Heavy metals and reproduction in the benthic carnivore, *Cominella glandiformis*.

By

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Abstract

Heavy metal pollution is a growing concern for aquatic ecosystems world-wide. Heavy metals are introduced to waterways via direct run off from land, storm drains and (for coastal environments) river and stream outflows. Offspring of species with benthic development have low dispersal potential, so are likely to experience the same conditions as their parents. Little is known about the effects of heavy metal pollution on these embryos in a marine context. I use the mud whelk *Cominella glandiformis* to examine the effects of the common heavy metals copper (Cu) and zinc (Zn) on aspects of foraging ability, condition and reproduction in adults, and growth and survival in encapsulated embryos and recently hatched juveniles. This species has an entirely benthic lifecycle, and occurs in a habitat prone to pollution, making it an ideal model species with which to examine the effects of ongoing heavy metal pollution.

In Chapter 2, I describe the reproductive patterns of *Cominella glandiformis* collected from two local sites and kept in the laboratory. Females from both sites laid a similar number of capsules and showed similar maternal investment. The average capsule was 1.06 mm^3 and contained 5 eggs of 0.28 mm diameter. Larger females had a higher fecundity than smaller females, producing larger capsules containing larger eggs. Previous research on *C. glandiformis* has relied on field-collected capsules, which could not be attributed to females. This chapter complements previous research on related rocky shore species (*C. maculosa* and *C. virgata*), finding consistent maternal investment across females. Further, this refines the existing understanding of reproduction in this species.

In Chapter 3, I examine the effects of pollutant mixture (Cu-only or Cu+Zn; 50 µg Cu L^{-1} and 100 µg Zn L^{-1}) and pathway (aqueous-only or aqueous+dietary) on aspects of adult whelk physiology and foraging ability. I exposed whelks to the above treatments for four weeks, measuring their respiration, food consumption and weight loss throughout. Pollutant pathway did not affect physiological stress, but pollutant mixture did. Copper polluted whelks reduced their food intake yet gained weight, suggesting that they prioritised present growth over future energy stores. After the four-week exposure, I tested the effects of metal exposure on foraging ability and preferences in a subset of whelks. Polluted whelks

preferred contaminated prey, showed impaired foraging efficiency. The pollution pathway had little effect on response variables in this study, though the addition of Zn may have reduced the growth-response seen in Cu-exposed whelks. Overall, these results indicate that pollution may leave whelks in poor condition prior to the breeding season, impairing their reproductive fitness.

In Chapter 4, I use *C. glandiformis* as a model species to examine the effects of heavy metals on the reproduction of a benthic carnivore with a benthic lifecycle. I exposed adult *C. glandiformis* to one of five heavy metal treatments (plus control) and examined their subsequent rates of capsule laying and indicators of maternal investment. Some continued development in the same pollution conditions as the female, allowing comparisons of survival, development time, and hatching size. Whelks exposed to >5 µg Cu L^{-1} began laying three weeks later than Control whelks, though a similar number of capsules were laid overall. Whelks showed similar maternal investment, laying similarly sized capsules containing approximately six eggs which developed in to six hatchlings. Eggs and hatchlings were similar sizes, though $\leq$3.5% of capsules hatched from the two most extreme treatments. The development time of capsules (i.e. time from laying to hatching) decreased through the season. Later laying by polluted whelks was associated with faster development, possibly due to warmer water temperatures. Consistent with previous *Cominella* research using starved females, these results suggest that females invest similarly in capsules, irrespective of adult stress experience.

In Chapter 5, I examine the effects of heavy metals on the development and post-hatching growth and survival of *C. glandiformis*. All embryos exposed to 50 µg Cu L^{-1} died at early multicellular stages within the first three weeks. Those exposed to 20 µg Cu L^{-1} (with or without 50 µg Zn L^{-1}) showed reduced growth rates compared to Controls and died before reaching the veliger stage. Juvenile whelks were unaffected by pollution, with similar survival and growth to Control juveniles after 5 weeks. This chapter agrees with previous findings that embryos are more sensitive than juveniles and suggests that recently hatched juveniles may be similarly tolerant of metals as adults.

My thesis uses laboratory-based experiments to assess the response of a benthic predator from a pollution-prone ecosystem to realistic heavy metal concentrations. Overall, *C. glandiformis* is highly tolerant of Zn. Pollution impaired adult condition and foraging ability,

which likely reduces reproductive output. My results suggest that mothers do not pre-empt the conditions that their offspring will experience, resulting in reduced embryonic growth and survival. Pollution-naïve juveniles may be similarly tolerant of moderate Cu and Zn as adults. Thus, as demonstrated in other species, the early, encapsulated stages are the most vulnerable life stage to pollution. Overall, my thesis represents one of few detailed examinations of reproduction in a marine benthic developer exposed to pollution. Further, it advances our understanding of the reproduction of this common, endemic intertidal predator.

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Table of Contents

Abstract i

Acknowledgements iv

Table of Contents v

List of Figures ix

List of Tables xi

Chapter 1 – General Introduction 2

1.1 Pollution in Coastal Ecosystems 2

1.2 Factors Affecting Metal Uptake 3

1.3 Toxicity and Protective Mechanisms 4

1.4 Route of Exposure and Trophic Interactions 6

1.5 Effects of Pollution on Reproduction and Early Life Stages 7

1.6 Intergenerational Effects 8

1.7 Benthic Development 10

1.8 Focal Species and System 12

1.9 Aims and structure of thesis 14

Chapter 2 – Reproduction in *Cominella glandiformis* from natural populations. 16

2.1 Introduction 16

2.2 Methodology 19

2.2.1 Study system 19

2.2.2 Study overview 19

2.2.3 Capsule laying trends. 19

2.2.4 Maternal investment between sites. 20

2.2.5 Capsule volume and female size 22

2.2.6 Capsule volume and number and size of eggs. 22

2.3 Results 23
2.3.1 Capsule laying trends 23
2.3.2 Maternal investment between sites. 24
2.3.3 Female size and capsule output and volume. 26
2.3.4 Relationships between the number and sizes of eggs and capsule volume. 28
2.4 Discussion 30
2.4.1 Comparisons with other endemic *Cominella* species 31
2.4.2 Conclusion 32
Chapter 3 – The effects of Cu and Zn pollution on the condition and foraging ability of a benthic carnivore 34
3.1 Introduction 34
3.2 Methodology 37
3.2.1 Study system 37
3.2.2 Experimental overview 37
3.2.3 Whelk collection and exposure to pollution: 38
3.2.4 Indicators of Physiological Stress (Experiment 1) 39
3.2.5 Chemosensation (Experiments 2, 3 and 4) 42
3.3 Results 46
3.3.1 Impacts of Pollution on Indicators of Physiological Stress 46
3.3.2 Chemosensation in Whelks Exposed to Prolonged Pollution 51
3.4 Discussion 58
3.4.1 Conclusion 60
Chapter 4 – Maternal investment of a benthic carnivore under Cu and Zn pollution 61
4.1 Introduction 61
4.2 Methodology 64
4.2.1 Study system 64

4.2.2 Experimental overview 64
4.2.3 Effects of Pollution on Capsule Output and Intracapsular Survival 67
4.2.4 Effects of Pollution on Maternal Investment 68
4.2.5 Effects of Pollution on the Timing of Egg Laying and Embryonic Development 69
4.3 Results 70
4.3.1 Effects of Pollution on Capsule Output and Intracapsular Survival 70
4.3.2 Effects of Pollution on Maternal Investment 71
4.3.3 Effects of Pollution on the Timing of Egg Laying and Embryonic Development 72
4.4 Discussion 74
4.4.1 Conclusion 76
Chapter 5 – Growth and survival of *Cominella glandiformis* offspring under Cu and Zn pollution. 78
5.1 Introduction 78
5.2 Methodology 81
5.2.1 Study system 81
5.2.2 Experimental overview 81
5.2.3 Heavy Metal Exposure During Development 82
5.2.4 Hatchling Experiment 83
5.2.5 Statistical Methodology 84
5.3 Results 86
5.3.1 Development 86
5.3.2 Hatchling Experiment 88
5.4 Discussion 90
5.4.1 Embryonic Development 90
5.4.2 Post-hatching survival and growth 92
5.4.3 Conclusion 92

Chapter 6 – Overall Discussion. 94

6.1 Summary of Major Findings 94

6.2 Pollution in Coastal Ecosystems 94

6.3 Zn regulation 96

6.4 Reproduction 97

6.5 Development 98

6.6 Pollution and juvenile performance 100

6.7 Conclusions 101

References 102

Appendix 117

List of Figures

Figure 1.1: *Cominella glandiformis* adult from Te Awarua-o-Porirua Harbour. (Photo courtesy of Melanie Dohner). 12
Figure 1.2: Location of Te Awarua-o-Porirua Harbour. 14
Figure 2.1: A capsule of Cominella glandiformis photographed under 2.5x magnification of a dissecting microscope. 21
Figure 2.2: Number of capsules laid per week by females from Browns Bay (pink) and Paremata Station (blue). 23
Figure 2.3: Capsule volumes of a subset of females from Browns Bay (BB) and Paremata Station (PS)..... 24
Figure 2.4: Egg diameters of a subset of females from each site.. 25
Figure 2.5: The number of eggs per capsule from a subset of females from each site. 25
Figure 2.6: The relationship between female shell length and the number of capsules laid (overall). 26
Figure 2.7: The relationship between female shell length and capsule volume from a subset of females. 27
Figure 2.8: The relationship between capsule volume and egg diameter from a subset of females. 28
Figure 2.9: The relationship between the number of eggs within a capsule and their diameter from a subset of females. 29
Figure 3.1: Example container used to enclose whelks. Dimensions: 97 x 97 x 80 mm (2 litres) with side panels (approx. 95 x 50 mm) covered with 3mm mesh. 39
Figure 3.2: Y-maze set up (photographed from below)..... 43
Figure 3.3: Respiration (mmol) of whelks in each treatment before (Week 0), during (Week 2) and after (Week 4) exposure to heavy metal pollution treatments. s. 47
Figure 3.4: Cockle tissue (g) consumed by whelks in each treatment over the four-week exposure. 49
Figure 3.5: Percent weight change in whelks from each treatment over 4-week experimental period.. 50
Figure 3.6: The number of trials in which whelks chose the arm containing the live, crushed cockle (green) or empty cockle shell (grey) across each of the five treatments in Experiment One.. 52
Figure 3.7: Time (seconds) taken to complete the Y-maze by whelks from each treatment in Experiment One. 53
Figure 3.8: Choice between unpolluted (green) or Cu+Zn polluted (grey) live, crushed cockle by whelks from two treatments (Control (unpolluted) and aqueous + dietary exposure to copper (Cu) and zinc (Zn)) in Experiment Two. 54
Figure 3.9: Time (seconds) taken to complete the Y-maze by whelks from each treatment in Experiments One (green) and Three (orange).. 57
Figure 4.1: The number of capsules laid by whelks from A) Control, B) Cu Low, C) Cu Med, D) Cu + Zn Med, E) Cu High, and F) Cu + Zn High treatments, over eight weeks.. 70
Figure 4.2: Proportion of capsules from each treatment that failed to develop (dark blue) or successfully hatched (light blue). 71
Figure 4.3: Proportion of female whelks laying capsules from six treatments across eight weeks. 72

Figure 4.4: Average development time (Date laid – hate hatched, days) of capsules from A) Control, B) Cu Low, C) Cu Med, D) Cu + Zn Med, and E) Cu + Zn High laid over 7 weeks. 73
Figure 5.1: Five pre-veliger embryos viewed through the capsule base. 83
Figure 5.2: Cumulative proportion of capsules alive (blue), hatched (yellow), and dead (grey) from the four treatments across six weeks. 86
Figure 5.3: Average size of pre-veliger embryos from three treatments. 87
Figure 5.4: Proportion of hatchlings alive (dark blue) or dead (light blue) in Control (left) and Cu+Zn (right) replicates after 1 and 5 weeks of treatment exposure. n = 6 jars per treatment. 88
Figure 5.5: Average growth (mm) of hatchlings in each of 6 replicate jars after five-week exposure to A) Control (unpolluted) and B) aqueous Cu+Zn pollution. 89

List of Tables

Table 2.1: Summary of maternal investment from four intertidal whelk species found in the Wellington Region (New Zealand). 33
Table 3.1: Aqueous pollutant concentrations used to pollute whelks and their food (*Austrovenus stutchburyi*) in treatments with dietary pollution. 38
Table 3.2: Results of a nested two-way ANOVA examining the effects of Week (before (Week 0), during (Week 2) and after (Week 4) exposure), Treatment and Replicate nested within Treatment on respiration of whelks from five treatments. 46
Table 3.3: Results of two-way ANOVA examining effects of Treatment and exposure duration (week number) on food consumption using non-nested model (Consumption ~ Treatment * Week). 48
Table 3.4: Results of a nested ANOVA examining the effects of Treatment, and Replicate nested within Treatment, on time taken by whelks to complete the Y-maze in Experiment One. 53
Table 3.5: Results of a nested ANOVA examining the effects of Treatment and Replicate nested within Treatment on time taken by whelks to complete the Y-maze in Experiment Two. 55
Table 3.6: Results of a nested two-way ANOVA examining the effects of Experiment number (representing both experience in maze and time starved), Treatment and Replicate nested within Treatment on time taken by whelks to complete the Y-maze in Experiments One and Three. 56
Table 4.1: Pollution regimes for experiments in 2019/20 (Ch. 4) and 2020/21 (Ch. 5) summers. Distilled water and stock solutions were added to 5L of filtered seawater from the Wellington South Coast (New Zealand). 65
Appendix-Table 3.1: Two-way ANOVA results examining effects of treatment and week number on food consumption using nested data (Consumption ~ Treatment (Replicate)*Week) (Chapter 3). 118
Appendix-Table 4.1: Average temperatures (^{o}C) of the Control replicates from the third week of females laying (end of October 2019) to the final week of hatchlings emerging (end of January 2020) (Chapter 4). 119
Appendix-Table 5.1: Average temperatures (oC) from all Control replicates during the development and hatchling experiments (Chapter 5). 120

Chapter 1 – General Introduction

1.1 Pollution in Coastal Ecosystems

The global urbanisation of coastlines is increasingly contributing to anthropogenic pressures on the marine environment and as global populations increase, our inputs are likewise set to climb. Urban areas are often situated close to coastal environments, with storm drains carrying anthropogenic wastes to these systems, such as heavy metals, nutrients, herbicides, insecticides, sediment, etc. (Grimm et al., 2008a; Kennish, 2002; Thrush et al., 2004). Sewage treatment stations can also introduce these contaminants into the systems following heavy rain (Grimm et al., 2008b; Risch et al., 2018). Historically, regulators thought water currents would be sufficient to carry away human waste (organic and inorganic), but this is not the case (Bryan, 1980). In some areas, pollutants that haven't been used for decades can still be found in sediments (Audry et al., 2004; Fabbri et al., 2001; Schäfer et al., 2002). Local governing bodies usually monitor coastal systems, aiming to keep anthropogenic inputs within guideline limits. These guidelines are largely based on concentrations that kill half of the population (LC_{50}) at multiple life stages of select species (Bryan, 1980). While useful as a broad point of comparison across species, these studies don't follow the life-stages of species through development and metamorphosis, or account for effects on the wider population or trophic interactions. The consequences of sub-lethal effects on long term population survival or adaptation are unclear.

Some heavy metals, such as copper (Cu), iron (Fe), and zinc (Zn), are 'biologically essential', meaning they are necessary in low doses for cellular functioning (e.g. enzymes, respiratory pigments) (Ansari et al., 2004; Bryan, 1980; Valavanidis & Vlachogianni, 2010). Non-essential metals, such as mercury (Hg), cadmium (Cd), or lead (Pb), are often toxic, even in low doses (Ansari et al., 2004; Valavanidis & Vlachogianni, 2010). All heavy metals can disrupt cellular functioning at high concentrations. Like many pollutants, these metals are introduced to the marine environment through direct run-off from land, streams, storm drains, and coastal erosion (Ansari et al., 2004; Botherway & Gardner, 2002; Valavanidis & Vlachogianni, 2010). Copper, Zn, Pb and Cd are amongst the most common heavy metals found in waterways (dos Santos et al., 2019; Li et al., 2012; Milne et al., 2009; Tomlinson et al., 1980). The most common sources of each metal are vehicle brake pad wear (Cu), leaching from old roofs (Zn,

Cu), and industrial exhaust (Zn, Cu and Pb) (Ansari et al., 2004; Charry et al., 2018; dos Santos et al., 2019; Gupta, 2020).

1.2 Factors Affecting Metal Uptake

Whether accompanied by fresh water or sediment inputs, multiple metals are often introduced to the environment simultaneously. The respective bioavailabilities to an organism can be influenced by its prior exposure. For example, pre-exposure of the clam *Ruditapes philippinarum* to Cd or Zn increased subsequent Zn uptake, whereas silver (Ag) had no effect (Ng & Wang, 2004). Pre-exposure to any of these three metals, however, resulted in substantial reductions in Hg uptake. It can be difficult to balance the limitations of metal-pollutant experiments (e.g. consuming non-polluted food, the absence of metal-binding sediment etc.) with maintaining a realistic study that is applicable to real-world conditions.

The abiotic conditions of an estuary can influence the bioavailability, and therefore toxicity, of metal pollutants to organisms, especially in the intertidal zone. These conditions may act on the metal, or influence how the organism responds to the metal stress (Bryan, 1979, 1980; Bryan & Langston, 1992). Temperature and salinity are two of the most influential abiotic factors on intertidal species (Harrison & Phizacklea, 1987; Ross et al., 2016), especially with regards to heavy metal pollution (Bryan, 1980; Bryan & Langston, 1992; Bryant et al., 1985; Jones, 1975).

Increased salinity (e.g. as the tide returns) reduces the bioavailability of Fe (reviewed by Bryan, 1980), but can increase that of Zn and Cd (Rainbow et al., 1993). Physiological responses of the organism experiencing these changes in salinity may also influence metal uptake. For example, Marsden and Rainbow (2004) demonstrated that the estuarine amphipod *Orchestia gammarellus* will experience increased Zn uptake until salinity reaches 25ppt, but uptake levels off below this salinity due to physiological responses to the low salinity. The interaction of such physiological stress and pollutant exposure can increase toxicity (see Velasco et al., 2019). Some studies, however, find no effect of salinity on metal toxicity (e.g. Mouneyrac et al., 1998).

Temperature influences metal toxicity through direct effects on the organism. Exposure of *Mytilus galloprovincialis* to 21^{o}C decreased metabolic activity, thereby reducing toxic effects

of subsequent mercury (Hg) exposure (Freitas et al., 2017). Warmer temperatures also speed up development time and growth rates in many species (McLaren, 2011; Przeslawski, 2004; Scheltema, 1967). For example, Kimberly and Salice (2013) exposed the benthic egg capsules of the fresh water snail *Physella pomilia* to cadmium (Cd, 5-25 µg L^{-1}) and temperature (25°C and 35 °C) in a factorial experiment. Hatching occurred sooner at the higher temperature without Cd but took longer and resulted in higher mortality when Cd was added. Other abiotic factors, such as inorganic sediment can also influence the bioavailability of heavy metals as a smaller grain size can increase binding of metals (Ansari et al., 2004; Campana et al., 2012). Nitrogen and phosphorus, common pollutants in estuarine systems (Fowler et al., 2013; Howarth, 2008), can also increase heavy metal bioavailability (Miranda et al., 2021).

1.3 Toxicity and Protective Mechanisms

Heavy metals disrupt a variety of cellular functions by denaturing enzymes, causing oxidative stress, and directly damaging DNA and membranes (Chiarelli & Roccheri, 2014; Janssens et al., 2009; Valavanidis & Vlachogianni, 2010). The effects can differ between species and metals along a shoreline (reviewed by Wang, 2002). These differences could be driven by a variety of factors, such as feeding method or rate, or prey choice. It can also be driven by the species-specific cellular response – the point at which defences are activated or overcome. For example, the mussel *Perna viridis* can regulate Zn accumulation if water contains <100 µg Zn L^{-1}. Beyond this Zn accumulates in tissues with death occurring at tissue concentrations of ~250 µg Zn g^{-1} (wet weight) (Chan, 1988). This species did not regulate Cu, Cd or Pb, however. Similar observations have been made in a freshwater snail (*Lymnara palustris* (Cœurdassier et al., 2005)), oysters (*Saccostrea cucullata*, *Magallana hongkongensis* and *M. angulata* (Cheung & Wang, 2008; Wang et al., 2011)), and the whelk *Reishia* (*Thais*) *clavigera* (Cheung & Wang 2008). Energy-related biomarkers can provide useful information on how organisms are coping with pollution, particularly in laboratory experiments. For example, Sokolova et al. (2012) and Brown et al. (2004) suggest using reproduction, metabolic rate (e.g. oxygen consumption, heart rate), and tissue energy status as biomarkers.

Some physical damage can be mitigated through the production of antioxidants (which neutralise reactive oxygen species produced by the metal) (Leonard et al., 2004; Stohs,

1995) and metallothionein (MT). Metallothionein is a low-weight peptide containing a thiol group, which binds to heavy metals, limiting cellular damage. The protein-metal complex can be transported to a lysosome for degradation, or stored in a metal rich granule (Amiard et al., 2006). Overall, the binding affinity shown by such proteins can be described as mercury (Hg) > copper (Cu) > silver (Ag) > bismuth (Bi) >> cadmium (Cd) > lead (Pb) > zinc (Zn) > cobalt (Co) (Vasak, 1991). Thus, the most toxic metals have a higher binding affinity and can displace metals of lower toxicity. The generalization and preferential binding allow organisms to adapt to metal fluctuations and reduce cellular damage.

The ability to excrete the bound metal will determine the concentration of metal found in bodily tissues. Most predatory marine organisms are able to regulate metal excretion, so that biomagnification is thought to be limited within marine food webs (Bryan, 1980). How rapidly a metal is incorporated into an organism's tissue is a major determinant of the metal's toxicity, as a slow enough uptake can be counteracted by growth, irrespective of excretion rate (Bryan, 1980). Bryan (1980) defined three metal excretion patterns: Type I) metal excretion equals intake, so body burden remains relatively constant or decreases with age; Type II) excretion is slower than accumulation, so body burden increases with age (these are the strongest bioaccumulators); Type III) rate of excretion is flexible, so can change to suit environmental conditions (to an extent). However, few invertebrates utilise Type III, probably due to the energetic requirements. For example, predatory whelks have demonstrated the ability to bioaccumulate heavy metal pollutants (Cheung & Wang, 2005; Wang, 2002; Wang & Ke, 2002), and some of these may be biomagnified within these benthic food webs (Wang, 2002).

