] ~~[Wild]~~ (1) ~~[Fish]~~ (1) ~~[Molluscs]~~ (1) ~~[Crustaceans] intended for quarantine in Great Britain'~~],

~~II.5~~ (1)(2) ~~**[Attestation for consignments originating from an area subject to disease control measures**~~

~~I, the undersigned official inspector, hereby certify that:~~

~~II.5.1 the animals referred to above originate from an area subject to disease control measures regarding (1) [Epizootic haematopoietic necrosis (EHN)] (1) [Viral haemorrhagic septicaemia (VHS)] (1) [Infectious haematopoietic necrosis (IHN)] (1) [Infectious salmon anaemia (ISA)] (1) [Koi herpes virus (KHV)] (1) [Bonamia exitiosa] (1) [Perkinsus marinus] (1) [Mikrocytos mackini] (1) [Marteilia refringens] (1) [Bonamia ostreae] (1) [Taura syndrome] (1) [Yellowhead disease] (1) [White spot disease] (1) [the following emerging disease: .....]],~~

~~II.5.2 the animals referred to above are allowed to be placed on the market according to the control measures laid down; and~~

~~II.5.3 the consignment is identified by a legible label on the exterior of the container, or when transported by well boat, in the ship's manifest, with the relevant information referred to in boxes I.8 to I.13 of Part 1 of this certificate, and the following statement:~~

~~'(1) [Wild] (1) [Fish] (1) [Molluscs] (1) [Crustaceans] originating from an area subject to disease control measures'.]~~

[redacted]

COUNTRY: BAILIWICK OF GUERNSEY

Placing on the market of aquaculture animals for farming, relaying, put and take fisheries, open ornamental facilities and restocking

II. Health information	I.2. Certificate reference number WK/202101223	I.2.a.

~~II.6~~ ~~(1)(9) [Requirements for species susceptible to Spring viraemia of carp (SVC), Bacterial kidney disease (BKD), Infectious pancreatic necrosis virus (IPN), Infection with Gyrodactylus salaris (GS) and Infections with salmonid alphavirus (SAV)~~

~~I, the undersigned official inspector, hereby certify that the aquaculture animals referred to above,~~

~~either~~ ~~(1) [originate from a country/territory, zone or compartment or part thereof:~~

~~(a) where (1) [SVC] (1) [GS] (1) [BKD] (1) [IPN] (1) [SAV] are notifiable to the competent authority and reports of suspicion of infection of the relevant disease must be immediately investigated by the competent authority,~~

~~(b) where all aquaculture animals of species susceptible to the relevant diseases introduced into Great Britain or part thereof comply with the requirements set out in II.6 of this certificate,~~

~~(c) where species susceptible to the relevant diseases are not vaccinated against the relevant diseases, and~~

~~(d) either (1) [which, in the case of (1) [IPN] (1) [BKD], complies with requirements for disease freedom equivalent to those laid down in Decision 2009/177.]~~

~~and/or (1) [which in the case of (1) [SVC] (1) [GS] (1) [SAV], comply with requirements for disease freedom laid down in the relevant OIE Standard.]~~

~~and/or (1) [which, in the case of (1) [SVC] (1) [IPN] (1) [BKD] (1) [SAV], comprises one individual farm which under the supervision of the competent authority:~~

~~(i) has been emptied, cleansed and disinfected, and followed in at least 6 weeks,~~

~~(ii) has been restocked with animals from areas certified free from the relevant disease by the competent authority.]]~~

~~and/or (1) [in the case of wild aquatic animals susceptible to (1) [SVC] (1) [IPN] (1) [BKD] (1) [SAV], have been subject to quarantine under conditions at least equivalent to those laid down in Decision 2008/946/EC],~~

~~and/or (1) [in the case of consignments for which GS requirements apply, have been held, immediately prior to the placing on the market, in water with a salinity of at least 25 parts per thousand for a continuous period of at least 14 days and no other live aquatic animals of the species susceptible to GS have been introduced during that period.]~~

~~and/or (1) [in the case of eyed fish eggs for which GS requirements apply, have been disinfected by a method demonstrated to be effective against GS.]]~~

II.7 (1)(10) **[Requirements for species susceptible to OsHV-1 µvar**

I, the undersigned official inspector, hereby certify that the aquaculture animals referred to above,

either (1) [originate from a country/territory, ~~zone or compartment~~:

(a) where OsHV-1 µvar is notifiable to the competent authority and reports of suspicion of infection of the relevant disease must be immediately investigated by the competent authority,

(b) where all aquaculture animals of species susceptible to OsHV-1 µvar introduced into Great Britain comply with the requirements set out in II.7 of this certificate,

(c) *either* (1) [which comply with requirements for disease freedom equivalent to those laid down in Decision 2009/177,]

[redacted]

COUNTRY: BAILIWICK OF GUERNSEY

Placing on the market of aquaculture animals for farming, relaying, put and take fisheries, open ornamental facilities and restocking

II. Health information	I.2. Certificate reference number WK/202101223	I.2.a.

~~and/or (1): [in the case of consignments intended for a zone or compartment with a surveillance or eradication programme, which itself is also covered by a surveillance or eradication programme,]~~

~~and/or~~ ~~(2): [have been subject to quarantine under conditions at least equivalent to those laid down in Decision 2008/946/EC.]~~

Notes

References to European Union legislation within this certificate are references to direct EU legislation which has been retained in Great Britain (retained EU law as defined in the European Union (Withdrawal) Act 2018).