Although these cellular mechanisms are useful in allowing organisms to deal with metal pollution to some degree, maintaining them is energetically expensive. This can result in reduced growth (Das & Khangarot, 2010; Pease et al., 2010; Plautz, Guest, et al., 2013; Regoli, 1998) and reproductive output (Das & Khangarot, 2010; Durou et al., 2008; Plautz, Guest, et al., 2013; Tlili et al., 2011). One of the simplest responses of mobile organisms to pollution is to simply move away (e.g *Hydrobia ulvae* (Araújo et al., 2012)). Alternatively, behavioural changes can reduce the degree of exposure. When experiencing Cu pollution, the aquatic amphipod *Hyalella azteca*, switches from feeding on coarse particulate organic matter (CPOM, associated with greater Cu binding) to fine particulate organic matter

(FPOM) (Tomczyk et al., 2018). It's believed that FPOM contains less Cu than CPOM, suggesting an active decision by *H. azteca* to reduce metal consumption. Behavioural changes can also expose the organism to other threats. Exposure to Cu-polluted sediment induces lower reburial rates in the bivalve *Indoaustriella lamprelli*, reducing body burden but also increasing the risk of predation (Hutchins et al. 2009).

1.4 Route of Exposure and Trophic Interactions

The likelihood of metals entering an organism can be largely attributed to the surrounding water chemistry and physical environment (see Bryan, 1980; Bryan & Langston, 1992). This has resulted in a debate over the relative importance of exposure via aqueous versus dietary pathways. The 'aqueous pathway' refers to metal accumulated from the water column, usually due to contact with gills, but some include the ingestion of contaminated water. Dietary exposure occurs when the organism's food source has become polluted. This could be contaminated CPOM for *Hyalella azteca* (Tomczyk et al., 2018), or contaminated tissue ingested by carnivores (e.g. whelks (Wang & Ke, 2002)). Despite a dominant focus on the aqueous pathway, studies have found significant effects of contaminated food on shrimp (Wallace et al., 1998), bivalves (Pan & Wang, 2009) and whelks (Cheung et al., 2006; Cheung & Wang, 2005, 2008; Wallace et al., 1998; Wang & Ke, 2002). It is important to understand the different effects and mechanisms of these pathways to better predict how species and communities will respond to anthropogenic pollutants.

In his 2002 review, Wang concluded that assimilation efficiencies (the amount of metal absorbed by an organism) in a benthic food web were driven more by the metal in question than by dietary exposure. Furthermore, bivalve species often differed in assimilation efficiencies depending on the metal of interest as well as aspects of digestion and food choice (reviewed by Wang, 2002). Several studies have demonstrated that whelks tend to bioaccumulate metals from their diet (Cheung & Wang, 2005; Wang, 2002; Wang & Ke, 2002). The cellular response of whelks to dietary metals is influenced by where the prey species store the metals within their tissues, and this can influence how much is taken up by a predator (Cheung & Wang, 2005, 2008; Chiarelli & Roccheri, 2014; Wang, 2002).

Heavy metals can impair organisms' ability to detect and/or accurately respond to chemical cues (Boyd, 2010; Kwan et al., 2015; Lurling & Scheffer, 2007). Further, pollutants do not

disrupt only one trophic level, but multiple levels and in different ways. Heavy metal exposure can indirectly alter interactions with predators and competitors of differing taxa (Lefcort et al., 1999). For example, the whelk *Urosalpinx cinerea* shows density dependent feeding on barnacle prey (i.e. more barnacles are consumed per whelk at lower whelk densities) in the absence of predators (Kwan et al., 2015). This feeding rate decreases in the presence of the whelk's crustacean predator (*Cancer productus*). This predator effect was absent above 50 μg Cu L^{-1}, indicating a loss of fear response in the whelks. Chemosensation appeared unimpacted in the crab, resulting in both crabs and whelks consuming prey at higher rates. Following a seven week (aqueous) Cu exposure, the freshwater pulmonate *Racesina luteola* consumed less food, and had reduced chemosensory ability and movement (Das & Khangarot, 2011).

1.5 Effects of Pollution on Reproduction and Early Life Stages

Larger females are generally expected to produce a greater number of offspring as they likely have greater energy reserves than smaller conspecifics (Chatzinikolaou & Richardson, 2010; Harding et al., 2008). Similarly, the energy reserves of a non-polluted female are likely to be higher than those of a polluted female, as the excretion of heavy metals can deplete energy reserves that could otherwise be invested in reproduction (Bi et al., 2016; Durou et al., 2008; Tlili et al., 2011).

Early developmental stages are often the most sensitive to heavy metals in the organism's lifecycle, with tolerance generally increasing with each developmental stage (Kimberly & Salice, 2013; Ringwood, 1990, 1991). The quality of the larvae is further reduced if the gametes that formed them were impaired by heavy metal pollutants (Bowen & Engel, 1996; Phillips & Rouchon, 2018; Watson et al., 2013), particularly sperm (Au et al., 2000; Au, Reunov, et al., 2001; Ringwood, 1991). The thick outer membrane that protects eggs from pollutants can also protect early embryo stages (Ringwood 1991 and references therein). Each developmental stage from this point on has a lower accumulation rate, usually attributed to the progressively lower metabolic rates through life stages (i.e. burden is highest in embryos because metabolic rate is highest). However, Ringwood (1991) demonstrated in the bivalve *Isognomon californicum* that this is in fact due to the surface area available for absorption. Interestingly, this species shows increasing tolerance through the stages (embryo < veliger < pediveliger < adult) (Ringwood 1990), but accumulation does

not follow the same trend, with pediveligers accumulating metals more rapidly than veligers. The author argued that this indicates that the veligers do not have the same cellular mechanisms (such as MTs), or perhaps cannot utilise them to the same extent, as pediveligers.

Larvae are sensitive to natural stressors such as temperature (Boukadida et al., 2016b), salinity (Gamain et al., 2016), and pH (Byrne, 2011) singularly and in combination (Byrne, 2012; Przeslawski et al., 2015; Ross et al., 2016). These stressors also increase the toxicity of heavy metal pollutants resulting in abnormal development (Boukadida et al., 2016b; Boukadida et al., 2017; Gamain et al., 2016; Kimberly & Salice, 2013, 2014a). Sub-lethal effects during these young life stages can permanently reduce the fitness of the organism (Chaparro et al., 2018; Rouchon & Phillips, 2017b). The consequences of sublethal exposure are often more extreme in early life stages, as reductions in growth rate increase the risk of subsequent mortality and reduce dispersal potential (Marshall & Keough, 2007, 2008).

1.6 Intergenerational Effects

There is some confusion in the use of the term 'transgenerational effect'. Ross et al. (2016) defined it as "a phenotypic change in offspring in response to the environmental stress experienced by a parent before fertilisation". This is the common usage among ecologists, but a more accurate view is perhaps that of Perez and Lehner (2019) who view it as effects that can be seen through two or more generations after the exposure occurred. They suggest that what Ross described be termed parental effects or intergenerational effects. Arguably, the two are interchangeable, but this review will ascribe to the suggestions of Perez and Lehner. It is worth noting that the mechanisms behind transgenerational effects and intergenerational effects are similar (Perez & Lehner, 2019).

Intergenerational effects occur when parental exposure to a pollutant is expressed in offspring that may have never experienced the pollutant themselves. Negative effects of this may be due to elevated pollutant concentrations in gonads (Phillips & Rouchon, 2018), physical damage in gametes (DNA damage by diuron (Barranger et al., 2014) or Cd (Au et al., 2000; Au, Lee, et al., 2001)) or changes to proteins (e.g. toposome in sea urchins. For example, *Biomphalaria glabrata* (a freshwater snail) raised with predation stress (predator and crushed conspecific) took longer to mature, and produced fewer and smaller offspring

than non-stressed conspecifics (Plautz, Guest, et al., 2013). A surprising by-product of this stress was an elevated tolerance to Cd in their offspring (Plautz, Guest, et al., 2013), a positive intergenerational effect.

Positive intergenerational effects are those where the offspring of stressor-exposed parents are born with some form of advantage in stressed conditions over those of naïve parents. There is some debate as to how much genetic adaptation occurs in natural populations living in polluted versus relatively clean environments. It is generally accepted that there is, at most, minimal genetic adaptation occurring. Vigneron et al. (2019) is one of the first studies to comprehensively demonstrate that tolerance to a pollutant in a naturally occurring population is the result of parentally induced phenotypic flexibility, rather than genetic change. They collected *Gammarus fossarum* from a Cd polluted river, and two reference populations, identifying females with hatched juveniles in their brooding pouch. These females, all exposed to the same non-polluted laboratory conditions, were isolated and monitored, and their broods followed as they hatched over two months. The first cohort was born only two weeks after arriving in the lab, whereas the third was born after 10 weeks. The third cohort showed no Cd tolerance, demonstrating that tolerance was triggered by the conditions experienced by the mother during brooding

Positive intergenerational effects are often an example of epigenetic inheritance, where gametes show DNA methylation and/or contain transcribed RNA or proteins that improve the survival of the resultant offspring through phenotypic change (Perez & Lehner, 2019). Males exposed to pollutants produce offspring with differing phenotypes and reduced survival, while female exposure tends to show greater effects (Au, Lee, et al., 2001; Bowen & Engel, 1996; Phillips & Rouchon, 2018). This is attributed to the larger size of ova, and the corresponding volume of proteins, etc that they contain (Foo & Byrne, 2016; Perez & Lehner, 2019). Maternal effects are a further subset of this, where advantages to offspring of pollutant-exposed females over naïve is often in the form of ready-made proteins or lipid reserves. These are energetically expensive to produce, and most commonly occur in the ova. Whether energy is invested in increasing reproductive output at the expense of offspring quality (increased fecundity) or quality at the expense of quantity (increased offspring fitness) is largely due to species and available resources (Marshall & Uller, 2007).

Magallana gigas eggs and larvae spawned in clean water, but from adults exposed to Cu and Zn pollution, had elevated tissue burdens and MT concentrations (Weng & Wang, 2014). It isn't clear whether elevated metal burden in gametes is a method for adults to reduce their burden by 'dumping' the metal, or if it is unintentional. Synthesising proteins for the gametes, thereby increasing the per-gamete energy required of the parent, suggests that dumping isn't occurring. When comparing larvae of exposed and non-exposed *M. hongkongensis* parents, all larvae initially grew at similar rates, and had similar tissue burdens as 2-day-old larvae (Weng & Wang, 2017). The authors attributed this to rapid efflux during this growth period as, from day three onward, growth rates became increasingly different, possibly due to cellular damage caused by the metals in gametes and larvae. Alternatively, the larvae may have utilised more of their energy reserves during the rapid growth phase, requiring a slower growth rate subsequently, or the females may have provisioned less due to their own energetic constraints (Weng & Wang, 2017). Either way, the larvae would be less likely to survive to adulthood etc. due to their smaller size, as discussed above.

1.7 Benthic Development

Most of the species discussed thus far have pelagic larval development, where offspring develop in the plankton before settling and metamorphosing into the adult form. Many larvae die before settlement due to biotic (e.g. predation, starvation) or abiotic (e.g. salinity, temperature) pressures (Mileikovsky, 1971; Pechenik, 1999). These adults produce large quantities of low energetic-cost offspring (Mileikovsky, 1971; Thorson, 1950; Vance, 1973). Species with benthic development exhibit higher per-offspring investment, with embryos brooded by the female or contained within a benthic mass (Vance, 1973). All nutrition required for embryos to develop must therefore be provided by the female, whereas most species with pelagic development can access nutrition from the water column (Pechenik, 1986; Thorson, 1950; Vance, 1973). Throughout my thesis, I use the term 'benthic development' to describe species which emerge as miniature versions of adults (though some species emerge from benthic capsules as later-stage larvae (see Pechenik, 1979)).

A recurring theme in reproductive biology is the trade-off between number and size of offspring – the same amount of energy can be invested in either many small (high parental fitness) or few large (high offspring fitness) offspring, irrespective of where development

occurs (i.e., pelagic, benthic or mixed) (Bernado, 1996; Kamel et al., 2010; Smith & Fretwell, 1974; Vance, 1973). Species with benthic development may invest further energy in their offspring, such as increasing nutrition supply in the form of larger eggs, or nurse eggs (Rivest, 1983; Spight, 1976b). Even within the same clutch, these resources can be partitioned unequally between capsules (Carrasco & Phillips, 2014; Carrasco et al., 2016; Chaparro et al., 1999; Marshall & Keough, 2007; Rivest, 1983; Spight, 1976b). This can be apparent in the number and sizes of capsules, eggs, nurse eggs, and hatchlings. Fewer siblings mean greater resources per-hatchling, resulting in larger sizes at hatching, thereby increasing their odds of survival (Carrasco et al., 2012; Marshall et al., 2006; Spight, 1976a). These differences in provisioning can be innate or triggered by environmental conditions (Allen et al., 2008; Carrasco et al., 2016; Jensen et al., 2014; Lloyd & Gosselin, 2007; Marshall, 2008; van der Sman et al., 2009).

An important difference between benthic and pelagic development is the environment that the offspring encounter. Where larvae are likely to disperse to other areas and experience different conditions than their parents (whether favourable or not), crawl-away hatchlings will likely remain in the same system as their parents. Maternal provisioning assumes that the female can accurately predict the conditions that her offspring will experience, despite the highly dynamic nature of the marine environment. Crean and Marshall (2009) argue that the best strategy for maternal provisioning in variable conditions is to generate a variety of phenotypes in the offspring, also known as 'bet-hedging'. It is therefore informative to determine how much variation occurs between clutches (i.e. same mother) and between females across treatments when investigating maternal provisioning experimentally. If the conditions are predictable, females may produce offspring with similar traits. For example, the brooding bryozoan *Bugula neritina* will produce larger larvae with more dispersive potential if intraspecific competition is high (Allen et al., 2008).

Whelks with benthic development provide an ideal case-study for the effects of heavy metal pollution on a subsequent generation, as whelks bioaccumulate metal from their diet, as well as aqueous surroundings (Blackmore & Wang, 2004a; Cheung et al., 2006; Cheung & Wang, 2005). Previous research has demonstrated that heavy metals may be transmitted from female to offspring via gametes in other species (Beeby & Richmond, 2001; Cazan & Klerks, 2014; Weng & Wang, 2017). Embryos can have elevated MT concentrations,

suggesting that it is either rapidly synthesized by embryos or maternally inherited (Damiens et al., 2006; Weng & Wang, 2014, 2017). While these offspring may be more tolerant of pollution (Piola & Johnston, 2006b; Plautz & Salice, 2013; Weng & Wang, 2014), their growth rates are often compromised (Marshall, 2008; Piola & Johnston, 2006b; Weng & Wang, 2017). There is also evidence of maternal transfer occurring for tributyltin (TBT) in the buccinoid *Buccinastrum deforme* (Averbuj et al., 2018) and volute *Pachycymbiola brasiliana* (Goldberg et al., 2004).

1.8 Focal Species and System

Cominella glandiformis (Figure 1.1) is an abundant, endemic, buccinoid whelk in New Zealand estuaries (Ansell, 2001; Morton & Miller, 1973). Females deposit benthic egg capsules in which embryos develop over several months to hatch out as small juveniles (Morley, 2013; Pilkington, 1974). Thus, the entire lifecycle of this species is benthic, and all stages reside within an ecosystem likely to experience metal pollution (Bryan, 1980; Kennish, 2002). As a benthic developer, this species had high levels of per-offspring investment, and previous research suggests that environmental factors can influence this (e.g. Lloyd & Gosselin, 2007; Plautz, Funkhouser, et al., 2013; Plautz, Guest, et al., 2013; Plautz & Salice, 2013; van der Sman et al., 2009). The degree of maternal investment can vary, resulting in fewer capsules or fewer embryos per capsule (Carrasco et al., 2012; Chatzinikolaou & Richardson, 2010; Cheung & Lam, 1999; van der Sman et al., 2009). In species with nurse eggs (non-viable, nutritive eggs), stressed females may allocate fewer nurse eggs per embryo, resulting in smaller hatching sizes (e.g. Lloyd & Gosselin, 2007).

Figure 1.1: *Cominella glandiformis* adult from Te Awarua-o-Porirua Harbour. (Photo courtesy of Melanie Dohner).

Previous descriptions of *C. glandiformis* reproduction have described the appearance and partial development of a few field-collected capsules (Morley, 2013; Pilkington, 1974). In contrast, the reproductive trends of two related species from the rocky-shore intertidal (*C. maculosa* and *C. virgata*) have been described in detail (Carrasco & Phillips, 2014; van der Sman, 2007). These species show different maternal investment (Carrasco & Phillips, 2014; Carrasco et al., 2016), which can be influenced by stress (van der Sman, 2007). Pilkington (1974) suggested that *C. glandiformis* females provide nurse eggs for extra-embryonic nutrition, but this is yet to be confirmed. While *C. glandiformis* in the South Island of New Zealand have been reported to lay capsules "throughout most of the year, particularly autumn" (Pilkington, 1974), those in the lower North Island appear to lay their eggs over late spring and summer (pers. obs.). This period was also observed for its rocky shore relatives *C. virgata* and *C. maculosa* nearby (Carrasco & Phillips, 2014; van der Sman et al., 2009).

Cominella glandiformis is commonly found clustered around *Austrovenus stutchbury*i and *Macomona liliana* (Ansell, 2001; Morton & Miller, 1973; Stewart & Creese, 2004). Recent studies into the effects of heavy metals (specifically Cu, Zn and Pb) on these infaunal bivalves have found reduced densities of *M. liliana* – the most sensitive of the two bivalves to this pollution (Fukunaga et al., 2010). *Austrovenus stutchburyi* only accumulates Cu when Zn was present, however, and appears to maintain limited Zn regulation, unlike *M. liliana* which tended to have the greatest body burden of all three metals (Fukunaga & Anderson 2011).

Te Awarua-o-Porirua Harbour (the Porirua Harbour) is located in the south of New Zealand's North Island (Figure 1.2). Comprised of two partially linked 'arms', this is one of the country's largest estuaries. Within each arm, streams and rivers deliver freshwater and pollutants from their catchments to the system. The Onepoto arm is surrounded by urban and industrial areas, with some farmland at the farthest end, where the Pauatahanui Arm receives run-off from forested, farmed, and urban land. *Cominella glandiformis* and *A. stutchburyi* are found throughout the harbour, with a large population at Paremata Station and Browns Bay (PS and BB in Figure 1.2(C)) (pers. obs.). The harbour has up to 2.7 times the Zn levels that the Auckland Regional Council Environmental Response Criteria (ARC ERC) deems as likely to cause ecological harm, and the Cu concentrations are at 'early warning'

trigger levels (ARC ERC, 2004; Sorensen & Milne, 2009). Since then, fine sediment loads (which often transport heavy metals into waterways) have doubled (Stevens & Forrest, 2020).

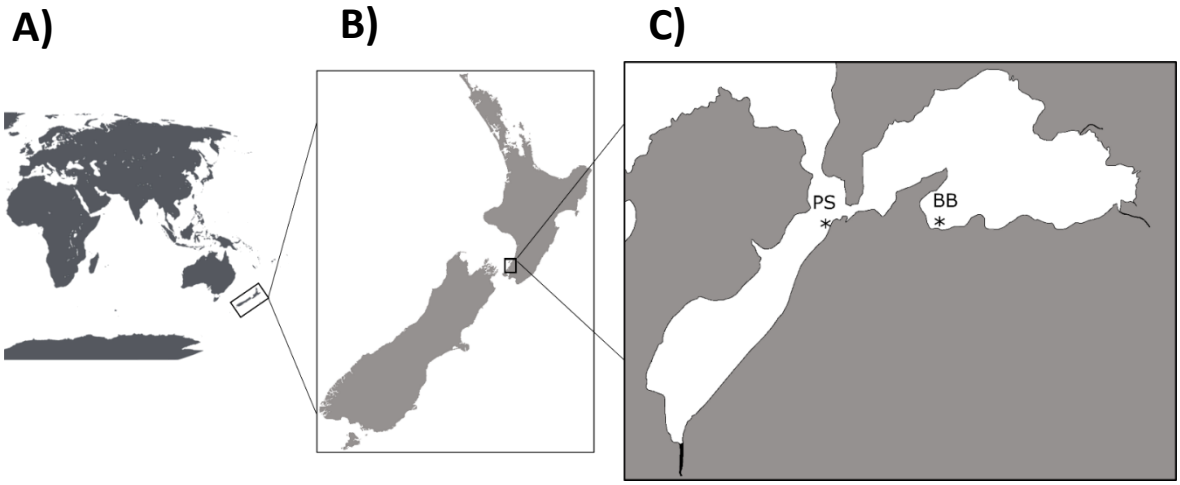

Figure 1.2: Location of Te Awarua-o-Porirua Harbour. A) a map of the word shows the location of B) New Zealand, with C) the Porirua Harbour (41°06'16.9"S 174°51'59.0"E), with two sites (Paremata Station (PS, 41°06'16.9"S 174°51'59.0"E) and Browns Bay (BB, 41°06'19.9"S 174°52'56.3"E)) containing high densities of *Cominella glandiformis.*

1.9 Aims and structure of thesis

My thesis uses *C. glandiformis* as a model organism to examine the effects of heavy metal pollution on multiple life stages in a benthic marine carnivore. The general reproductive trends of this species are unclear, but research with other species suggests that maternal stress may impact offspring performance. Research on benthic development under heavy metal pollution has used aquatic pulmonates, which likely have different toxicity thresholds and responses than marine species.

In Chapter 2, I describe the maternal investment of *C. glandiformis* from two sites with large populations of both whelks and their prey (*A. stutchburyi*). In Chapter 3, I examine the effects of pollutant mixture and pathway on the physiology and foraging ability of *C. glandiformis* after one-month exposure. Chapter 4 builds on this, examining the maternal investment of females exposed for one month prior to the laying season. The developmental mortality of their (likewise polluted) offspring is also reported. In Chapter 5, I follow the growth and survival of encapsulated embryos and recently hatched juveniles exposed to pollutants. I summarise and discuss the results of these experiments in Chapter 6.

Chapter 2 – Reproduction in *Cominella glandiformis* from natural populations.

2.1 Introduction

Benthic development, where embryos develop within a physical structure and hatch as miniature adults (i.e. no pelagic stage), requires a high per-offspring investment. The female provides the energy necessary to complete development, as well as manufacturing a protective structure (hereafter 'capsule') (Averbuj et al., 2017; Kideys et al., 1993; Miloslavich, 1996; Perron, 1981; Vance, 1973). Embryos with access to greater yolk reserves are expected to develop into larger hatchlings (Chaparro et al., 1999; Chaparro & Paschke, 1990; Lloyd & Gosselin, 2007; McGinley et al., 1987; Rivest, 1983; Smith & Fretwell, 1974; Spight, 1976a, 1976b). Larger offspring can have greater energy stores (Moran & Emlet, 2001), faster growth rates (Marshall & Keough, 2007, 2008; Moran & Emlet, 2001) and are more likely to survive (Carrasco & Phillips, 2012; Marshall & Keough, 2008; Moran & Emlet, 2001; Rawlings, 1994; Spight, 1976a), but require greater maternal investment than a smaller hatchling. Due to the finite energy reserves of females, a trade-off between the number and size of offspring is expected (Bernado, 1996; Kamel et al., 2010; Smith & Fretwell, 1974; Vance, 1973).

Larger females often lay more capsules than smaller conspecifics, likely due to greater energy reserves (Chatzinikolaou & Richardson, 2010; Chung et al., 2013; Harding et al., 2007; Ilano et al., 2004). As larger females have larger reproductive organs (e.g., capsule gland and pallial oviduct) (Fretter & Graham, 1994), there is often a relationship between female and capsule size (Chatzinikolaou & Richardson, 2010; Chung et al., 2013; Ilano et al., 2004; Nasution et al., 2010). These larger capsules usually contain more and/or larger offspring (Avaca et al., 2021; Chatzinikolaou & Richardson, 2010; Harding et al., 2007; Smith & Thatje, 2013), making capsule volume a useful indicator of maternal investment.