Part I:

- Box I.12: If appropriate, use the authorisation number for the farm or the mollusc farming area in question. Use 'other' if wild aquatic animals.
- Box I.13: If appropriate, use the authorisation number for the farm or the mollusc farming area in question. Use 'other' if intended for restocking.
- Box I.19: Use the appropriate HS codes: 0301, 0306, 0307, 030110 or 030270.
- Box I.20 and I.31: As regards quantity, give the total number.
- Box I.25: Use the option 'Breeding' if intended for farming, 'Relaying' if intended for relaying, 'Pets' if intended for open ornamental facilities, 'Game restocking' if intended for restocking, 'Quarantine' if the aquaculture animals are intended for a quarantine facility, and 'Other' if intended for put and take fisheries.

Part II:

(1) Keep as appropriate.

(2) The 24-hour option applies only to consignments of aquaculture animals which according to Article 8 of Regulation (EC) No 1251/2008 must be accompanied by a certificate and which in compliance with the placing on the market requirements are allowed by the competent authority to leave an area subject to control provisions or a country/territory, zone or compartment with an eradication programme approved in accordance with Article 44(2) of that Directive. In all other cases the 72-hour option applies.

(3) Only applicable to consignments of aquaculture animals caught in the wild and immediately transported to a farm or mollusc farming area without any temporary storage.

(4) Part II.2 of this certificate applies to species susceptible to one or more of the diseases referred to in the title. Susceptible species are listed in Annex 1A to Regulation (EC) 1251/2008.

(5) Consignments of wild aquatic animals may be placed on the market regardless of the requirements in Part II.2 of this certificate if they are intended for a quarantine facility complying with the requirements laid down in Decision 2008/946/EC.

(6) To be authorised into Great Britain, zone or compartment declared free from VHS, IHN, ISA, KHV, Marteilia refringens, Bonamia ostreae, or Whitespot disease or with a surveillance or eradication programme, one of these statements must be kept if the consignment contain susceptible or vector species to the disease(s) for which disease freedom or programmes(s) apply(ies). Data on the disease status of each farm and mollusc farming area in Great Britain are accessible at:

https://www.gov.uk/government/groups/fish-health-inspectorate#disease-status-of-fish-shellfish-and-crustacea-in-england-and-wales
https://www.gov.scot/publications/registers-of-authorised-aquaculture-production-businesses-and-authorised-processing-establishments/
https://www.gov.scot/publications/health-status-of-fish-and-shellfish-diseases-in-scotland/

[redacted]

COUNTRY: BAILIWICK OF GUERNSEY

Placing on the market of aquaculture animals for farming, relaying, put and take fisheries, open ornamental facilities and restocking

II. Health information	II.a. Certificate reference number WK/202101223	II.b

(7) Part II.3 of this certificate applies to vector species to one or more of the diseases referred to in the title. Possible vector species and the conditions, under which consignments of such species are to be considered vector species, are listed in Annex 1 to Regulation (EC) No 1251/2008. Consignments of possible vector species may be placed on the market regardless of the requirements in Part II.3 if the conditions set out in Column 4 of the table in Annex 1 to Regulation (EC) No 1251/2008 are not fulfilled or they are intended for a quarantine facility complying with the requirements laid down in Decision 2008/946/EC.

(8) Part II.5 of this certificate applies to consignments of aquaculture animals which according to Article 8 of Regulation (EC) No 1251/2008 must be accompanied by a certificate and which in compliance with the placing on the market requirements are allowed by the competent authority to leave an area subject to control provisions or a country/territory, zone or compartment with an eradication programme.

(9) Part II.6 of this certificate only applies to consignments intended for Great Britain or part thereof which is regarded as disease-free, or subject to a surveillance or eradication programme for SVC, BKD, IPN, GS or SAV (see Schedule 1 to the Aquatic Animal Health (England and Wales) Regulations 2009 or the Aquatic Animal Health (Scotland) Regulations 2009, as the case may be, and the consignment comprises species listed in Annex 1A to Regulation (EC) 1251/2008 as susceptible to the disease(s) for which the disease-free status or programme(s) apply(ies).

Part II.6 shall also apply to consignments of fish of any species originating from waters where species listed in Schedule 1 to the Aquatic Animal Health (England and Wales) Regulations 2009 or the Aquatic Animal Health (Scotland) Regulations 2009 (as the case may be) as species susceptible to infection with GS, are present, and where those consignments are intended destinations listed in that Schedule.

Consignments of wild aquatic animals for which SVC, SAV, IPN and/or BKD related requirements are applicable, may be placed on the market regardless of the requirements in Part II.6 of this certificate if they are intended for a quarantine facility complying with the requirements laid down in Decision 2008/946/EC.

(10) Part II.7 of this certificate only applies to consignments intended for Great Britain, a zone or compartment which is regarded as disease-free, or with a surveillance or eradication programme , and the consignment comprises species listed in Schedule 1 to The Aquatic Animal Health (England and Wales) Regulations 2009 and The Aquatic Animal Health (Scotland) Regulations 2009 as susceptible to OsHV-1 µvar.

The requirements set out in part II.7 shall not apply to consignments intended for a quarantine facility complying with the requirements at least equivalent to those laid down in Decision 2008/946/EC.

Official Veterinarian

Name (in capital letters): GRACE HODGKINSON

Qualification and title: DVM MRCVS, DCQ(V)-EX
Deputy States Veterinary Officer

Date: 17/05/2021

Stamp: States of Guernsey GG011 Endorsed Veterinarian

Signature: [redacted]

en 6/ 6

Figure C3: Health certificate for imported M. gigas

Methods

Gamete extraction

Following overnight holding, mature oysters were individually submerged in approximately 1 litre of 35 ppt ASW at 20 °C for one hour to revitalise them. Oysters were separated by sex, and males sequentially shucked to reveal the gonad, and their gametes extracted from the gonad using a glass Pasteur pipette. Sperm from the five males were mixed into a single 7 ml glass bijou and stored 'dry' on ice. The same process was then repeated on the female oysters to extract the eggs into a separate 7 ml glass bijou.

Preparation of gamete suspensions

The cell density of the dry sperm and egg mixtures was determined using cell counts conducted using a haemocytometer and Sedgewick rafter cell respectively. Counts were conducted on 0.5 µl gamete samples diluted 60,000-fold. These counts were used to calculate dilution factors required to achieve the desired cell densities.

Dry gamete solutions were individually diluted in 35 ppt ASW at ratios of 1:1000 and 1:10 for sperm and eggs respectively and stirred gently to homogeneity. Each solution was then filtered through a clean 90 µm nylon sieve to remove any gonad tissue debris.

To achieve experimental sperm solutions of the desired concentration, 600 µl of the diluted sperm solution was further diluted into 1 litre of ASW at each of the experimental salinities. Likewise, 930 µl of the diluted egg solution was diluted into 1 litre ASW at each experimental salinity. These procedures resulted in cell densities of 200 cells ml^{-1} and 20,000 cells ml^{-1} respectively.

Resulting solutions were stirred regularly with glass rods to keep the gametes suspended and maintain homogeneous cell density. Gamete solutions were allowed to age for 45 minutes prior to use to enable activation of the gametes.

Results

Sound exposures

A)

Sound pressure level (dB_{RMS} re 1 µPa)

Ambient				Ferry				Piling			
119.6	119.9	119.8	119.5	146.2	139.2	137.0	141.6	140.0	135.0	133.9	140.3
119.5	119.5	119.7	119.6	140.3	139.3	137.2	140.6	139.2	132.8	133.3	135.7
119.6	119.7	119.9	119.6	136.9	141.7	141.5	138.8	141.1	135.0	137.4	142.2
119.6	119.8	120.1	119.6	136.9	141.9	141.3	138.4	143.9	134.1	137.6	142.8
119.3	119.7	119.8	119.6	139.1	138.4	139.0	142.7	137.5	134.0	135.9	138.7
119.3	119.6	119.4	119.3	145.7	139.1	136.2	144.3	140.2	136.2	136.2	140.7

B)

Particle motion (dB_{RMS} re 1 µm s^{-2})

56.6	56.7	56.6	56.6	72.1	69.4	69.4	70.5	64.6	66.4	65.5	65.8
56.7	56.7	56.6	56.6	70.8	70.1	68.7	68.6	63.5	63.4	62.7	65.5
56.7	56.7	56.8	56.7	72.1	71.4	72.7	73.6	66.3	64.9	63.6	67.5
56.7	56.8	56.6	56.6	73.6	71.3	71.0	73.3	66.8	65.5	63.2	68.5
56.8	56.8	56.9	56.8	71.2	69.1	70.8	70.6	64.8	63.9	62.9	67.5
56.6	56.8	56.8	56.6	73.1	68.3	67.8	68.0	64.9	64.1	65.7	64.9

Figure C4: Heatmap of received sound levels in each sound treatment. *As measured within a proxy vessel located at each location on the exposure system. A) sound pressure; B) particle motion. All measurements are absolute values taken at each location.*