Localised stressors, such as food scarcity, pollution, tidal exposure, etc., can reduce the energy available for reproduction and cause females from neighbouring areas to differ in reproductive trends (Avaca et al., 2015; Chatzinikolaou & Richardson, 2010; Collin & Ochoa,

2016; McGinley et al., 1987). For example, van der Sman (2007) collected *Cominella maculosa* from sites with differing food availability and kept them under either starved or sated food treatments prior to the laying season. The number of hatchlings per capsule did not vary, but the number and sizes of capsules laid showed site and treatment -specific patterns: Sated whelks from the high-prey abundance site laid more, though similarly sized, capsules than starved whelks. Sated whelks from the low-prey-abundance site produced half the number of capsules as the starved whelks, but the capsule volume was larger.

Cominella maculosa, C. virgata and *C. glandiformis* are three species of whelks common in New Zealand (Morton & Miller, 1973). Both *C. maculosa* and *C. virgata* occupy the rocky-shore intertidal area while *C. glandiformis* is an estuarine species. Despite occupying the rocky-shore intertidal, the reproductive strategies implemented *C. maculosa* and *C. virgata* vary. Where *C. maculosa* encapsulate multiple embryos within each capsule (all of which develop), *C. virgata* encapsulates a single embryo (Carrasco & Phillips, 2014). In *C. maculosa*, larger capsules contained more embryos, but the number of hatchlings was negatively correlated with their size (Carrasco & Phillips, 2014). As expected from a trade-off between the number and size of offspring, the individually encapsulated embryos of *C. virgata* hatched at twice the size of *C. maculosa* (Carrasco & Phillips, 2014). This larger hatching size and the subsequent growth rates enabled *C. virgata* juveniles to outgrow the claw size of one of their main predators within 2 months (Carrasco & Phillips, 2012).

Although there are some descriptions of a few field-collected capsules (Graham, 1941; Morley, 2013; Pilkington, 1974), little is known about reproduction in *C. glandiformis*. The tolerances and adaptations required for survival in an estuarine environment differ from those of the rocky shore, particularly for salinity and anoxia (e.g. González-Oreja & Saiz-Salinas, 1998; Teske & Wooldridge, 2003; Ysebaert et al., 1998). Comparisons between reproductive trends in estuarine (present study) and rocky-shore (Carrasco & Phillips, 2014) relatives are likely to reflect this.

This chapter examines the variation in maternal investment seen in *Cominella glandiformis* from two sites within the same harbour. Specifically, I ask:

1) Do whelks from different sites show a) different laying patterns (e.g. timing, number laid) or b) differences in maternal investment (volume of capsule, and number and size of eggs)?
2) Are the number and volume of capsules related to female shell length?
3) What is the relationship between a) capsule volume and the number or size of eggs, and b) the number and sizes of eggs?

2.2 Methodology

2.2.1 Study system

I focus on the iteroparous, estuarine whelk *Cominella glandiformis*. This species reproduces over late spring and throughout the summer. Females lay capsules on available surfaces such as empty shells or seagrass (*Zostera muelleri*) blades. Each capsule contains multiple eggs that all develop into crawl-away juveniles (hatchlings) over several months (i.e. no nurse eggs, pers. obs). The source location of my study animals is the Te Awarua-o-Porirua Harbour (Wellington, New Zealand), a large estuary comprised of two partially linked arms (Figure 1.2(C)). I chose two sites within this harbour – Browns Bay (BB) within the Pāuatahanui Arm (41°06'19.9"S 174°52'56.3"E) and Paremata Station (PS) within the Onepoto arm (41°06'16.9"S 174°51'59.0"E). Both sites contain large whelk populations, as well as *Austrovenus stutchburyi* (cockles), a preferred food source of the whelks (Ansell, 2001; Morton & Miller, 1973; Stewart & Creese, 2004).

2.2.2 Study overview

I examine reproductive patterns of whelks from two proximal sites to establish trends in capsule laying and indicators of maternal investment. I collected whelks from BB and PS sites on two occasions (late August and early October) in the lead-up to the reproductive season. I placed each female in her own container so that capsules could be linked to their mother. Whelks were kept in a seatable with flowing filtered (≤1 µm) seawater (FSW). I recorded the number of capsules laid by each female over four months to evaluate patterns of variation in capsule production, among individuals collected from different locations, and through time. Additionally, I sub-sampled capsules to evaluate patterns of variation in capsule volume, and numbers and sizes of eggs contained within.

2.2.3 Capsule laying trends.

I collected adult *C. glandiformis* of varying sizes from the two sites in late August 2018 (14 females from PS and 5 females from BB) and early October 2018 (7 females from each site). I maintained whelks at the Victoria University Coastal Ecology Lab, in flowing, filtered (≤1 µm) seawater (FSW) for an initial acclimation period of 14 days. During this time, I determined the sex of each individual (indicated by the presence/absence of penis), and I tagged all females using bee tags affixed with super glue. I measured the shell lengths of all

females (shell apex to siphonal notch) to the nearest 0.01mm using Vernier callipers.). I allocated a random male to each container to ensure fertilisation. Twice a week I pooled whelks from each site into a 2L container to feed on live, crushed *A. stutchburyi* collected from the same site. I recorded the number of capsules laid in each container Sunday through Friday, from September 2018 to mid-January 2019.

Statistical Analyses:

I conducted all statistical tests in R (V. 4.0.3) using RStudio (V 1.3.1093; RStudio Team (2020)). I visually checked data using histogram, Fitted vs. Residual and QQ plots produced by the plot() and hist() functions of the graphics package, and qqnorm() and qqline() functions of the stats package (R Core Team, 2020).

To determine whether weekly capsule production varied between females from the two sites, or through the season, I performed a generalised linear mixed model (GLMM) with Poisson distribution. This model examined the number of capsules laid per week by each female, with site and week (and their interaction) as fixed factors, and collection group (whether female was collected in late August or early October), and female ID as random factors. This used the glmer() function from the lme4 package (Bates et al., 2014).

2.2.4 Maternal investment between sites.

I haphazardly selected capsules to be preserved in 70% ethanol on the day they were laid. Once the laying season was finished, I created a list of females that laid consistently throughout the season. From this list, I haphazardly selected seven females from each site, and examined their capsules for indicators of maternal investment. To determine capsule volume and egg diameter, I either photographed the specimens using a Canon EOS 70D(w) mounted on a dissecting microscope (Olympus S261), or manually measured them under a dissecting microscope. I first determined the capsule volume then dissected out the eggs within to determine their size (4.5x mag) and number. Capsules are the shape of a truncated cone (Figure 2.1), with a narrower top than base. I estimated capsule volume by measuring the height (1.5x mag) and the diameters of plug and base (2.5x mag) using Equation 2.1. For photographed capsules and eggs, I used ImageJ (Schneider et al., 2012) to take measurements.

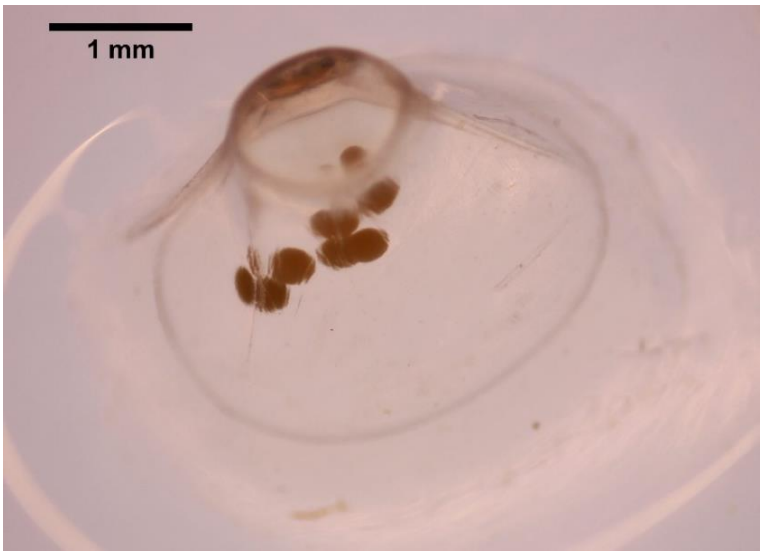

Figure 2.1: A capsule of *Cominella glandiformis* photographed under 2.5x magnification of a dissecting microscope.

$$V = \frac{1}{3} \times \pi \times h\,(r_1^2 + r_{1\times}\, r_2 + r_2^2) \qquad \text{Equation 2.1}$$

Where r_1 is the radius of the capsule base, r_2 is the radius of the plug, and h is the height of the capsule.

Statistical Analyses:

Using linear mixed effect models (LMMs), I examined whether capsule volume and the number and size of eggs within varied between females from the two sites (fixed factor). For capsule volume and the number of eggs per capsule, I included female ID as a random effect in a LMM (site as fixed factor). When examining the size of eggs, I included female ID and capsule ID as random effects. I performed these linear mixed models using the lmer() function from the lme4 package (Bates et al., 2014). The lmer() function doesn't produce p-values, so I used the anova() function from the lmerTest package. This estimates p-values using the Satterthwaite approximation, and is considered more reliable than others (e.g. likelihood ratio tests) (Luke, 2017).

2.2.5 Capsule volume and female size

Female size can determine total capsule output and volume in other species. As maternal investment trends didn't vary between sites, I pooled data to examine the relationship between female size and capsule trends.

Statistical Analyses:

I used the lm() function from the stats package (R Core Team, 2020) to examine the relationship between female shell length and the total number of capsules laid, and capsule volume.

2.2.6 Capsule volume and number and size of eggs.

A larger capsule volume could contain more and/or larger eggs, yet a trade-off is usually expected between the number and size of eggs. Using data pooled across sites, I examined the relationships between these capsule traits.

Statistical Analyses:

I used LMM's to examine the relationships between capsule volume and shell length and capsule volume and the number of eggs. Both include Female ID as a random effect. I included Capsule ID and Female ID as random effects in the LMM examining the relationship between capsule volume and size of eggs as multiple eggs occur in each capsule. I also examined the relationship between the number and size of eggs, with Capsule ID and Female ID as random effects.

2.3 Results

2.3.1 Capsule laying trends

Overall, whelks from both sites laid more capsules as the season progressed (GLMM with Poisson distribution (Female and Collection Group as random factors): Chisq = 64.43, 11 df, $p < 0.0001$), though there was no overall difference between sites (Chisq = 0.41, 1 df; $p = 0.5204$). There was a significant interaction between Week and Site (Chisq = 28.33, 11 df; $p < 0.0029$), with increasing numbers of capsules laid over the first four weeks by whelks from both sites, and a decline in week five (Figure 2.2). Between weeks six and eight, the average BB female laid 1.2 times as many capsules as a PS conspecific (Figure 2.2). The final 4 weeks, however, saw this reversed, with the average PS female laying 1.2 times more than a BB conspecific (Figure 2.2).

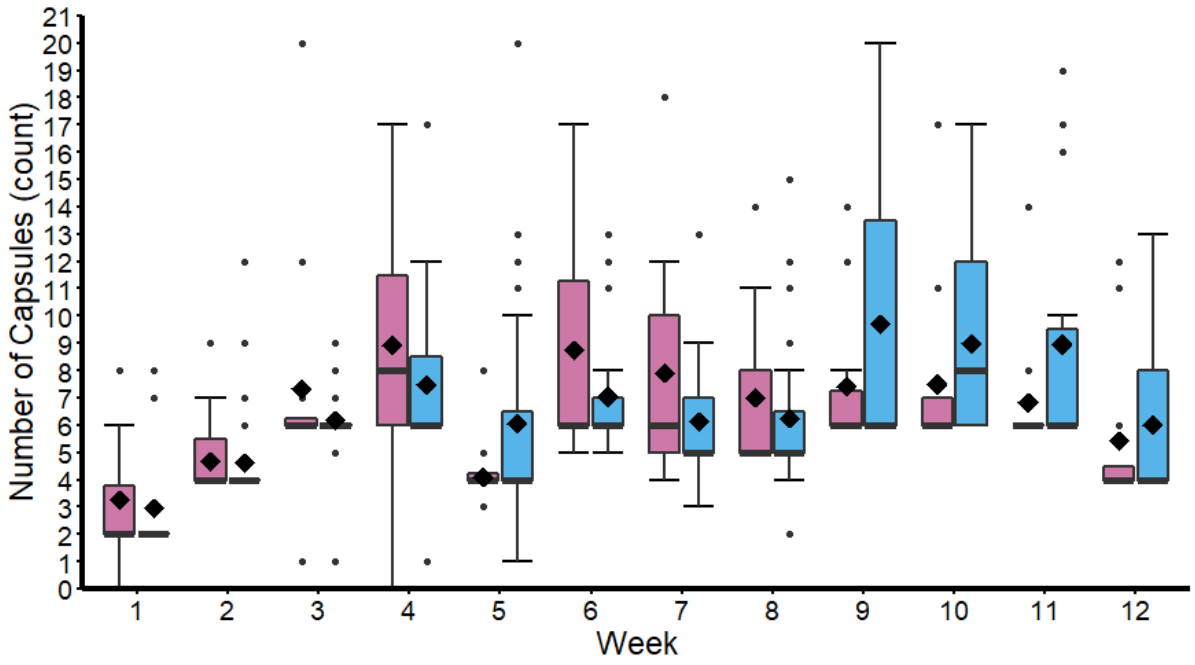

Figure 2.2: Number of capsules laid per week by females from Browns Bay (pink) and Paremata Station (blue). The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR (interquartile range) of the box. Mean represented by black diamond. n = 12 whelks from BB, 23 whelks from PS.

2.3.2 Maternal investment between sites.

Capsules had similar volumes across sites (LMM (Female and week laid as random effects): F(1, 15.1) = 0.1081, p = 0.7469), averaging 6.7 µL (+/- 1.8 stdev) (Figure 2.3). These capsules contained 5.1 (+/- 1.6 std. dev) eggs (LMM (Female as random effect): F(1, 12.2) = 1.192, p = 0.296), though capsules of PS whelks contained a wider range (Figure 2.4). Eggs were similarly sized (LMM (Female and week laid as random effects): F (1, 12.6) = 0.1030, p = 0.7535), averaging 0.28 mm diameter (+/- 0.04 std. dev), though a wider range in the number of eggs occurred in the capsules of PS whelks (Figure 2.5). A high proportion (44%) of the variation in egg diameter occurred within and between capsules.

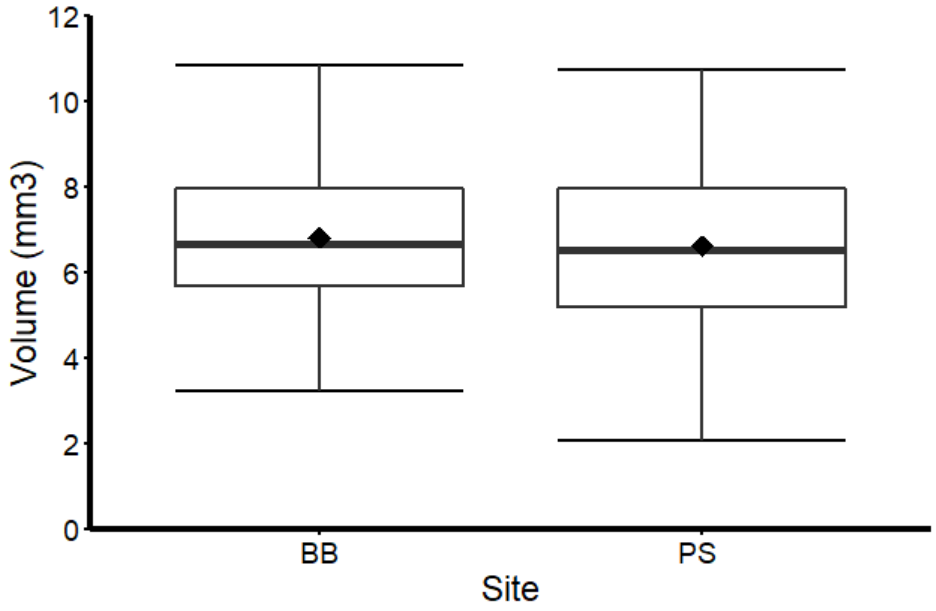

Figure 2.3: Capsule volumes of a subset of females from Browns Bay (BB) and Paremata Station (PS). The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR of the box. Mean represented by black diamond. n = 109 capsules from 7 BB females, 128 capsules from 7 PS females.

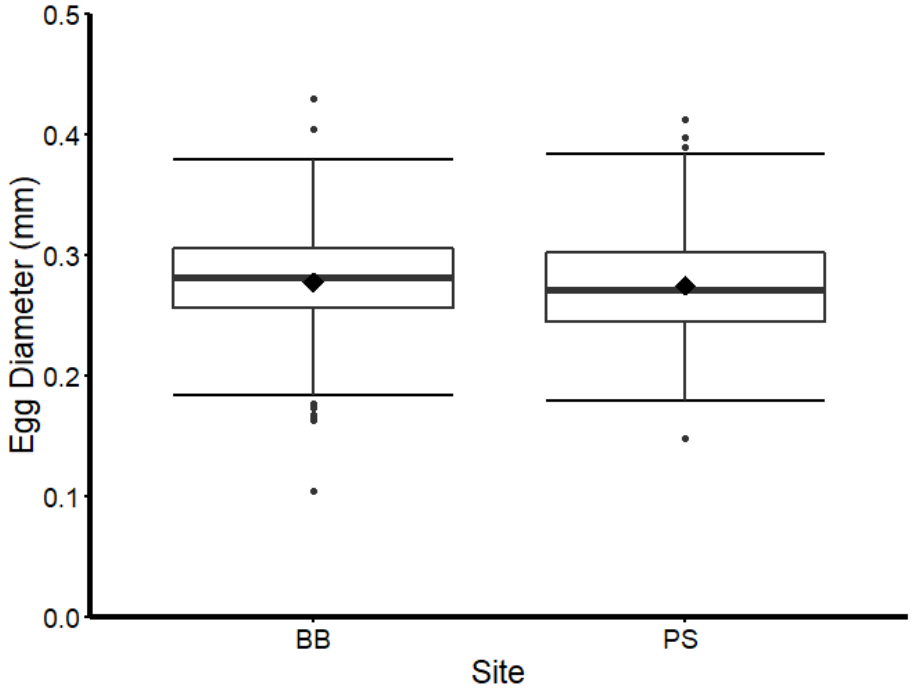

Figure 2.4: Egg diameters of a subset of females from each site. The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR of the box. Mean represented by black diamond. n = 439 eggs in 109 capsules from 7 BB females, 427 eggs in 128 capsules from 7 PS females.

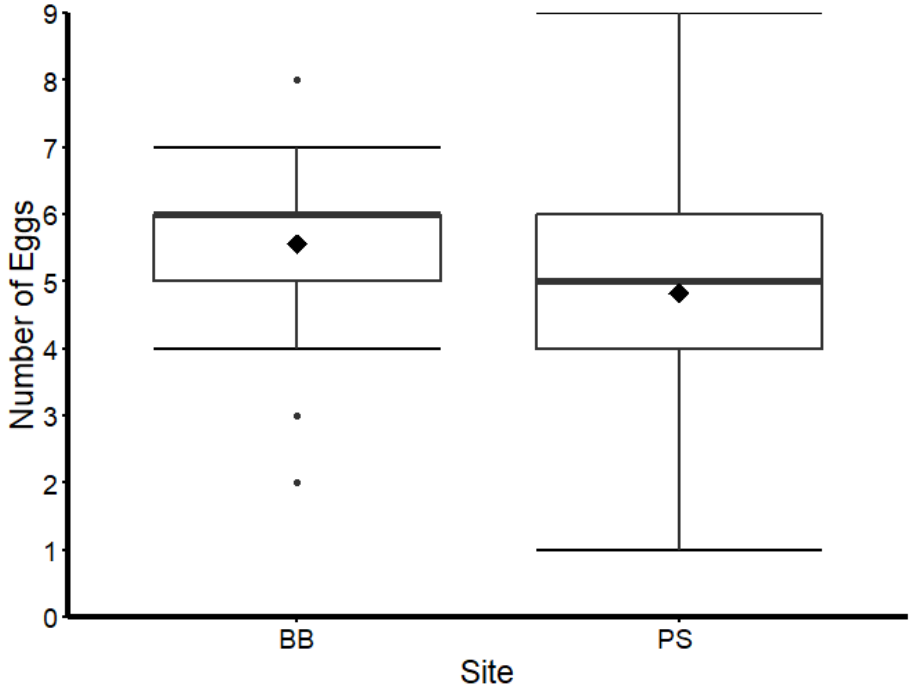

Figure 2.5: The number of eggs per capsule from a subset of females from each site. The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR of the box. Mean represented by black diamond. n = 109 capsules from 7 BB females, 128 capsules from 7 PS females.

2.3.3 Female size and capsule output and volume.

Larger females laid more capsules, with an additional 3.3 (+/- 1.1 std. errors) more capsules laid for each 1 mm increase in female shell length (linear regression: $F_{1,33} = 9.35$, $p = 0.0044$) (Figure 2.6). They also laid larger capsules, with volume increasing by 0.42 (+/- 0.1 std. errors) mm^3 for each additional millimetre in shell length (linear regression: $F_{1, 231} = 15.84$, $p < 0.0001$) (Figure 2.7).

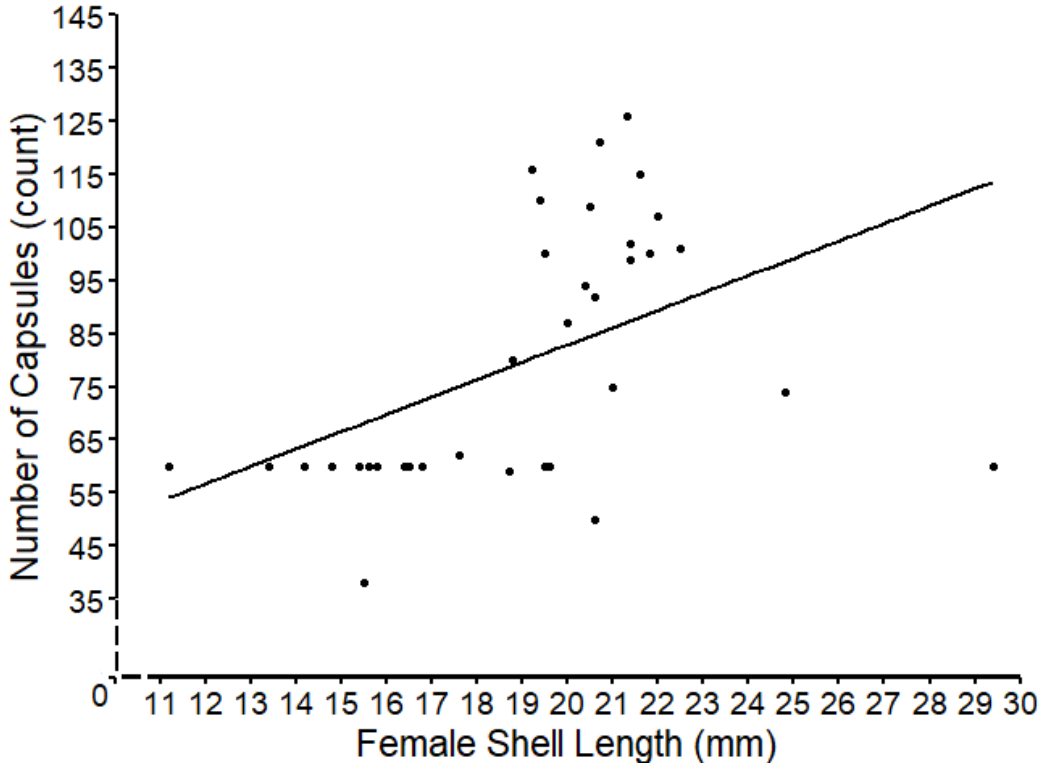

Figure 2.6: The relationship between female shell length and the number of capsules laid (overall). Data is pooled across sites. n = 35 whelks. Y = 17.54 + 3.27x.

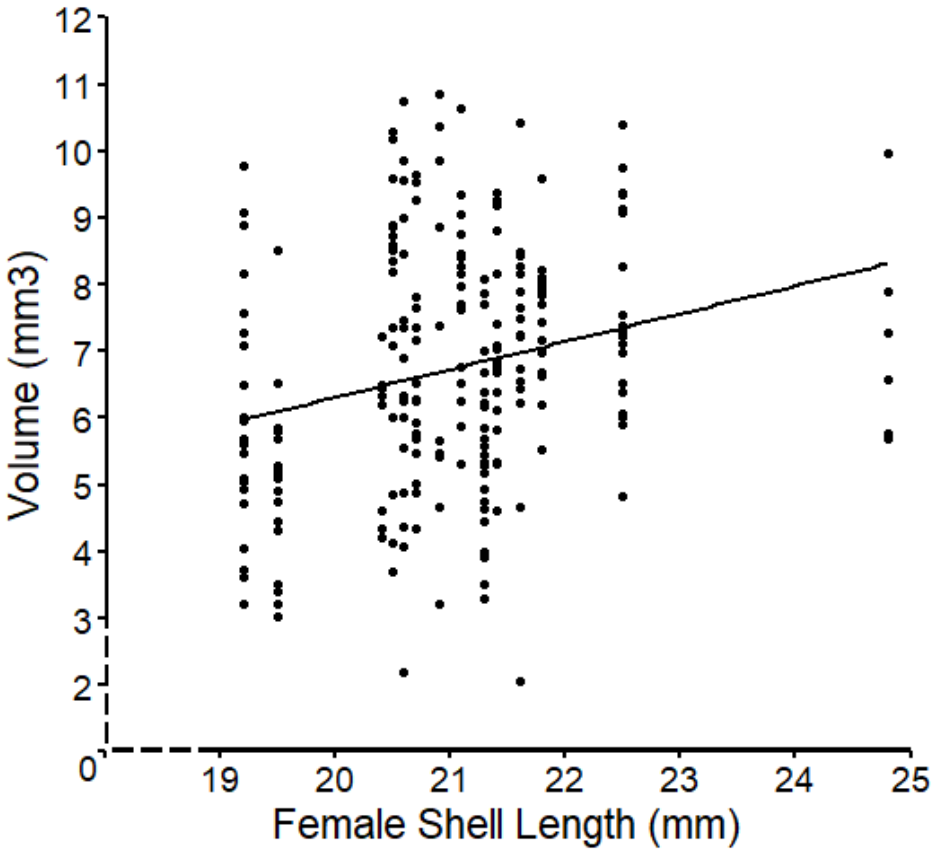

Figure 2.7: The relationship between female shell length and capsule volume from a subset of females. Data is pooled across sites. n = 14 whelks. Y = -2.04 + 0.42x

2.3.4 Relationships between the number and sizes of eggs and capsule volume.

When pooled across sites, capsule volume and the number of eggs per capsule were unrelated (LMM (Female as random effect): F(1, 223.5) = 2.17, p = 0.1423). For every 1uL increase in capsule volume, egg diameter increased by 0.003 mm (+/- 0.001 se) (LMM (Capsule ID and Female ID as random effects): F(1, 141.5) = 5.58, p = 0.0196) (Figure 2.8). The average egg diameter is 0.002 mm smaller (+/- 0.0006 m std. error) with each additional egg in a capsule (LMM (Capsule ID and Female ID as random effects): F(1, 730.2) = 10.64, p = 0.0012) (Figure 2.9).

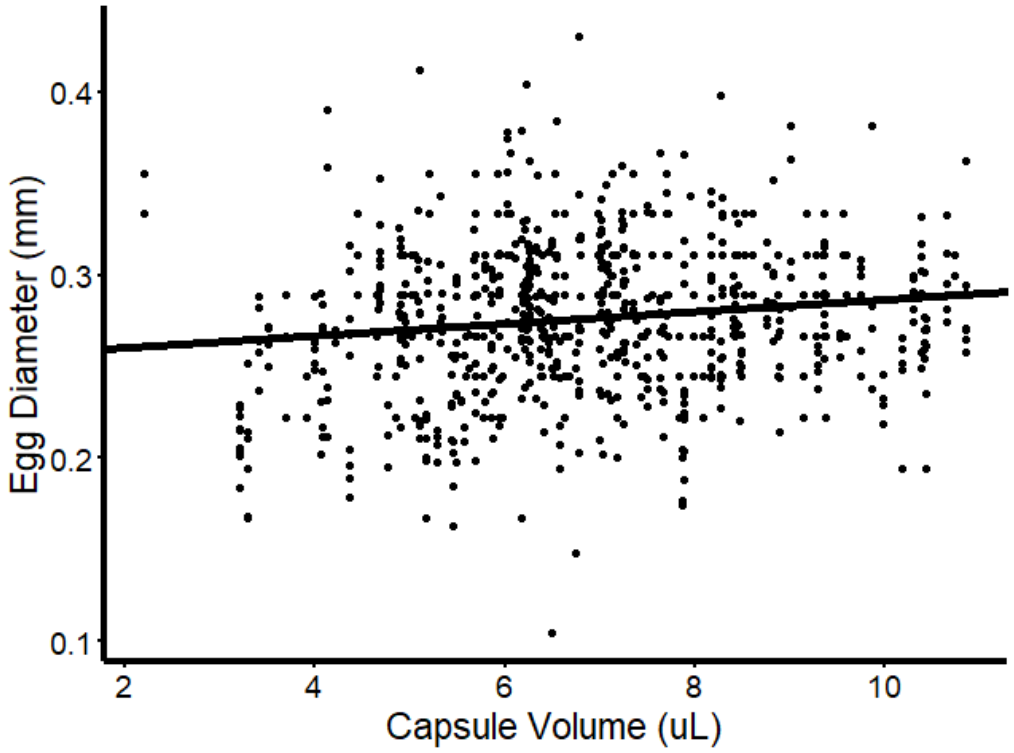

Figure 2.8: The relationship between capsule volume and egg diameter from a subset of females. Data is pooled across sites. n = 171 capsules from 14 whelks.
Y = 0.249 + 0.0039x.

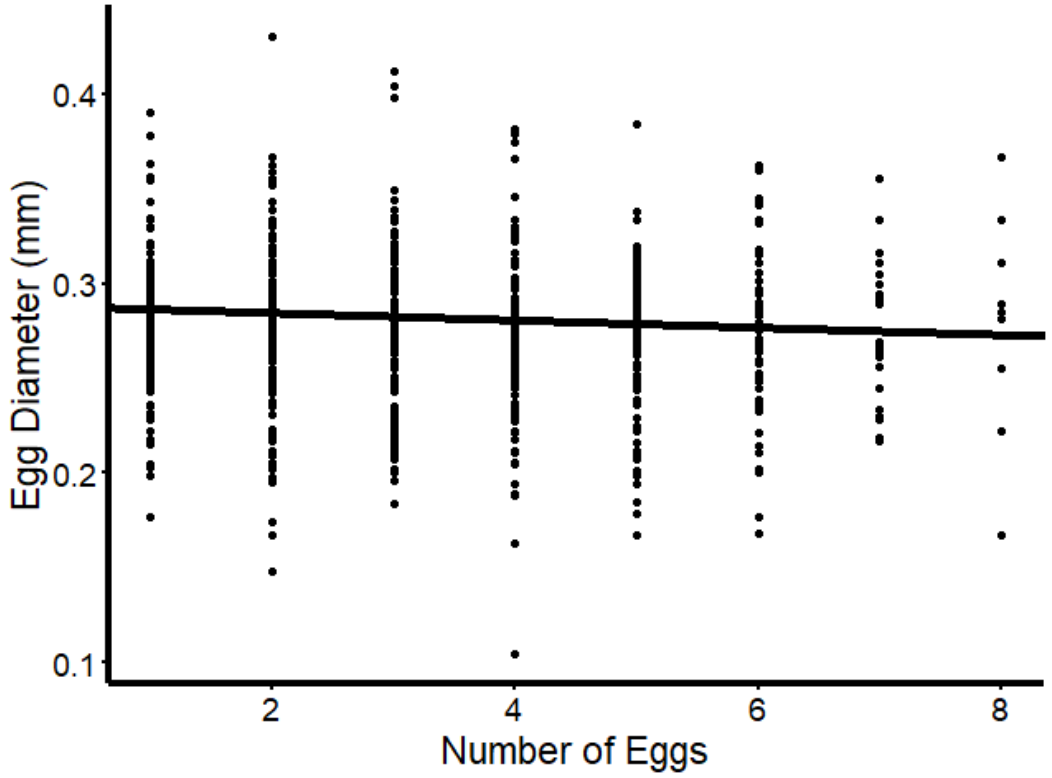

Figure 2.9: The relationship between the number of eggs within a capsule and their diameter from a subset of females. Data is pooled across sites. n = 172 capsules from 14 whelks.
Y = 0.2822 - 0.0019x

2.4 Discussion

This study is the first to characterise the reproductive cycle of *C. glandiformis*. Overall, whelks from both sites showed similar reproductive patterns, laying more capsules as the season progressed and investing similarly in each capsule. Despite experiencing the same laboratory conditions, weekly capsule laying by whelks from Paremata Station peaked three weeks after Browns Bay whelks. This suggests that site-specific experiences prior to collection may influence reproduction almost two months later. Environmental cues such as temperature and daylength often signal the start of the laying season in whelks (Chatzinikolaou & Richardson, 2010; Cumplido et al., 2010; Harding et al., 2008; Penchaszadeh et al., 2009), and are likely the reason for increasing capsule laying through the season.

Larger females laid more capsules over the season than their smaller conspecifics. Female size was also related to capsule volume, likely due to the increased size of reproductive organs and these capsules also had a larger volume. (Fretter & Graham, 1994). Further, smaller individuals tend to invest more energy in growth than reproduction (Chatzinikolaou & Richardson, 2010). Though this can't be confirmed in the present study as females were not re-measured at the end of the season.

Larger capsules contained larger eggs, which are expected to have higher energy reserves, enabling larger hatching sizes (Chaparro et al., 1999; Chaparro & Paschke, 1990; Lloyd & Gosselin, 2007; McGinley et al., 1987; Rivest, 1983; Smith & Fretwell, 1974; Spight, 1976a, 1976b). A negative relationship between the number and size of eggs occurred in *C. glandiformis* capsules, consistent with the expected trade-off between number and size of offspring (Kamel et al., 2010; Smith & Fretwell, 1974). Like its rocky shore relatives (*C. maculosa* and *C. virgata*), *C. glandiformis* encapsulates a consistent number of eggs to each capsule, none of which act as nurse eggs (Chapter 4; Chapter 5; Carrasco & Phillips 2014). Consistent with this observation is the lack of relationship between capsule volume and the number of eggs within. A positive correlation between these variables is often found in species with nurse eggs (e.g. Avaca et al. 2012; Chatzinikolaou & Richardson 2010; Nasution et al. 2010).

2.4.1 Comparisons with other endemic *Cominella* species

Previous descriptions of *C. glandiformis* reproduction (Graham, 1941; Morley, 2013; Pilkington, 1974) rely on few field-collected capsules, and were therefore unable to describe reproductive trends. Five to seven embryos per capsule were reported by these studies, consistent with the average 5.1 in the present study (range: 1-9 eggs). Pilkington (1974) hypothesised that capsules may contain nurse eggs, but this was not observed in the present study (data not shown), or by Morley. Further, Pilkington (1974) reported seeing *C. glandiformis* capsules throughout the year in Otago Harbour, but none were observed prior to September in the present study. This is supported by capsules found on seagrass blades in summer, but not winter in the Porirua Harbour (Duncan, 2017). Previous studies of rocky shore *Cominella* have reported laying seasons beginning in September or October (Carrasco & Phillips, 2014; van der Sman, 2007; van der Sman et al., 2009). The timing of the reproductive season is assumed to be related to day length and/or warming temperatures, as found in other species (e.g. Avaca et al., 2015; Cumplido et al., 2010; Harding et al., 2008).

The average *C. glandiformis* capsule was 6.7 µL, can contained five 0.28 mm (diameter) eggs. Previous descriptions did not describe egg sizes, though Morley (2013) describes the capsules as “measuring less than 2 mm in diameter”, and “3mm full width”, which agrees with the current study. Rocky shore *Cominella* also encapsulate a consistent number and size of eggs, the average capsule containing 7.7 (+/- 0.2) and one embryo, respectively (Table 2.1). This investment continued even when starved, though fewer capsules were laid (van der Sman, 2007). Further, offspring of starved *C. virgata* emerged at a similar size to those of sated adults but had slower post-hatching growth rates (van der Sman et al., 2009). It is unknown whether *Cominella glandiformis* in poor condition likewise show similar investment in number and sizes of offspring by compromising total reproductive output.

As summarised in Table 2.1, *C. glandiformis* lays much smaller capsules, yet containing slightly larger eggs, than reported in related rocky shore species or *Haustrum scobina* (whose capsules have a closer morphology than the other *Cominella*). This is likely due to a combination of the smaller shell of *C. glandiformis* (Table 2.1) and the more ‘squat’ shape of the capsules. Though not examined in the present study, these smaller, squatter capsules may have a higher surface area to volume ratio. This could enable more efficient diffusion of

oxygen in an ecosystem prone to anoxic conditions. The larger egg diameter could indicate greater per-embryo yolk supplies, increasing the nutrition available to each embryo (Clarke, 1993)

2.4.2 Conclusion

Cominella glandiformis show similar trends to those previously reported in rocky shore *Cominella* in New Zealand. Females show consistent per-capsule investment, though further research should examine whether this continues under stress. Females from different sites laid similar numbers of capsules, though output peaked at slightly different times. This suggests lingering effects of site-specific experiences, even after >1 month in a laboratory setting. Larger females had a higher fecundity than smaller females, producing more capsules over the season. As capsule size increased, so did the number and size of eggs within. As predicted by life history theory, egg diameter decreased with each additional egg.

Table 2.1: Summary of maternal investment from four intertidal whelk species found in the Wellington Region (New Zealand). Data is presented as average (+/- standard error) for Carrasco & Phillips (2014) data, and average (+/- standard deviation) for the present study. No standard error was presented for van der Sman (2007) data.

Species	Female shell length (mm)	Capsule volume (µL)	Number of eggs per capsule	Size of eggs (2-cell stage in Carrasco study)	Source
Haustrum scobina	n/r	18.6 (+/- 1.7)	235 (+/- 17)	0.23 (+/- 0.001)	Carrasco & Phillips (2014)
	15.2 - 25.3	10.2 (high food) 11.5 (low food)			van der Sman (2007)
Cominella virgata	n/r	23.1 (+/- 0.97)	1	0.22 (+/- 0.01)	Carrasco & Phillips (2014)
	27.3-40.1	23.6 (high food) 22.4 (low food)			van der Sman (2007)
Cominella maculosa	n/r	29.7 (+/- 0.86)	7.7 (+/-0.3)	0.24 (+/- 0.01 SE)	Carrasco & Phillips (2014)
	32 - 47.5	40.2 (high food) 37.5 (low food)			van der Sman (2007)
Cominella glandiformis	11.2 – 29.4	6.7 (+/- 1.8 stdev)	5.1 (+/- 1.6 std dev)	0.28 (+/- 0.04 std dev)	Present study

Chapter 3 – The effects of Cu and Zn pollution on the condition and foraging ability of a benthic carnivore

3.1 Introduction

Heavy metal pollution is a widespread and well-recognised threat to ecosystems (Ali et al., 2019; Ansari et al., 2004; Audry et al., 2004; Bryan & Langston, 1992; Nicolaus et al., 2022; Zhang & Shao, 2013). Marine invertebrates are exposed to pollution via their diet and surrounding seawater and sediment. Heavy metal exposure can cause a variety of responses, from the cellular-level (e.g. production of metallothionein (MT), a metal-binding protein) (Boldina-Cosqueric et al., 2010; Brown et al., 2004; Valavanidis & Vlachogianni, 2010) to behavioural (e.g. moving away, reduced food consumption) (Guo et al., 2013; Roper & Hickey, 1994; Zidar et al., 2005).

The pathway (aqueous, dietary or sediment) and mixture of pollutants can influence the observed responses (both physiological and behavioural) of organisms (Campana et al., 2013; DeForest & Meyer, 2015; Lapointe et al., 2011; Sobrino-Figueroa & Caceres-Martinez, 2009; Vedamanikam & Hayimad, 2013; Wang & Ke, 2002). A lot of research has focused on understanding the effects of each pathway in isolation (i.e., aqueous-only, or dietary-only). For example, the concentration of MT can influence the uptake of diet-borne heavy metals in the whelk *Reishia (Thais) clavigera*, but has no effect on aqueous uptake (Blackmore & Wang, 2004a). While single-pathway studies improve our understanding of how organisms respond to this stress, experiencing dietary-only pollution is unlikely to occur in nature.

Many studies have noted that organisms will reduce their feeding rate when presented with polluted food (Cain et al., 2016; Guo et al., 2013; Pease et al., 2010; Zidar et al., 2005; Zidar et al., 2003; Zidar et al., 2004). This could indicate an active choice to avoid pollutant exposure (e.g. Zidar et al., 2004), or a passive choice due to appetite loss (e.g. Cain et al., 2016). Either way, the energy available to the organism is reduced, which can impact subsequent growth and reproduction (e.g. Cheung et al., 2008; Cheung & Lam, 1999; Das &

Khangarot, 2011; van der Sman, 2007). Food consumption can also be impacted if the organism cannot locate prey. Scavengers, such as whelks and crabs, respond to water-borne odours to find food. Copper (Cu) pollution has been reported to impair chemosensation in several taxa (Krang & Ekerholm, 2006; Lurling & Scheffer, 2007; McIntyre et al., 2008; Wang et al., 2013). This can make them more vulnerable to predation, impede foraging efficiency, and interfere with social cues (Boyd, 2010; Kwan et al., 2015).

Estuaries are particularly prone to pollution because materials from a larger watershed may be concentrated and retained in sediments. Copper and zinc (Zn) are common pollutants in these systems (dos Santos et al., 2019; Li et al., 2012; Milne et al., 2009). While both metals are biologically essential, Cu especially can cause significant damage to cell membranes (Letelier et al., 2005; Rozsa & Salanki, 1990; Valavanidis & Vlachogianni, 2010). Heavy metals can be biomagnified in benthic food webs "with gastropods as top predators" (Wang, 2002), as dietary pollution is the predominant pathway for heavy metal pollution of whelks (Blackmore, 2000; Blackmore & Morton, 2002; Blackmore & Wang, 2004; Wang & Ke, 2002).

Elevated Cu, and especially Zn, concentrations in the sediment of the Porirua Harbour have previously been reported (ARC ERC, 2004; Sorensen & Milne, 2009), Fine sediment loads, which often transport heavy metals into waterways, have since doubled (Stevens & Forrest, 2020). *Cominella glandiformis*, a common whelk found in New Zealand estuaries, consumes the infaunal bivalves *Austrovenus stutchburyi* and *Macomona liliana* (Ansell, 2001; Morton & Miller, 1973; Stewart & Creese, 2004). These bivalves can bioaccumulate heavy metals from surrounding sediment (Fukunaga & Anderson, 2011), and may therefore expose *C. glandiformis* to dietary pollutants.

I aimed to investigate the effects of pollutants (copper-only or copper+ zinc) and exposure pathway (aqueous-only or aqueous + dietary) on the physiology and foraging abilities of *Cominella glandiformis*. Specifically, I address the following questions: 1) How does pollution affect indicators of physiological condition? 2) Does prolonged exposure to pollution impair foraging ability? 3) Does the exposure pathway matter? 4) Do foraging preferences mediate exposure to pollutants? Both Cu and Zn can act as neuro-inhibitors, so I expected exposure to both would cause the strongest reduction in whelks' chemosensory abilities. The dietary pathway is an important route of heavy metal accumulation in whelks, so I expected whelks to choose unpolluted prey over polluted. Further, I expected exposure to simultaneous

aqueous and dietary pollutants to cause greater physiological stress (e.g. greater weight loss and respiration rate) than aqueous-only exposure. Similarly, I expected exposure to two heavy metals to be more physiologically taxing than exposure to one.

3.2 Methodology

3.2.1 Study system

I focus on the common mud whelk *Cominella glandiformis*. Though these whelks are scavengers, but they also predate *A. stutchburyi* (Stewart & Creese, 2004). An iteroparous species, *C. glandiformis* lay benthic egg capsules over the austral spring/summer (late September to January (Chapter 2)), and there is evidence that this is affected by heavy metal pollution (Chapter 4). I collected whelks from Paremata Station (41°06'16.9"S 174°51'59.0"E), a site within the Porirua Harbour (Figure 1.2(C)). This site contains a high density of *C. glandiformis* and *A. stutchburyi* and is likely well-flushed with each tidal cycle.

3.2.2 Experimental overview

I examine the effects of Cu or Cu + Zn (Table 3.1) exposure via either aqueous-only or aqueous + dietary pathways on *C. glandiformis* adults in a set of laboratory experiments. In Experiment 1, I used respiration, food consumption, and weight change as indicators of physiological stress over a month-long exposure to pollution (Aim 1, Aim 3). In Experiment 2, following the month-long exposure, I examined the foraging ability of whelks presented with a food item or empty shell (the blank) in a Y-maze (Aim 2, Aim 3). In Experiment 3, I tested whelk preference for polluted or unpolluted prey (Aim 3, Aim 4). By this point, whelks had been starved for a further week, so Experiment 4 re-tested their foraging ability in a food/empty shell Y-maze.

I chose the Cu and Zn concentrations (Table 3.1) to reflect high but realistic exposure levels. Exposure to these concentrations reduced reproductive output in whelks (see Chapter 4). I added stock solutions of $CuSO_4$ (0.09753 g $CuSO_4$ in 1L distilled water) and $ZnCl_2$ (0.10485 g $ZnCl_2$ in 1L distilled water) to approximately 5L of seawater to create 'aqueous' pollution treatments (Table 3.1). Seawater was taken from Wellington's South Coast. I polluted cockles (*A. stutchburyi*) using the same aqueous pollution treatments to create the 'dietary' pollution treatments. I changed the water three times a week and froze the cockles at -4^{o}C after two weeks.

Table 3.1: Aqueous pollutant concentrations used to pollute whelks and their food (*Austrovenus stutchburyi*) in treatments with dietary pollution.

Treatment	Distilled water only	Cu stock solution (0.09753 g $CuSO_4$ per L distilled water)	Zn stock solution (0.10485 g $ZnCl_2$ per L distilled water)	Approximate concentrations in 5L of seawater.
Control	20 mL	-	-	-
Cu High	10 mL	10 mL	-	50 µg $Cu2^+$
Cu+Zn High	-	10 mL	10 mL	50 µg Cu^{2+}, 100 µg Zn^-

3.2.3 Whelk collection and exposure to pollution:

I collected 220 whelks (~1.5 to 2.3 cm in length) from a single site within the Porirua Harbour (Paremata Station) on 16/06/2020 from the mid to low tidal area. I also collected additional cockles to provide food for the whelks during the 20-day acclimation period. I tagged whelks with uniquely numbered tags (i.e., bee-tags) affixed with superglue, measured their length (siphonal notch to apex) with vernier callipers to nearest 0.01mm, sexed them (presence/absence of penis) and weighed each whelk to the nearest 0.0001g. For acclimation, whelks were kept in a 40L Perspex tank (dimensions: H: 30.5, L: 60.5; W: 29.6 cm) with flowing raw seawater and regularly fed with live, crushed cockles.

After the acclimation period, I allocated whelks to one of five treatments comprising: four different metal exposures (Table 1) and a Control using only filtered (≤1 µm) seawater. I randomly assigned 10 whelks (except for one replicate which mistakenly had only 9), with similar sex ratios and shell lengths to each of 20 containers. I used 2L (97 x 97 x 80 mm) plastic containers with 3mm mesh side panels in two sides and in the lid to ensure water circulation (Figure 3.1). I placed each container in a bucket filled with 5L of filtered (≤1 µm) seawater and aerated by an air-stone fed by an air pump (Resun LP-100 Airpump). Each treatment had 4 replicate buckets. I kept whelks in these conditions for 31 days (06/07 to 06/08/2020). I measured the water temperature of the Control buckets once per day, Monday to Friday. I positioned buckets in a stratified-random pattern, ensuring that two buckets of the same treatment were not side by side. At the end of the four-week exposure I re-weighed the whelks. I tested a subset of 16 whelks per treatment in Y-maze

experiments (described below) over the subsequent two weeks. I euthanised the remaining whelks.

Figure 3.1: Example container used to enclose whelks. Dimensions: 97 x 97 x 80 mm (2 litres) with side panels (approx. 95 x 50 mm) covered with 3mm mesh. Lid has ~45 mm diameter hole, usually covered in 3mm mesh (absent in photo).

3.2.4 Indicators of Physiological Stress (Experiment 1)

Respiration

To determine whether pollutant mixture or pathway affected the respiration rate of whelks, I quantified their oxygen consumption over a 90-minute period before exposure (Wk 0) and at two points during the experiment (Wks 2 and 4). I selected a subset of 11 whelks from each of the five treatments to test. I used six 60mL screw top containers for respiration chambers, allowing one whelk per treatment plus a blank in each 90-minute incubation. To ensure water circulation, I placed a stirrer bar at the bottom of each chamber. To keep the whelk separated from the stirrer bar, I inserted a wire mesh platform, allowing approx. 1/3 of the container height for the stirrer bar, 2/3 for the whelk. I submerged a magnetic stirrer pad in a tray containing approximately 50 L of water which was continually pumped through a Hailea chiller (Model: HC150A), cooling water to approximately 10°C. I checked the water bath temperature before each incubation to ensure it was <11°C.

For each incubation I placed the containers and lids in a bucket containing approx. 5L of ≤1 µm filtered seawater and removed any air bubbles. I quantified the temperature, pH and oxygen saturation of the water in the bucket, and these are the 'initial' measurements for each of the chambers. I haphazardly placed five whelks, one from each treatment, into each of 5 submerged containers. The sixth container acted as a blank. I placed whelks on the mesh platform, operculum-up and allowed them to acclimate for 5 minutes, or until the whelk had fully extended its foot. I observed that prolonged foot extension released any air bubbles that may have formed during transfer from the treatments. I secured the lid to each container, keeping both fully submerged. I then placed each chamber on a magnetic stirrer pad for the 90-minute incubation, under fluorescent lighting.

At the end of the incubation, I removed and unsealed each chamber to obtain the 'final' oxygen saturation and temperature. I assumed that the pH of the water was constant over the 90-minute period, so it was not remeasured. I then returned whelks to their respective treatments, and thoroughly rinsed the chambers with tap water then seawater before the next incubation. While the respiration of three whelks per replicate (12 per treatment) was intended to be assessed, some whelks were inadvertently missed in both Week 0 and Week 4. Thus, eleven whelks per treatment were assessed in Weeks 0 and 2, but 6 of these whelks were missed in Week 4 (one from each treatment except Aqueous-only Cu which lost two).

I converted all measurements of oxygen saturation (O_2%), from the bucket (initial) and chambers (final) to µmol O_2 L^{-1} using the methods of (Garcia & Gordon, 1992) (see appendix for details; Appendix-Equations 3.1 to 3.6). Respiration (i.e., oxygen consumed) was calculated using Equation 3.1.

$$Respiration = Conc_{initial} - Conc_{final} \quad \text{Equation 3.1}$$

Where $Conc_{initial}$ represents the calculated initial O_2 concentration (µmol L^{-1}), and $Conc_{final}$ represents that of the chamber at the end of the 90-minute incubation.

Food Consumption

To determine whether pollutant mixture or pathway affected whelk appetite, I presented whelks with known quantities of food for three hours then reweighed remaining tissue (described in detail below). To ensure whelks were uniformly close to the food, I stood

containers on their side with the bubbler placed in front of lid mesh to ensure water movement and oxygenation continued. I later repeated this process without whelks in the containers, to check for tissue loss due to water movement or other means unrelated to whelk action.

I fed whelks twice a week during the experiment – unpolluted cockles in Aqueous-only treatments, polluted cockles in Aqueous + Dietary Treatments. After a week of trial and error (not described here) to determine how much and how best to present the cockle meat, I fed the whelks in the following way. For all (except the first) feeding events, I removed cockles from the freezer to defrost in the morning, then the adductor muscles were broken by fully opening the shell, and the tissue (including gill) and surrounding shell were blotted dry with a paper towel and weighed. I placed each cockle in a labelled Petri dish which corresponded to a replicate bucket. I presented whelks with this pre-weighed food, and they were allowed to feed for 3 hours. At the end of the feeding time, most of the tissue remained in easily removed pieces. To determine how much tissue was consumed, I carefully checked the container for any remaining pieces of tissue using forceps. I blotted dry and re-weighed the shell and any remaining tissue to compare to the initial tissue + shell weight.

Weight Change

To determine whether pollutant mixture or pathway affected the live weight of whelks, I weighed whelks before (Wk 0) and after (Wk 4) a month-long exposure period. I calculated percent weight change for each whelk Equation 3.2. Values greater than 0% indicate weight loss, while a value less than 0 indicates weight gain. I mistakenly did not record the initial weights of two whelks, so these were excluded from the data set.

$$\%\, change = \left(\frac{initial - \ final\ weight}{initial\ weight}\right) \times\ 100 \qquad \text{Equation 3.2}$$

Statistical Testing

I conducted all statistical tests in R (V. 4.0.3) using RStudio (V 1.3.1093; RStudio Team (2020)). I visually checked data for normality, homogeneity and outliers using the autoplot function (package: ggplot (Kassambara, 2020)) which produces Fitted vs. Residual, QQ,

Scale-Location and Constant Leverages plots. I used the lm function from the stats package (R Core Team, 2013) to perform the ANOVAs described in this section.

To examine whether prolonged exposure to pollutants and pathways caused increased oxygen consumption (mmol), I performed a nested two-way ANOVA on the effects of Treatment (with nested replicates) and exposure time (week). To test for a reduction in appetite due to pollution-associated stress, I performed a two-way ANOVA examining the effects of Treatment and exposure time (week number) on food consumed (g) per week. I compared the fit of a nested model (replicate within Treatment) to the above non-nested model using an F-test to compare model residuals using the anova() function of the stats package (R Core Team, 2013). Where appetite examines food intake at the replicate level, changes in the weights of individual whelks between pre- and post- exposure can give a more fine-scale indication of physiological stress. Due to the non-normal distribution of the data, I compared each whelk's initial and final weights using the wilcox.test function from the stats package (R Core Team, 2013). This non-normality was largely driven by five whelks which gained or lost substantive weight. I used a Kruskal Wallis test (kruskal.wallis function from the stats package (R Core Team, 2013)) to examine whether percent weight loss varied between treatments, and the pairwise.wilcox.test function (stats package, R Core Team (2013)) to perform a non-parametric post-hoc test.

3.2.5 Chemosensation (Experiments 2, 3 and 4)

Experiments 2, 3 and 4 assess whether pollutants and pathways affected the whelks' ability to find food, and how quickly they moved towards the stimulus. For Experiment 2, I used 16 whelks from all five treatments, while I only tested whelks from the most extreme pollution treatment (Cu+Zn, aqueous+dietary) against those from Control for Experiments 3 and 4. I chose to compare only the most polluted whelks to unpolluted as a treatment effect would be best detected between these two disparate treatments. I starved the whelks for one week prior to Experiment 2 and did not feed them subsequently.

I positioned the Y-maze on an upturned Perspex tank with a camera (GoPro Hero) mounted underneath, capturing whelk movement from beneath the Y-maze. A hose delivered filtered (≤1 µm) seawater to the Y-maze. The feed then split between the two maze arms at a rate of approximately 13 cm3 sec-1 (26 cm3 sec-1 speed at base of the maze) (Figure 3.2). I used two Y-mazes so that I could test a whelk in one while flushing the other with seawater.

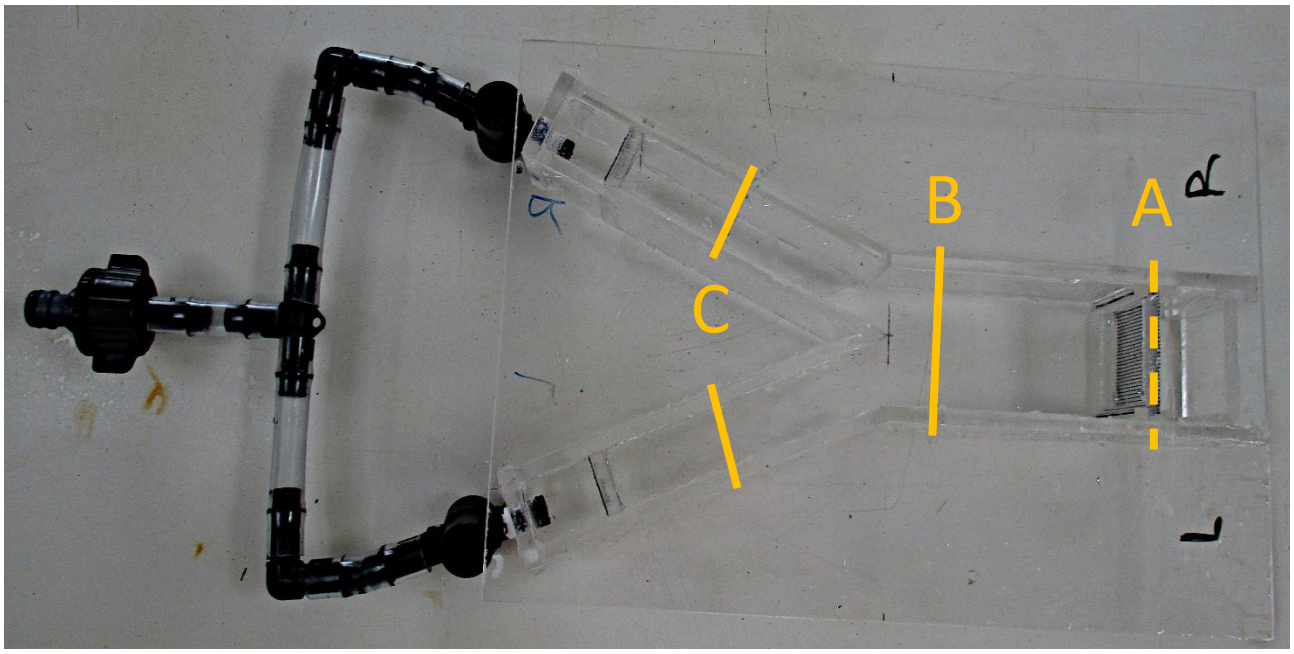

Figure 3.2: Y-maze set up (photographed from below). A indicates acclimation barrier placement; B the 'halfway line', 2 cm from where arms meet; C the 'decision line', 5 cm from end of arm.

Once I positioned the GoPro Hero under the Y-maze, I placed a whelk in the acclimation area (area to the left of 'A' in Figure 2). I removed the barrier (A in Figure 2) once the whelk righted itself. I recorded the arm chosen once the whelk reached the decision line (C in Figure 2). As the current whelk ran the maze, I removed the next whelk from its container and placed in a large petri dish with filtered seawater. I considered it a null trial if a whelk took longer than 5 minutes to right itself, or >12 minutes to reach the halfway line (2cm from where the arms met; B in Figure 2). I re-tested the whelk after at least three hours, but usually the next day. I used GoPro footage to determine how long it took for the whelk to run the maze. This time began when they passed the acclimation barrier housing (A in Figure 2) and concluded when they reached the 'decision' line, 5cm from the end of the arm (C in Figure 2).

Experiments 2 and 4 (described below) required a 'blank' stimulus. I used a series of cockle shells collected from the upper intertidal of Browns Bay where they were unlikely to have

been in recent contact with dead tissue and were not covered in algae. To ensure that there were no traces of dead tissue scent, I soaked the shells in freshwater, roughly scrubbed them under a freshwater tap, then placed them in a new bucket of fresh water. I selected a new shell for each trial from this bucket, so any cues remaining from the field would have been consistent across treatments and trials.

Pre-experiment trials indicated that rinsing the maze with tap water, wiping it with paper towel, and flushing the maze with filtered sea water for 5 minutes or more removed waterborne cues of the previous whelk and cockles. These trials also indicated that the flow from both arms was similar. To ensure that each of the two mazes didn't only use left (L) or right (R) arms, and whelks from the same treatment didn't encounter the cockle in the same arm every time, the arm with the stimulus (crushed cockle) was alternated in the pattern R, L, R, R, L, L for the 5-treatment experiment, and R, R, L, L for both 2-treatment experiments.

Locating Food (Experiments 2 and 4)

In Experiment 2, I tested whether the differing pollutants and pollution pathways affected whelks' ability to locate, and move to, food. I selected a subset of 16 whelks from each of the five treatments to run the Y-maze. I placed a live, crushed cockle (food item) in one arm of the Y-maze, and a blank (empty cockle shell) in the other. Once all trials were completed, whelks from both Cu exposure routes and aqueous-only Cu+Zn were euthanised, leaving Unpolluted and aqueous + dietary Cu + Zn exposed whelks for the remaining experiments.

In Experiment 4, I tested whether additional starvation (18-19d total) would change the success and time taken by whelks to locate food. This experiment was conducted identically to Experiment 1, but with whelks from only Control and Cu + Zn aqueous + dietary treatments. At the end of these trials, all whelks were euthanised.

Food Preference (Experiment 3)

I tested whelks' preference for polluted or unpolluted cockles with the Y-maze, and whether this preference was affected by the whelk's pollution experience. I kept cockles in either polluted (Cu + Zn High) or unpolluted seawater for 5 days. Previous studies examining Cu-pollution on mussels have found cellular-level effects of heavy metals in similar time frames (3-7 days in *Mytilus galloprovincialis* exposed to 10 µg Cu L^{-1} (Gomes et al., 2012); 2-9 days in *Mytilus edulis* exposed to 40 µg Cu L^{-1} (Zorita et al., 2007). If whelks can detect a

difference there is likely a chemical cue released by the cockle, which I expect to have emerged within 5 days. As in the experiment above, the choice and time taken to make this choice were recorded for each whelk. In this experiment, however, each arm contained either a polluted or unpolluted crushed cockle.

Statistical Testing

Due to some whelks failing to be retested (or failing the re-test), I performed statistical analyses on 12 (rather than 16) whelks from each treatment. This also meant that I could not directly compare the speeds of two Control whelks between the first and final Y-maze experiments. Using whelks from the Control treatment in the first Y-maze experiment, I tested for a left/right arm bias with the fisher.test function from the stats package (R Core Team, 2013). I also used this function to test whether stimulus choice (i.e. whelk chose food item vs. blank, or polluted vs. unpolluted food item) differed between treatments in each of the three Y-maze experiments.

While all whelks made a clear final choice of arm, six whelks initially entered the other arm. I removed these whelks from the dataset before examining treatment effects on time taken to choose an arm. As this data was non-normal for all three experiments, I performed a natural log transformation. I examined the effect of treatment (with nested replicates) on time taken to choose an arm using a nested ANOVA for each of the three experiments. For Experiment One, which had whelks from all five treatments, I used an ANCOVA to examine the relationship between (natural log transformed) time to run maze and size or weight of whelk once treatment effects were controlled for. I used the lm function from the stats package (R Core Team, 2013) for both the ANOVA and ANCOVA.

3.3 Results

3.3.1 Impacts of Pollution on Indicators of Physiological Stress

Effects of Pollution on Respiration

Chambers containing whelks differed from empty chambers (blanks) (One way ANOVA: $F_{5, 189} = 29.06$, p-value <0.0001). Average whelk respiration was similar across treatments (Table 3.2), though increased between week 0 (before exposure) and weeks 2 and 4 (middle and end of exposure) by 1.3 and 1.5 times, respectively (Table 3.2, Tukey HSD test: p <0.0001, Figure 3.3).

Table 3.2: Results of a nested two-way ANOVA examining the effects of Week (before (Week 0), during (Week 2) and after (Week 4) exposure), Treatment and Replicate nested within Treatment on respiration of whelks from five treatments.

Source of Variation	df	Sum of Squares	F-value	p-value
Week	2	2.2541	10.7678	<0.0001
Treatment	4	0.0876	0.2092	0.9328
Week*Treatment	8	0.2622	0.3131	0.9595
Week*Treatment(Replicate)	45	3.3142	0.7036	0.9054
Residuals	99	10.3623		

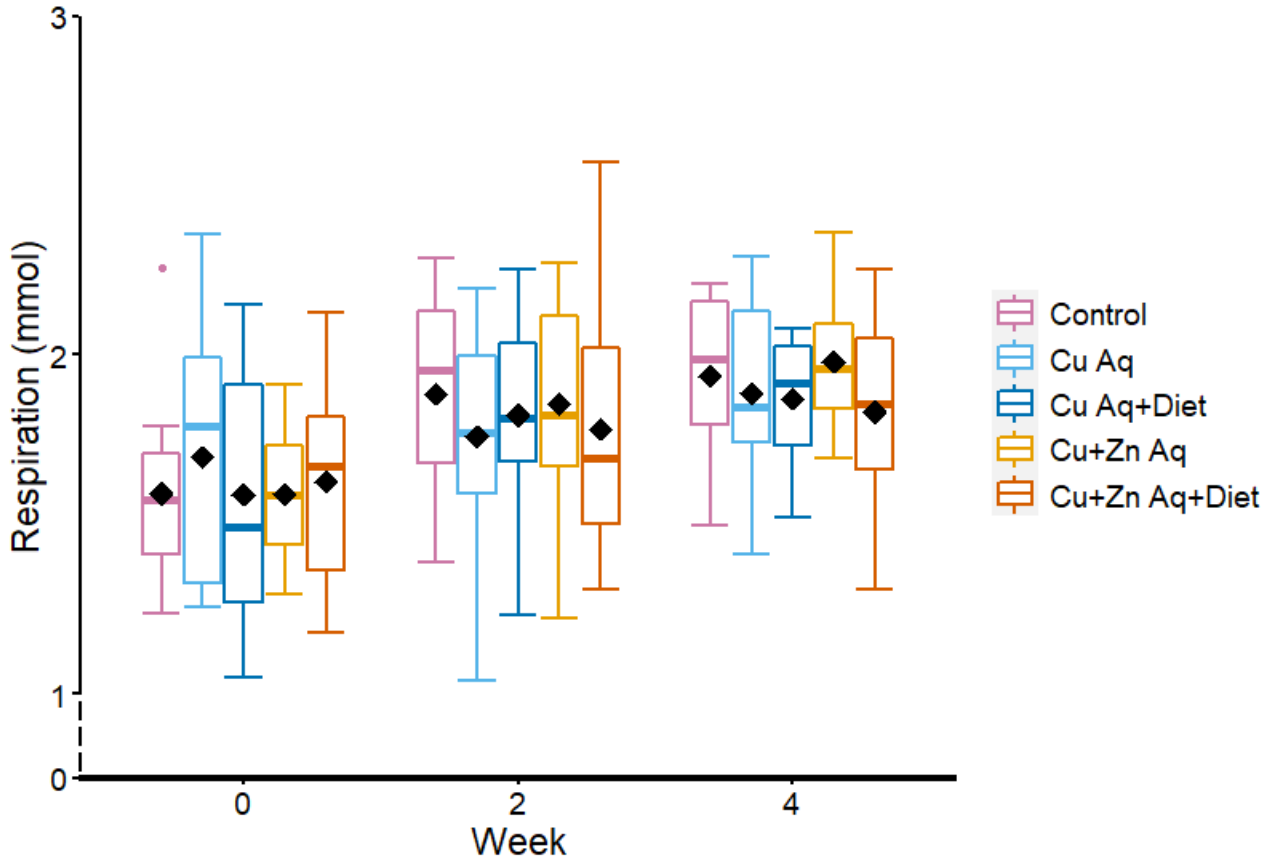

Figure 3.3: Respiration (mmol) of whelks in each treatment before (Week 0), during (Week 2) and after (Week 4) exposure to heavy metal pollution treatments. The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR (interquartile range) of the box. Mean represented by black diamond. n = 11 whelks per treatment for Weeks 0 and 2; All treatments except Cu-aqueous had 10 whelks in Week 4, and 9 whelks in Cu-aqueous.

Effects of Pollution on Food Consumption

In all treatments, whelks consumed a similar amount of tissue in the first week (Table 3.3, Figure 3.1). Whelks from pollution treatments (i.e., potentially stressed whelks) consumed less tissue than those from Control, though this disparity decreased between weeks 2 and 4 (Table 3.3, Figure 3.4). Whelks experiencing pollution consumed similar amounts of tissue irrespective of pollutant mixture (Cu or Cu+Zn) or pathway (Figure 3.4). Across treatments, whelks in each replicate repeatedly consumed more tissue one week than they did the next. As these fluctuations did not occur at the same time across the replicates, this may help explain the high variation seen in Figure 3.4. An alternate model was run in which replicates were nested within Treatments (i.e. Consumption ~ Treatment (Replicate)*Week; Appendix-Table 3.1). This model was compared to that of Table 3.3 and found to be a poorer fit (H_0: simple model is best; F-test: $F_{60} = 0.6649$; p=0.9416).

Table 3.3: Results of two-way ANOVA examining effects of Treatment and exposure duration (week number) on food consumption using non-nested model (Consumption ~ Treatment * Week).

Source of Variation	df	Sum of Squares	F-value	p-value
Treatment	4	7.6038	11.8123	<0.0001
Week	3	1.3661	2.8296	0.0414
Treatment*Week	12	5.7673	2.9864	0.0011
Residuals	120	19.3115		

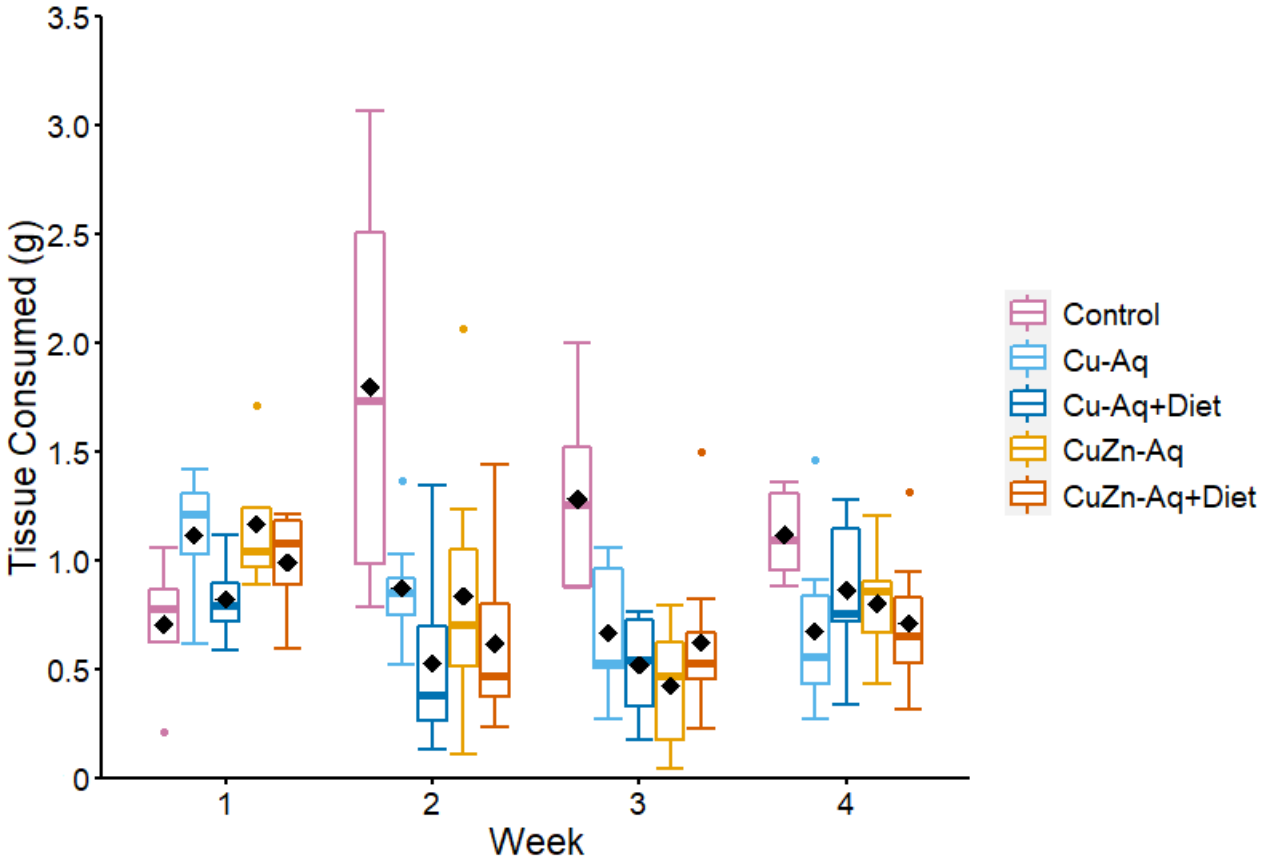

Figure 3.4: Cockle tissue (g) consumed by whelks in each treatment over the four-week exposure. The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR (interquartile range) of the box. Mean represented by black diamond. n = one feeding event across three replicates per treatment for Week 1; n = two feeding events across three replicates per treatment for Weeks 2 – 4.

Effects of Pollution on Weight

Overall, whelks gained weight during the experiment (Wilcox test: V = 15693, p<0.0001), with an average 1.31% (+/- 6.60% SD) increase. Weight change did not significantly differ between pollution treatments (Pairwise Wilcoxon Test: p > 0.58). However, the average whelk experiencing aqueous-only Cu pollution gained 2% (+/- 2.79 std dev) more weight than Control whelks (Pairwise Wilcoxon Test: p = 0.0063; Figure 3.5). The average weight change in Cu aqueous + diet whelks almost differed from Control whelks (Pairwise Wilcoxon Test: p = 0.0511) at 2.75% weight gain, but with a large standard deviation of 8.69 (Figure 3.5). There were several whelks which lost or gained much more weight than most, creating five large outliers which prohibited parametric tests. These outliers are informative, so they were not removed for statistical tests.

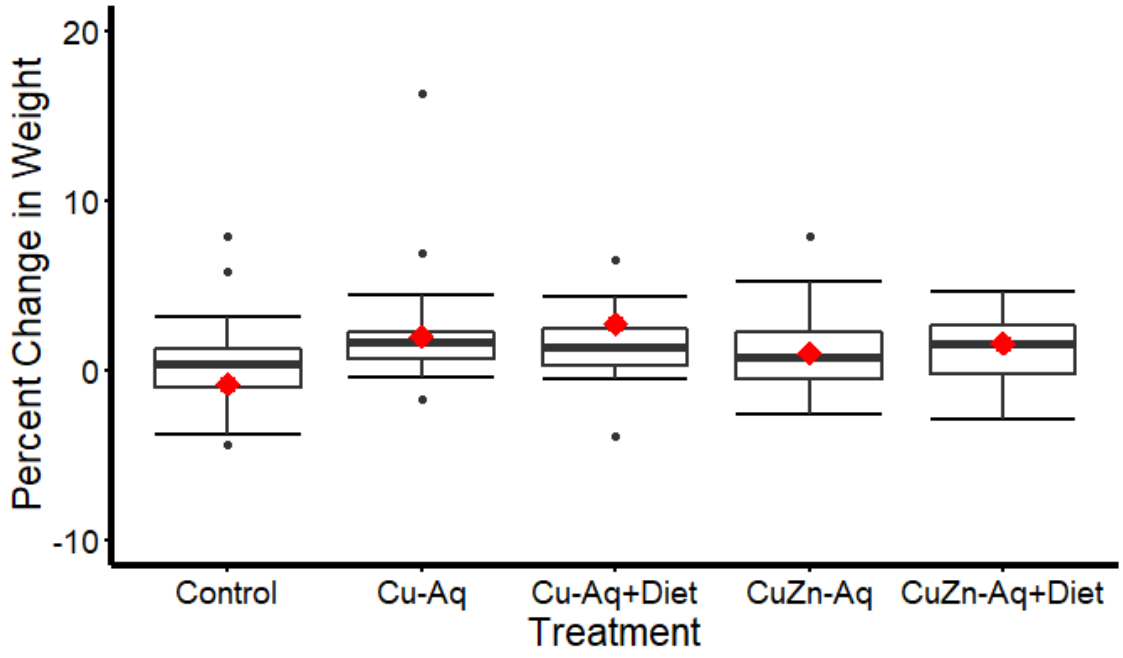

Figure 3.5: Percent weight change in whelks from each treatment over 4-week experimental period. The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR (interquartile range) of the box. Mean represented by red diamond. The five substantive outliers were removed for graph readability; n = 38 from Control; n= 38 from aqueous Cu; n = 39 from aqueous + dietary Cu; n = 40 aqueous Cu+Zn; n = 38 aqueous + dietary Cu+Zn.

3.3.2 Chemosensation in Whelks Exposed to Prolonged Pollution

Control whelks in Experiment 1 always chose the arm containing the cockle, so were used to confirm the absence of an arm bias. Eight of the 14 unpolluted whelk trials had the cockle in the left arm, six in trials in the right. There was no evidence of an evidence of an arm bias ($p = 1.0$).

Ability of Whelks to Locate Food

All 12 Control whelks (100%) chose the arm containing the live, crushed cockle, compared to 9 of 12 (75%) from both exposure treatments of Cu, and the Aqueous-only Cu + Zn, and 7 of 12 (58%) from Aqueous+Dietary Cu+Zn (Figure 3.6), although this was not significant (Fisher's Exact Test: p-value =0.1647). All whelks made a clear final choice of arm, though 10% initially entered the other arm. These, and one whelk which was identified as an extreme statistical outlier, were removed from the dataset when examining time taken to make a choice. Of whelks that made a single choice of arm, Control whelks completed the Y-maze, on average, 1.4 and 1.6 times faster than whelks experiencing aqueous-only and aqueous+dietary Cu pollution pathways, respectively (Table 3.4; Tukey Test: $p < 0.02$; Figure 3.7). Neither shell length (ANCOVA: Adjusted $R^2 = 0.19$, $F_{4, 42} = 0.1712$, $p = 0.9549$) nor whelk weight (ANCOVA: Adjusted $R^2 = 0.21$, $F_{4, 42} = 0.4229$, $p = 0.791188$) influenced the time taken to complete the Y-maze, regardless of treatment.

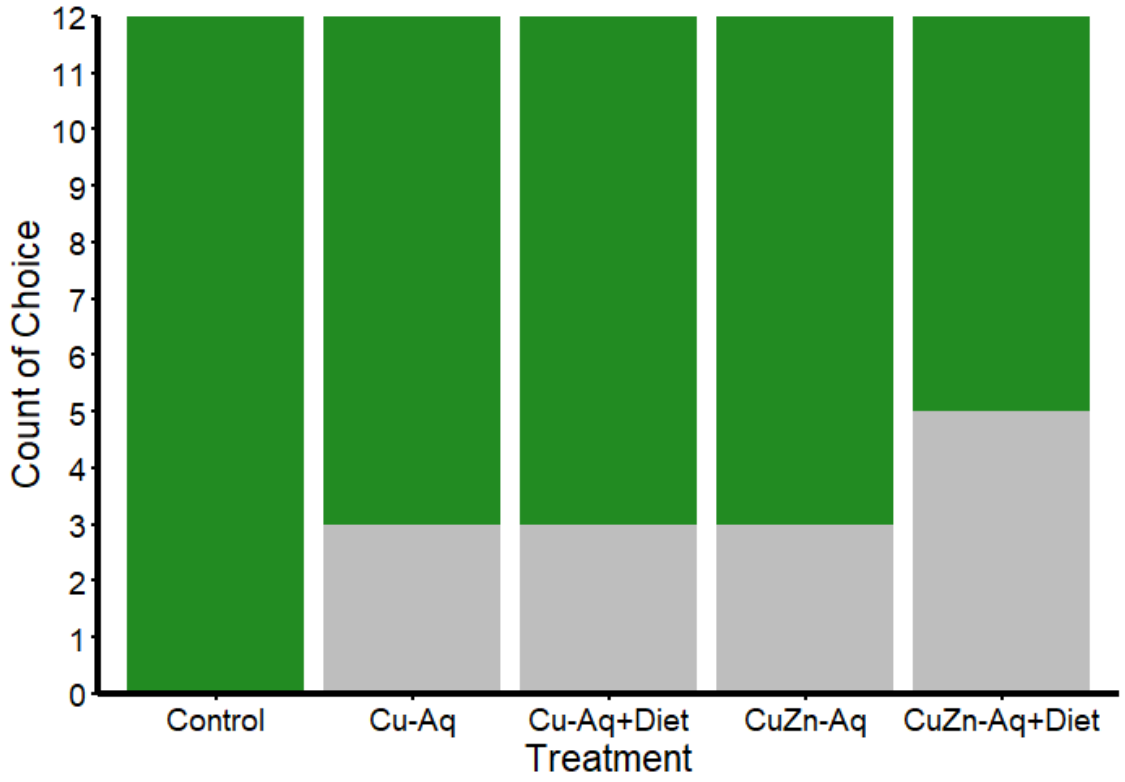

Figure 3.6: The number of trials in which whelks chose the arm containing the live, crushed cockle (green) or empty cockle shell (grey) across each of the five treatments in Experiment One. n= 12 whelks per treatment.

Table 3.4: Results of a nested ANOVA examining the effects of Treatment, and Replicate nested within Treatment, on time taken by whelks to complete the Y-maze in Experiment One.

Source of Variation	df	Sum of Squares	F-value	p-value
Treatment	4	1.6667	3.9507	0.01023
Treatment(Replicate)	15	0.6036	0.545	0.8938
Residuals	32	2.3624		

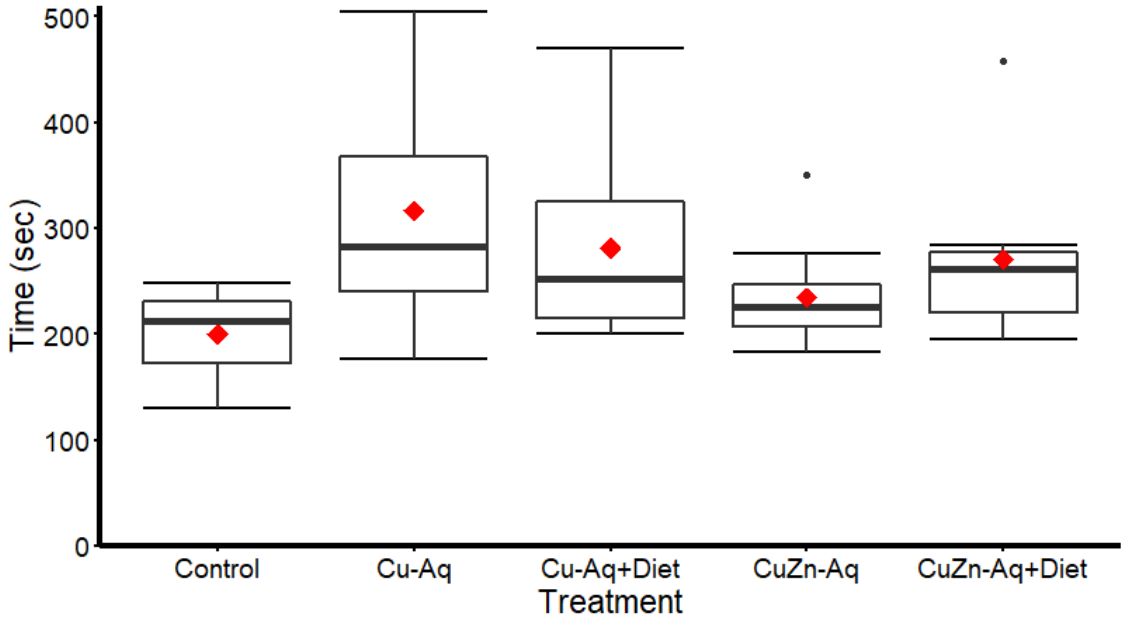

Figure 3.7: Time (seconds) taken to complete the Y-maze by whelks from each treatment in Experiment One. The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR (interquartile range) of the box. Mean represented by red diamond. n = 12 whelks per treatment.

Whelks' Preference for Polluted or Unpolluted Food Item

67% (8 of 12) of Control whelks chose the unpolluted cockle (rather than the polluted cockle), compared to 17% (2 of 12) of whelks exposed to Cu+Zn (aqueous + dietary) pollution (Fisher's Exact Test: p = 0.0361; Figure 3.8). All whelks chose the first arm they entered as their final decision during this experiment. Control and polluted whelks took similarly long to complete the Y-maze (Table 3.5).

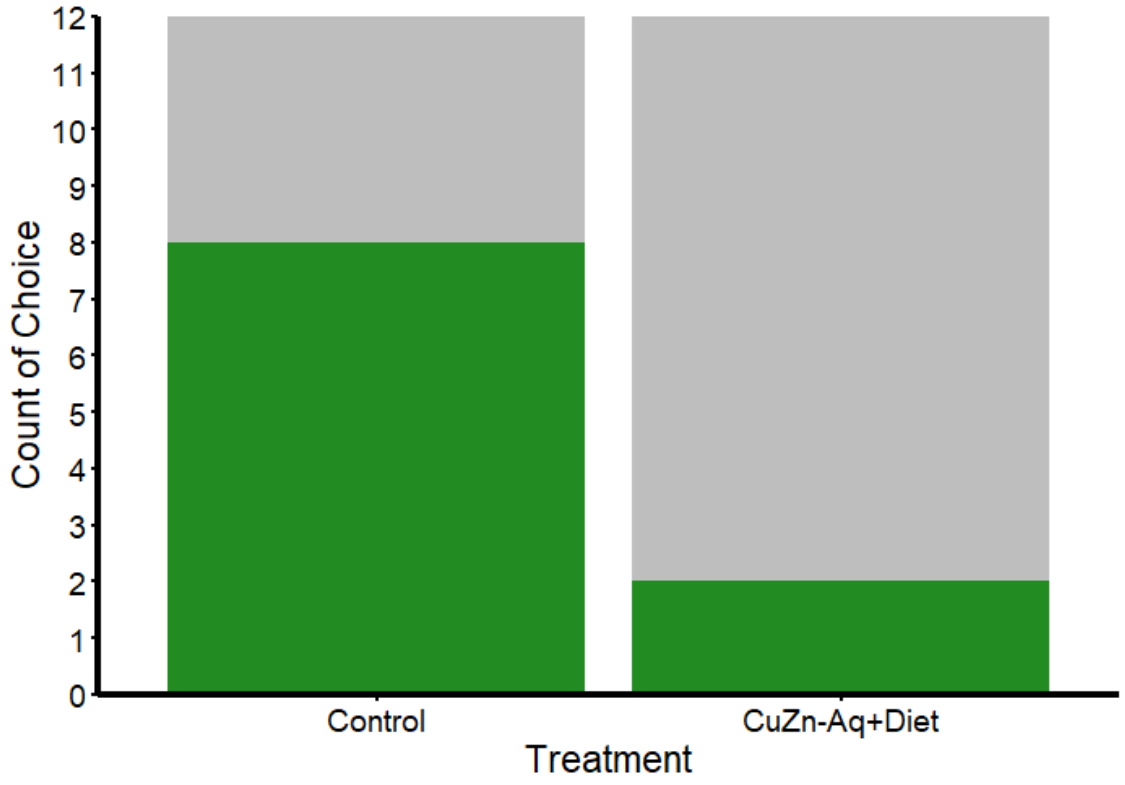

Figure 3.8: Choice between unpolluted (green) or Cu+Zn polluted (grey) live, crushed cockle by whelks from two treatments (Control (unpolluted) and aqueous + dietary exposure to copper (Cu) and zinc (Zn)) in Experiment Two. n= 12 whelks per treatment.

Table 3.5: Results of a nested ANOVA examining the effects of Treatment and Replicate nested within Treatment on time taken by whelks to complete the Y-maze in Experiment Two.

Source of Variation	df	Sum of Squares	F-value	p-value
Treatment	1	0.0113	0.1182	0.7358
Treatment(Replicate)	6	0.5602	0.9737	0.4757
Residuals	15	1.4385		

Ability of Whelks to Locate Food Following Additional Starvation.

A similar number of unpolluted (100%, 12 of 12) and polluted (67%, 8 of 12) whelks chose the maze arm containing the live, crushed cockle (Fisher's Exact Test: p-value =0.0932), however none of the Control whelks chose the 'blank' arm compared to four polluted whelks. One more polluted whelk than in Experiment One chose the arm with the food. Only one whelk initially explored a different arm in Experiment 3, compared to four (two from each of Control and Cu+Zn Aqueous+Diet) in Experiment One. One whelk was excluded from the Experiment 1 data as an extreme statistical outlier, leaving 19 whelks from Experiment One (Control and Cu+Zn Aqueous+Dietary treatments only) and 23 whelks from Experiment Three. Time to complete the maze (using whelks which explored only one arm) was similar within treatments in Experiments One and Three (Table 3.6). Pooled across experiments, unpolluted whelks completed the maze 1.36 times faster than polluted whelks (Table 3.6; Figure 3.9).

Table 3.6: Results of a nested two-way ANOVA examining the effects of Experiment number (representing both experience in maze and time starved), Treatment and Replicate nested within Treatment on time taken by whelks to complete the Y-maze in Experiments One and Three.

Source of Variation	df	Sum of Squares	F-value	p-value
Experiment No.	1	0.01364	0.3155	0.5791
Treatment	1	0.93103	21.5380	<0.0001
Experiment*Treatment	1	0.00023	0.0054	0.9421
Experiment*Treatment(Replicate)	12	0.86954	1.6763	0.1311
Residuals	25	1.12391		

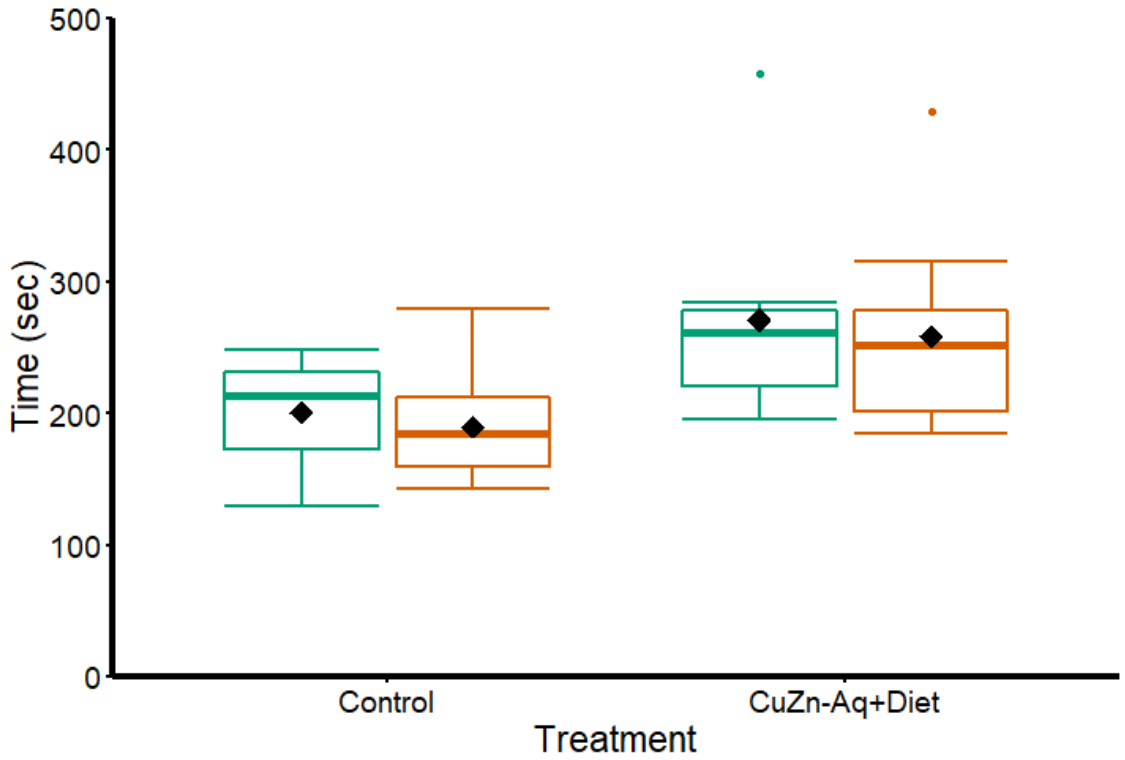

Figure 3.9: Time (seconds) taken to complete the Y-maze by whelks from each treatment in Experiments One (green) and Three (orange). The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR (interquartile range) of the box. Mean represented by black diamond. n = 19 whelks from Experiment One (Control = 10, Cu+Zn = 9); n = 23 whelks from Experiment Three (Control = 11, Cu+Zn = 12).

3.4 Discussion

Polluted whelks evinced greater physiological stress and reduced chemosensory abilities compared to Controls. Pollutant mixture and pathway didn't influence indicators of physiological stress as much as was hypothesised. For example, neither appeared to influence food consumption or respiration. Despite consuming less food than Control whelks, those in the Cu-only treatments gained more weight during the experiment. This species may therefore increase its growth rate in response to pollution (e.g. Type II accumulation in Bryan, 1980), though further research is required to confirm this.

While whelks from all treatments were able to sense and locate food (though some polluted whelks did not), Control whelks reached the prey item faster. This was observed in both Experiments 2 (all treatments) and 4 (Control and Cu+Zn Aq+Diet whelks only), indicating that the further starvation and exposure had no effect on performance. When presented with the choice between contaminated and uncontaminated cockles, however, all whelks took a similar amount of time to choose. Interestingly, Control whelks tended to choose uncontaminated cockles where Polluted whelks chose contaminated.

Polluted whelks consumed less food than their unpolluted conspecifics, irrespective of pollutant pathway or mixture. A similar pattern was observed in *Nassarius siquijorensis* from Xiamen, China. These whelks consumed less tissue when presented with oysters from highly Cu and Zn polluted estuaries (Guo et al., 2013). While this significantly reduced the whelks' exposure to dietary pollution these whelks also had a poorer condition index (CI) at the end of the study. Interestingly, whelks fed moderately polluted oysters consumed them at a similar rate to those fed unpolluted oysters, exposing whelks to higher dietary pollution rates than those fed highly polluted oysters. As Guo et al. (2013) saw a reduction in ingestion rate from the first week, they concluded that this was an avoidance behaviour, rather than "adverse metabolic effects of ingested metals". In the present study, however, whelks experiencing both aqueous-only and aqueous + dietary pollution evinced reduced appetites compared to Controls. This suggests that the 'pickiness' observed is likely due to cellular damage leading to appetite loss, as was seen in *Lymnaea stagnalis* by Cain et al. (2016).

The physiological effects of heavy metals vary between species and can be influenced by co-occurring metals. For example, the co-occurrence of Zn can increase (e.g. Fukunaga et al., 2011) or decrease (e.g. Rouchon & Phillips, 2017a) the toxicity or physiological indicators observed. Responses from polluted whelks did not differ much between mixture treatments. The clearest effect of co-occurring Zn can be seen in the weight changes, as the slight weight gain in whelks from Cu+Zn treatments did not differ from that of the Control whelks. Since all polluted whelks were exposed to the same aqueous Cu concentrations, Zn may reduce the need for a growth response in whelks. The reduced food consumption seen in all pollution treatments suggests that whelks were likely in poorer condition by the end of the experiment.

The Week 0 respiration tests were taken at the end of the laboratory-acclimation period, prior to heavy metal exposure. I did not examine respiration during the initial (<24 hrs) exposure period as this study was interested in the physiological effects of ongoing pollution, not the initial stages of acclimation. Control whelks were kept under almost identical conditions in Week 0 as Weeks 2 and 4, but with the addition of 20mL distilled water. This would cause a negligible change to 5L of seawater, so another explanation for why respiration rates increased across treatments is required. Gametogenesis, the metabolically expensive formation of mature gametes, may be an explanation for the increase in respiration rate between Weeks 0 and 2. Initial results suggest that *C. glandiformis* males may have fully developed gametes year-round, whereas females may begin gametogenesis in July (S. Sudarsky, personal communication). For the females, this varies from whelk-to-whelk, with size, condition and stress unpredictably impacting when the process starts. As the respiration tests were conducted in July, this seems a plausible explanation.

Cominella glandiformis occurs in a habitat prone to Cu and Zn pollution, and their preferred food source bioaccumulates these (and other) metals (Fukunaga & Anderson, 2011). Whelks used in the preference Y-maze experiment had been starved for 18 days and may have ignored any 'unfavourable' cues to obtain sustenance. This is supported by whelks making a single choice – whichever arm they initially entered was their final choice. Starvation can make organisms less fussy (Kasumyan, 2019; Perry, 1987), and can override response to alarm cues (e.g. in fish (Kasumyan, 2019)).

3.4.1 Conclusion

This study used ecologically realistic Cu and Zn concentrations to examine the effects of prolonged (>4 weeks) pollution on adult *C. glandiformis* physiology and chemosensory abilities. Copper pollution impaired foraging ability and likely reduced the condition of whelks, irrespective of pollutant pathway or mixture. Further, polluted whelks appear likely to increase their dietary exposure by preferential consumption of contaminated prey. Such prey items are likely to be in poor condition themselves, thus reducing nutritional gain to whelks. These results suggest that whelks could start the reproductive season in poor condition. This could reduce the number of offspring produced, and even the post-hatching performance of their offspring.

Chapter 4 – Maternal investment of a benthic carnivore under Cu and Zn pollution

4.1 Introduction

Physiological stress has been repeatedly linked to lower fecundity in iteroparous invertebrates (*Daphnia magna*: Bae et al., 2016, Marshall & Uller, 2007; *Tritia reticulata:* Chatzinikolaou & Richardson, 2010*; Nassarius spp.*:, Cheung, 1997, Cheung et al., 2008; *Turbo sarmaticus*: Foster et al., 1999; *Magallana sikamea*: Weng & Wang, 2014; review by Sokolova et al., 2012). This lower reproductive output is often compounded by reduced offspring survival, as early developmental stages are highly sensitive to stressors (Boukadida et al., 2016a; Przeslawski, 2004; Przeslawski et al., 2015; Rouchon & Phillips, 2017a). Even a short, low-dose, sublethal exposure during the larval phase can cause reduced growth rates after settlement. Moreover, initial exposures can cause increased sensitivity to future exposure events (Rouchon & Phillips, 2017b).

Some species will expend energy to 'front foot' their offspring to withstand unfavourable conditions. This 'higher quality' may be an immediate advantage, such as larger larval size, or a subtler longer-term advantage. For example, larger larvae are more resilient to stressors and are more dispersive (Marshall, 2008; McKenzie et al., 2011), though may be poorer performing after settlement (Allen et al., 2008; Marshall, 2008; Piola & Johnston, 2006a). Copper (Cu) polluted *M. sikamea* females produce eggs with elevated body burdens, triggering embryos to rapidly synthesise metal-binding proteins (metallothionein, MT) (Weng & Wang, 2014). These pollution-exposed larvae are more tolerant of Cu pollution than naïve conspecifics (Weng & Wang, 2014) but have slower growth rates, likely due to energy expended on MT synthesis (Weng & Wang, 2017).

Many species with benthic development encapsulate their embryos within a protective physical structure (hereafter, 'capsule'). The energetic cost of encapsulating embryos increases the per-offspring investment of females compared to free-spawning species, but also decreases pre-recruitment mortality (Kideys et al., 1993; Pechenik, 1979, 1999). Capsules reduce embryonic exposure to abiotic stressors such as desiccation, UV,

fluctuations in salinity, and pollutants (Pechenik, 1982; Pechenik & Miller, 1983; Rawlings, 1999; Untersee, 2007), and protect against generalist predators (Harmon & Allen, 2018; Rawlings, 1994; Schwab & Allen, 2014). Stressed females often lay fewer capsules, sometimes varying the number and/or size of eggs within. For example, the whelk *Nassarius festivus* lays fewer capsules, containing fewer eggs, when experiencing increasing hypoxia (Cheung et al., 2008). Similarly, starved *N. reticulatus* lay fewer, smaller capsules containing fewer eggs (Chatzinikolaou & Richardson, 2010). Both studies concluded that females expended less energy on reproduction to ensure that they would survive to lay again in the breeding season.

Theoretically, varying the number or size of eggs allows an energetic trade-off between the number and 'quality' of offspring (Bernado, 1996; Kamel et al., 2010; Smith & Fretwell, 1974; Vance, 1973). The number and size of emergent juveniles ('hatchlings') can depend on egg and capsule characteristics (Nasution et al., 2010; Pechenik et al., 1984; Spight, 1976b), with larger hatchlings considered more likely to survive (Carrasco & Phillips, 2012; Moran & Emlet, 2001; Rivest, 1983; Salas-Yanquin et al., 2021; Spight, 1976b). Stressed females may lay capsules that take longer to develop to hatch (Chaparro et al., 2014; Khangarot & Das, 2010), or produce hatchlings which exhibit slower growth rates (Chaparro et al., 2014; van der Sman et al., 2009) and/or lower survival (Averbuj et al., 2018; Carrasco et al., 2012; Coeurdassier et al., 2003). Stress experienced during development can also influence embryo survival (Gallardo & Cancino, 2009; Khangarot & Das, 2010; Untersee, 2007) and hatching size (Montory et al., 2009; Salas-Yanquin et al., 2021) of embryos.

Heavy metal pollution is closely associated with human activity and has regularly increased over the last century (Crain et al., 2009; Lu et al., 2018; Zaynab et al., 2022). Species with larval development are often used to examine the effects of heavy metals, as these sensitive early stages occur in direct contact with water-borne pollutants. In nature, these larvae are likely to disperse away from parental conditions, so need only survive pollution for a short period. Benthic developers, however, will continue to experience parental conditions as they are 'trapped' within the capsule (Pechenik, 1979).

Cominella glandiformis is a predator and scavenger, common in intertidal estuaries (Ansell, 2001; Morton & Miller, 1973). Previous research on other *Cominella* spp. in New Zealand has reported similar numbers and sizes of offspring per capsule (Carrasco & Phillips, 2014;

van der Sman, 2007). Capsule volume may be related to the number or size of eggs or hatchlings of gastropods with encapsulated development (Chaparro et al., 1999; Chatzinikolaou & Richardson, 2010; Nasution et al., 2010), so can be used as an indicator of maternal investment.

This study aimed to explore the effects of heavy metal pollution on the reproductive output and maternal provisioning of a benthic estuarine carnivore with completely benthic lifecycle. Specifically, I ask:

1) Does pollutant exposure reduce the number of potential recruits through a) reduced number of capsules laid over the season and b) reduced intracapsular survival?
2) Does maternal investment (indicated by capsule volume and number & size of eggs or hatchlings) decrease with pollution?
3) Does pollutant exposure influence the timing of embryonic development through a) changes to the timing of the laying season and b) development time of encapsulated embryos?

4.2 Methodology

4.2.1 Study system

I focused on the whelk *Cominella glandiformis*, an iteroparous species that lays capsules containing multiple eggs over late spring through summer. These eggs develop into hatchlings in approximately 40 days. The context for my experiments is the Porirua Harbour, Wellington, New Zealand (Figure 1.2(C)). I collected *C. glandiformis* from Paremata Station (41°06'16.9"S 174°51'59.0"E), a site with a high density of *Cominella glandiformis*, and likely well-flushed with each tidal cycle.

4.2.2 Experimental overview

I addressed the effects of exposure to Cu or Cu + Zn at environmentally realistic concentrations (Table 4.1) on reproduction in *Cominella glandiformis* in a set of laboratory experiments. Both metals are potential pollutants in the focal system, though their effects remain unknown. I exposed adult whelks to these treatments in the lead up to and during their reproductive season. I used pollution regimes that may occur: (1) commonly in the system (Cu Low), (2) more episodically following heavy rain in New Zealand systems (Cu-Med, Cu+Zn Med), or (3) reflect higher concentrations of pollution measured elsewhere (Cu High, Cu+Zn High). I recorded the number of capsules laid by each female over three months (Aims 1a, 3a). I examined some of these capsules to quantify volume and number and size of eggs, while I allowed others to develop and hatch for examination of the number and size of hatchlings (Aims 2, 3b). I subsequently examined the proportion of capsules that survived to hatch (Aim 1b).

Table 4.1: Pollution regimes for experiments in 2019/20 (Ch. 4) and 2020/21 (Ch. 5) summers. Distilled water and stock solutions were added to 5L of filtered seawater from the Wellington South Coast (New Zealand).

Treatment	Distilled water only	Cu stock solution (0.09753 g $CuSO_4$ per L distilled water)	Zn stock solution (0.10485 g $ZnCl_2$ per L distilled water)	Approximate concentrations in 5L of seawater.
Control	20 mL	-	-	-
Cu-Low (Ch 4)	19 mL	1 mL	-	5 µg Cu2+
Cu-Med (Ch 4, 5)	16 mL	4 mL	-	20 µg Cu2+
Cu+Zn Med (Ch 4, 5)	11 mL	4 mL	5 mL	20 µg Cu2+, 50 µg Zn-
Cu High (Ch 4, 5)	10 mL	10 mL	-	50 µg Cu2+
Cu+Zn High (Ch 4)	-	10 mL	10 mL	50 µg Cu2+, 100 µg Zn-

In early September 2019, I collected adult *C. glandiformis* from Paremata Station and transported them to the Victoria University Coastal Ecology Lab. I kept whelks in flowing, filtered (≤1 µm) seawater for 14 days to acclimate. During this time, I determined the sex of each (presence/absence of penis) and tagged all females using bee tags affixed with super glue. I measured the shell lengths of all females (shell apex to siphonal notch) to the nearest 0.01mm using Vernier callipers. I fed whelks with cockle meat twice a week for two to four hours. I was not interested in paternal effects in this study, so males were not tagged nor consistently assigned to females. I allocated whelks to each of the six treatments (Table 4.1) consisting of three replicate trays, containing four females in individual containers (12 whelks per treatment). I filled each 10L clear plastic tray with ~5L filtered (≤1 µm) seawater (plus 20mL treatment aliquots; Table 4.1). I placed each female (plus a random male) in a clear plastic container (745 mL, 99 x 99 x 76 mm) so that capsules could be linked to their mother. Two sides (plus the lid) of each container had 400µm mesh windows. I used an air-stone fed by an air pump (Resun LP-100 Airpump) to aerate the water in each tray.

I fed whelks with cockles exposed to the same aqueous treatment twice a week. As described in Chapter 3, I collected cockles by hand from Paremata Station in the Porirua Harbour and froze them after two weeks of pollution. I pooled whelks within each tray into a 2L (97 x 97 x 80 mm) plastic container with 3mm mesh panels in two sides (+ lid) containing two cockles. This also meant that females were paired with multiple males over the course of the experiment. I changed the water in each tray at the end of feeding days. The temperature of the Control trays was taken around 11:50am 5 days a week from the end of October (Appendix-Table 4.1).

I checked each container for egg capsules daily, Sunday through Friday, recording the number of capsules laid each day for two months (14/10/2019 to 06/12/2019). I either preserved the capsules in 70% ethanol or sealed them in a small mesh (400µm) bag. I kept the bagged capsules in 10L buckets, under the same pollutant conditions as their parents. Depending on how difficult it would be to separate the capsules, bags contained between 1 and 10 capsules (3.68 $\pm$ 2.07 SD). As capsule fouling can limit oxygen diffusion and smother the embryos (Cancino et al., 2000; Cohen & Strathmann, 1996), I monitored algal growth in the buckets. To reduce fouling, I covered the buckets with shade cloth, changed the water three times per week and at the same time wiped down the buckets, air-stones, and tubes. I

regularly checked bags for signs of algal overgrowth and development. If a bag showed signs of algal fouling I would remove the capsule, check that the embryos were alive, and place in a new bag. As with the adults, I took the temperature at approximately midday on weekdays from Control buckets.

Hatchlings darken as they develop. I could observe this darkening through the transparent capsule, and this allowed me to monitor development without opening the bag. I checked the bags for hatchlings 5 days a week initially but increased this to six days a week at the height of hatching. Based on previous experience and similar accounts in the literature, I selected 50 days as a cut off for hatchling development. It appears that capsules do not experience sufficient mechanical abrasion in a laboratory setting. I preserved the capsule + hatchlings in 70% ethanol, recording them as hatched (alive, with brown or dark brown shells), dead (no movement), or under-developed (alive but the shell had not darkened).

4.2.3 Effects of Pollution on Capsule Output and Intracapsular Survival

I conducted all statistical tests in R (V. 4.0.3) using RStudio (V 1.3.1093; (RStudio Team, 2020)). I visually checked data using histogram, Fitted vs. Residual and QQ plots produced by the plot() and hist() functions of the graphics package, and qqnorm() and qqline() functions of the stats package (R Core Team, 2020). To evaluate the effects of pollutants on capsule output, I examined the number laid by each female across the six treatments. I often recorded no eggs from females in the daily counts, resulting in 71.6% of the data being zeroes. Data was therefore pooled for each female to a weekly count (34.2% zeroes). I fitted a generalised linear mixed model (GLMM) with Poisson distribution to examine the effects of Treatment, Week (i.e. when capsule was laid), and the interaction of the two, on the number of capsules laid by each female per week. I used the glmer() function from the lme4 package (Bates et al., 2014), and included Female as a random effect to reduce the likelihood of pseudoreplication. I used the Anova() function from the car package (Fox & Weisberg, 2019) to obtain p-values.

To evaluate the effects of pollutants on intracapsular survival, I compared the number of capsules initially placed in bags with the number that successfully hatched. I compared the proportion of capsules that survived to hatch from each bag across the six treatments using the glmer() function with binomial distribution and logit-link function. I performed post-hoc

Tukey comparisons using the glht function from the multcomp package (Hothorn et al., 2008).

4.2.4 Effects of Pollution on Maternal Investment

I selected 5 whelks with similar laying patterns from each treatment for examination of the capsule, egg, and hatchling attributes. These females laid multiple capsules every week and, for the Control and Cu Low whelks, were not the females with the highest capsule output. Capsules laid in the first and final two weeks, with opportunistic sampling in between, were examined for capsule volume and egg size. All hatchlings that survived to hatch, if attributable to a capsule, were examined. To determine capsule volume and sizes of eggs and hatchlings, I either photographed the specimens using a Canon (EOS 70D (w)) camera mounted on a Olympus S261 dissecting microscope, taking measurements using ImageJ (Schneider et al., 2012), or else I manually measured them under a dissecting microscope. I measured the shell length (siphonal notch to apex) of hatchlings (2.5x mag), and I recorded the total number of hatchlings per capsule. For the capsules containing eggs, I first determined the capsule volume then dissected out the eggs within to quantify egg diameter (4.5x mag) and number. Capsules are the shape of a truncated cone (Figure 2.1:), with a narrower top than base. I estimated capsule volume by measuring the height (1.5x mag) and the diameters of plug and base (2.5x mag) using Equation 4.1.

$$V = \frac{1}{3} \times \pi \times h\,(r_1^2 + r_1 r_2 + r_2^2) \qquad \text{Equation 4.1}$$

Where r_1 is the radius of the capsule base, r_2 is the radius of the plug, and h is the height of the capsule.

Reduced maternal investment can be indicated by smaller capsule volume. To examine whether this occurred under pollution stress, I fitted a linear mixed model (LMM) with fixed effect of Treatment, and random effect of Female. Changes in the number and sizes of eggs can also indicate altered maternal investment, with a greater number of smaller eggs representative of a lower investment per offspring. The number of embryos that survive to hatch, and their size, can indicate whether capsules contained the necessary qualities (e.g. lipids within eggs or capsule albumen-type matrix) to grow all embryos to maturity. I fitted a GLMM to examine the effect of Treatment (and random effect of female) on the number of

eggs or hatchlings per capsule. I fitted a similar model to explore the effects of pollution on the sizes of these eggs and hatchlings, with the random effects of female and capsule ID. I used the lme() function for all above tests.

4.2.5 Effects of Pollution on the Timing of Egg Laying and Embryonic Development

While warmer water allows faster embryonic development (Cancino et al., 2003; Gallardo & Cancino, 2009; Spight, 1975), it also increases the toxicity of heavy metal pollutants (Boukadida et al., 2016a; Hajimad & Vedamanikam, 2013; Kimberly & Salice, 2013; McLusky et al., 1986). To examine whether heavy metal pollution influenced the timing of laying, I compared the number of females laying or not laying each day for each treatment. I examined the effects of Treatment and Week on these proportions by fitting a generalised linear model (GLM) with binomial distribution and logit-link function from the lme4 package (Bates et al., 2014).

I examined the development time of embryos from five of the six treatments, as only one bag in the Cu High treatment (50 µg Cu L^{-1}) survived to hatch. Bags that contained multiple capsules hatched on the same day. I therefore examined development time for each bag, rather than each capsule. I fitted an LMM to examine the effects of Treatment and Week on the development time of embryos across the five available treatments. I used the lme() function of the nlme package (Pinheiro J, 2020) and included the random effect of Female to reduce the likelihood of pseudoreplication. I performed post-hoc Tukey comparisons using the glht function from the multcomp package (Hothorn et al., 2008).

4.3 Results

4.3.1 Effects of Pollution on Capsule Output and Intracapsular Survival

Exposure to heavy metals did not influence the number of capsules laid by females (GLMM with Poisson distribution: $\chi^2 = 9.05$, 1 df, $p = 0.107$), though Week ($\chi^2 = 538.1$, 7 df, $p < 0.001$), and the interaction of Week and Treatment ($\chi^2 = 465.7$, 35 df, $p < 0.001$) did. The first three weeks had the lowest average capsule production overall, due to low (or absent) laying by whelks in the Cu+Zn Med and high Cu treatments (Figure 4.1). Within each treatment, the number of capsules laid varied from week to week, with a general increase in capsule production through the season (Figure 4.1). All pollution treatments appear to peak in Week 7 (late November; Figure 4.1). Capsule production from the Control treatment, however, peaked three weeks earlier (first week of November) (Figure 4.1). Though not statistically different, the Control whelks had the highest average total output (77.42 +/- 19.09 SD), and Cu High and Cu+Zn High whelks the lowest (35.33 +/-20.19, 34.17 +/-21.76, respectively).

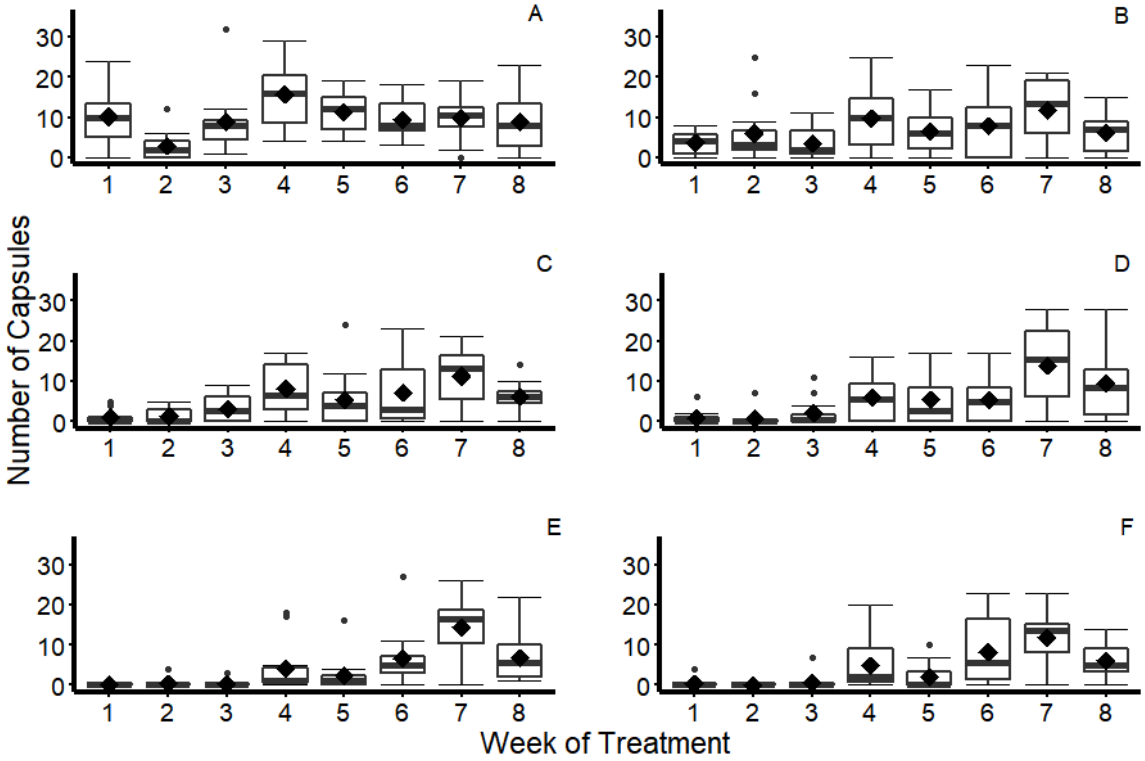

Figure 4.1: The number of capsules laid by whelks from A) Control, B) Cu Low, C) Cu Med, D) Cu + Zn Med, E) Cu High, and F) Cu + Zn High treatments, over eight weeks. The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR (interquartile range) of the box. Mean represented by black diamond. n = 12 whelks per treatment.

Intracapsular survival was 57.3 and 23.1 times lower in the high Cu treatments (without and with Zn, respectively) than Control (GLM with binomial distribution: $\chi^2 = 108.66$, 5 df, $p < 0.0001$; Tukey: $p < 0.015$). The treatments with 50 µg Cu L^{-1} had the lowest proportions of surviving capsules, with only 1.4% (2 of 141, Cu High) and 3.5% (5 of 142, Cu+Zn High) capsules hatching (Figure 4.2). A similar proportion of capsules survived to hatch in the Control (81.3%, 388/477), Cu Low (87.5%, 281/321) and Cu Med (67.2%, 117/174) treatments (Tukey: $p > 0.05$).

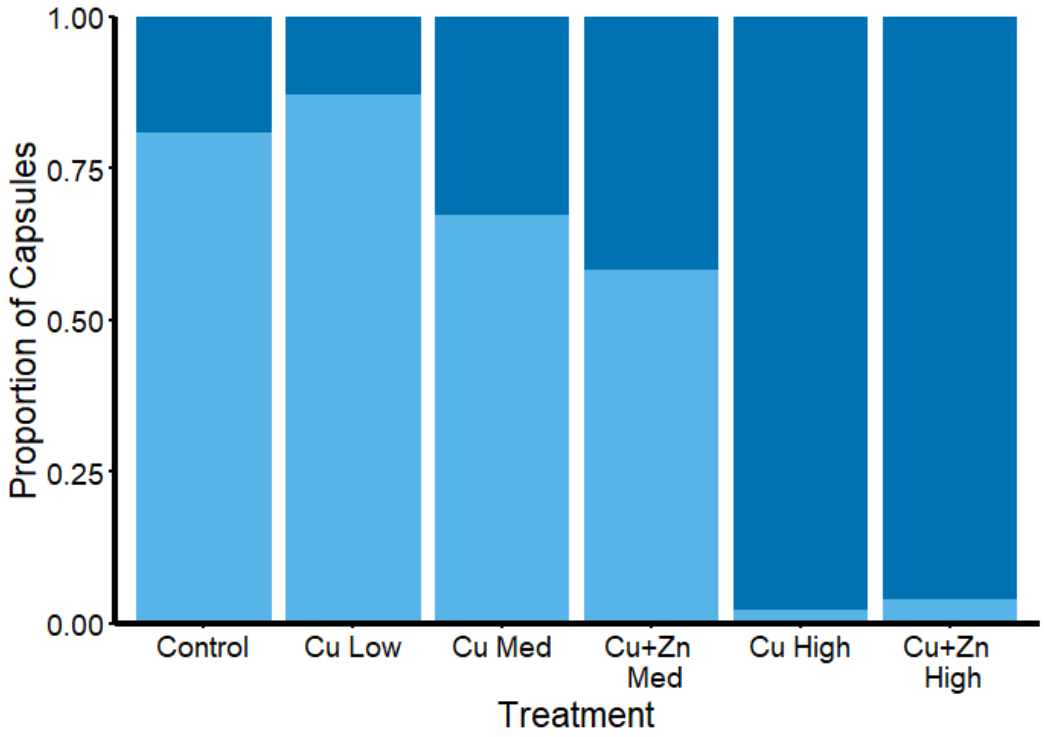

Figure 4.2: Proportion of capsules from each treatment that failed to develop (dark blue) or successfully hatched (light blue). n = 477 capsules from Control, 321 capsules from Cu Low, 174 capsules from Cu Med, 141 capsules from Cu High, 174 capsules from Cu+Zn Med, and 142 capsules from Cu+Zn High.

4.3.2 Effects of Pollution on Maternal Investment

Females exposed to different concentrations and combinations of Cu and Zn did not appear to vary their maternal investment on a per-capsule basis. Specifically, capsules contained 5.92 eggs on average (+/- 1.27 SD; linear mixed model: $F_{5, 24} = 0.9578$; $p = 0.4628$) and 5.57 hatchlings (+/- 1.29 SD; linear mixed model: F4, 15 = 0.9357, p = 0.4699). Sizes of eggs (linear mixed model: $F_{5, 24} = 0.9578$; $p = 0.6428$) and hatchlings (linear mixed model: $F_{5, 24} = 0.371$, $p = 0.8634$) were also similar across treatments, with the average egg growing from a diameter of 0.30 mm (+/- 0.04 SD) to a hatching shell length of 1.12 mm (+/- 0.09 SD).

Capsule volume also did not vary across treatments (LMM (Female as random effect): $F_{5, 24.6} = 1.61$; $p = 0.1938$), averaging 6.76 µL (+/- 1.40 SD).

4.3.3 Effects of Pollution on the Timing of Egg Laying and Embryonic Development

As seen for capsule production (Figure 4.2), exposure to Cu (with or without Zn) delayed the start of the laying season (GLM with binomial distribution, Treatment-Week interaction: $\chi^2 = 57.2$, 5 df, $p < 0.0001$). Females from Cu+ Zn Med and both high Cu treatments laid few or no capsules during the first three weeks of the experiment (Figure 4.3). Overall, the proportion of females producing capsules increased through time ($\chi^2 = 22.2$, 1 df, $p < 0.0001$), and was usually highest in treatments with $\leq$5 µg Cu L^{-1}($\chi^2 = 118.1$, 5 df, $p < 0.0001$). By the final week of the experiment, all six treatments had a similar proportion of females laying capsules (Figure 4.3)

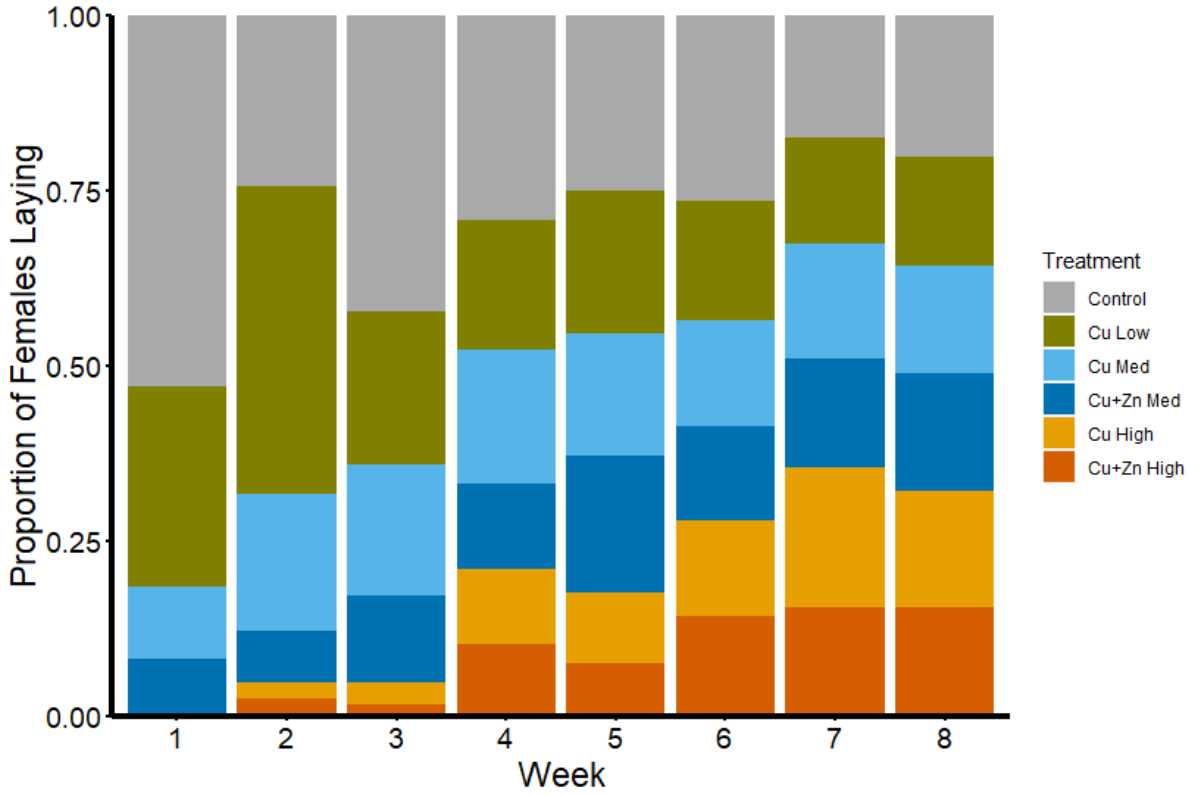

Figure 4.3: Proportion of female whelks laying capsules from six treatments across eight weeks.

The concentration and combination of pollutants did not affect development time of capsules (Nested ANOVA: $F_{4, 33} = 2.275$, $p = 0.0821$). Instead, the week in which capsules were laid dictated development time (Nested ANOVA: $F_{6, 189} = 36.487$, $p < 0.0001$, with the average duration significantly decreasing every other week (Tukey HSD: <0.002; Figure 4.4). On average, bags with capsules laid in the first week took 1.2 times longer to hatch than those in the fourth week, and 1.3 times longer to hatch than those in the seventh week (Tukey HSD < 0.0001; Figure 4.4).

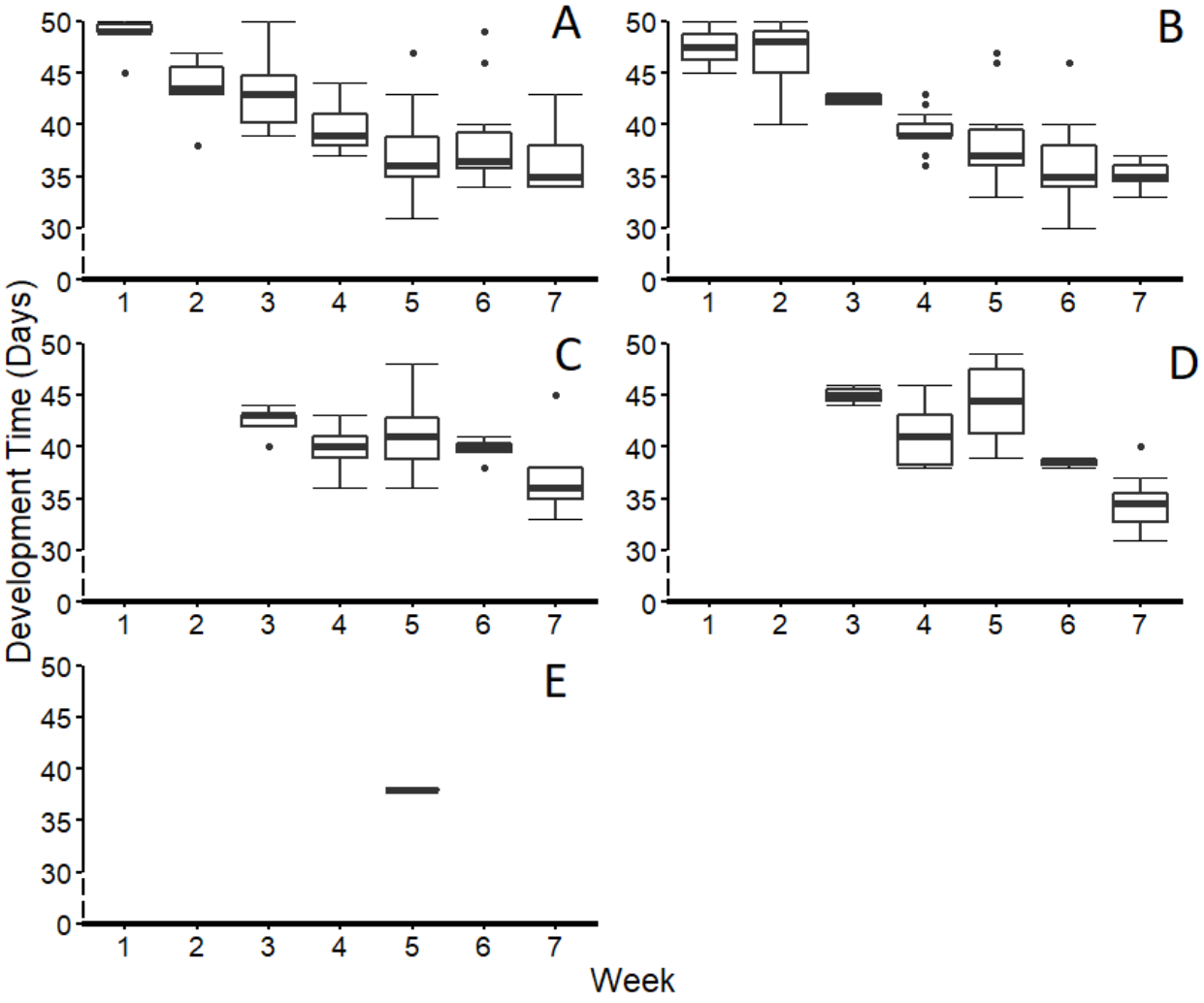

Figure 4.4: Average development time (Date laid – hate hatched, days) of capsules from A) Control, B) Cu Low, C) Cu Med, D) Cu + Zn Med, and E) Cu + Zn High laid over 7 weeks. The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR (interquartile range) of the box. Mean represented by black diamond. n = 101 bags from Control, 73 bags from Cu Low, 36 from Cu Med, 29 from Cu+Zn Med, and 1 from Cu+Zn High treatments (the only capsule to develop, explaining the absence of any distribution in panel E).

4.4 Discussion

Exposure to higher (≥20 µg) concentrations of Cu (with or without Zn) delayed capsule laying by several weeks, with fewer capsules hatching as the concentration increased. Females don't appear to alter maternal investment, with similar capsule and offspring traits across treatments. Approximately 6 eggs developed into 6 hatchlings, with little variation in sizes. Development time decreased through the season, likely due to warming water temperatures (Appendix-Table 4.1).

Due to the three-week head start, females from the Control and Cu Low treatments produced more capsules than those of higher treatments (though this was not statistically significant). It is difficult to determine whether this was a stress-induced effect or an active choice by polluted females. Either way, the delay resulted in embryos developing in warmer water temperatures. Warming water temperatures increase reproductive output in whelks (Chatzinikolaou & Richardson, 2010; Harding et al., 2008). Perhaps the polluted females required slightly higher temperatures to support reproduction than their less polluted counterparts. As I did not follow these females until they stopped laying, it is unclear whether these females have a shortened reproductive season, or simply offset. Harding et al. (2008) saw *Rapana venosa* capsule production decline in late summer, corresponding with higher water temperature and daylength. A shorter laying season would significantly reduce the raw reproductive output of polluted females, even before accounting for the reduced survival of embryos. If the delay represents an 'offset' season, then polluted females could produce a similar number of capsules to non-polluted ones. Either way, a greater proportion of embryos will experience higher temperatures during development.

Within physiological limits, warmer water temperatures increase developmental rates but a concomitant reduction in hatching success is often also reported (Kimberly & Salice, 2013; Pechenik et al., 2003; Salih et al., 1981; Smith et al., 2013). Heavy metals can also become more toxic as temperature increases (Boukadida et al., 2016a; Kimberly & Salice, 2014a; McLusky et al., 1986), causing further impacts. For example, *Physella pomilia* kept at 35^{o}C developed faster than those at 25^{o}C (Kimberly & Salice, 2013). If simultaneously exposed to cadmium (Cd), however, embryos took longer to develop and experienced higher mortality rates. Early development is often the most vulnerable life stage to stressors (Chaparro et al.,

2014; Kimberly & Salice, 2013; Pandori & Sorte, 2019; Pineda et al., 2012), and sublethal effects during development can manifest in later life stages (Chaparro et al., 2018; Pechenik, 2006; Pechenik et al., 2003; Salas-Yanquin et al., 2022).

Due to the energetic demands of tolerating heavy metal pollution during the reproductive season, let alone any 'front-footing' of offspring by females, trade-offs are expected between the number and size of offspring (Bernado, 1996; Kamel et al., 2010; Smith & Fretwell, 1974; Vance, 1973). The number and size of eggs appears to be genetically determined in *Cominella*. Natural history studies indicate that *C. maculosa* and *C. virgata* package consistent numbers and sizes of eggs (Carrasco & Phillips, 2014; van der Sman, 2007), as does *C. glandiformis* (Chapter 2). Size at hatching may be similarly fixed, though maternal stress may influence post-hatching performance. For example, starved *C. virgata* produced hatchlings of similar size to those of well-fed mothers, but the post-hatching growth rates were much lower (van der Sman et al., 2009). In the present study, all juveniles emerged at a similar size, despite increased mortality with higher Cu concentrations. Stress during development has been linked to poorer performance as juveniles and adults (Ellis et al., 2009; Kimberly & Salice, 2014b; Lurling & Scheffer, 2007; Pechenik, 2006; Rouchon & Phillips, 2017b; Salas-Yanquin et al., 2022).

Embryos may be exposed to pollutants that diffuse across capsule walls, or 'inherit' pollution from their mother. Goldberg et al. (2004) found TBT (tributyltin) in the eggs of *Pachycymbiola brasiliana*, a volute with benthic development. While there was evidence that TBT had permeated the capsule walls, the concentrations found in the eggs indicated maternal inheritance. Maternally inherited Cu has been found in oyster larvae (Weng & Wang, 2017), which also inherit the metal-binding protein metallothionein (MT) (Weng & Wang, 2014). These larvae show a higher tolerance to Cu pollution than naïve counterparts, likely due to the MT (Weng & Wang, 2014, 2017). Further research should examine whether this is also common in species with benthic development when exposed to heavy metals.

In the present study, many capsules exposed to 50 µg Cu L^{-1} died during early developmental stages (pers. obs.), suggesting that development stalled early on. Though surviving to hatch, *Racesina luteola* embryos exposed to concentrations $\geq$ 32 µg Cu L^{-1} had developmental abnormalities in shell gland, tentacle, and eye development (Das & Khangarot, 2011). Embryos of this species were more tolerant than *C. glandiformis*, hatching

in 52 µg Cu L^{-1}, though no embryos survived exposure to 100 µg Cu L^{-1}. Both this and the present studies saw no effects on adults or embryos exposed to 5 µg Cu L^{-1}.

The combination of Zn with Cu had little effect, suggesting that this species is highly Zn tolerant. Rouchon and Phillips (2017a) found that, in a sea urchin, the toxicity of Cu decreased if it co-occurred with Pb or Zn, which may also explain this trend. The >58% survival in 20 µg Cu L^{-1} treatments is very high compared to similar experiments with larvae. For example, *Evechinus chloroticus* larvae show stunted growth at 5 µg Cu L^{-1}, and do not develop beyond the gastrula stage when exposed to 20 µg Cu L^{-1} (Rouchon & Phillips, 2017a). This conforms with the expectation that capsules protect developing embryos from waterborne stressors (Averbuj et al., 2018; Untersee, 2007). This may be due to a naturally high tolerance in this species, the capsule providing some protection against heavy metal exposure, or a combination of the two (Untersee 2007; Rawlings 1999).

Whelks showed reduced food consumption in another study on this species (Chapter 3), and polluted whelks seemed similarly disinterested in the present study (pers. obs.). Most of the cockle tissue remained after feedings in polluted treatments, whereas little remained from Controls. Starved *C. virgata* produced a similar number of smaller capsules compared to their well-fed counterparts (van der Sman et al., 2009). Further, maternally inherited lipid content is theorised to decline when females are starved (Carrasco et al., 2016), which would presumably impact hatchling size. Therefore, females in this study may be compromising the quality (e.g. nutrition) of their offspring in favour of maintaining capsule laying rates. The offspring of both starved (van der Sman et al., 2009) and polluted (present study) females hatch at similar sizes to those of non-stressed females. This suggests a selective pressure for maintaining initial hatching size, even if their subsequent growth rates are lower (van der Sman et al., 2009).

4.4.1 Conclusion

Benthic food webs, particularly those with whelks as top predators, tend to bioaccumulate heavy metals (Wang, 2002). *Cominella glandiformis*, with an entirely benthic lifecycle and occurring in a habitat prone to pollution, appears to be very tolerant at the adult stage. Even as developing embryos, this species appears to be less sensitive than the larval species examined in similar studies. Environmentally realistic Cu and Zn pollution resulted in

females laying slightly fewer capsules, but these experienced a significant increase in developmental mortality. While there was no evidence of changes in maternal investment, laying capsules later in the season may have been a 'strategic choice' by polluted females to reduce the development time of their offspring. Species with benthic development have limited dispersal abilities (Dohner, 2016), so offspring tolerance to persistent stressors through development is important for maintaining local populations.

Chapter 5 – Growth and survival of *Cominella glandiformis* offspring under Cu and Zn pollution.

5.1 Introduction

The earliest developmental stages of organisms are often the most sensitive to stressors (Gomot, 1998; Pineda et al., 2012; Kimberly & Salice, 2013). Stress can result in reduced development rates, developmental abnormalities, and increased mortality (Chaparro et al., 2018; Chaparro et al., 2014; Das & Khangarot, 2011; Gallardo & Cancino, 2009; Przeslawski et al., 2015). In species with planktonic development, these early stages occur in direct contact with water-borne pollutants. A lot of research has focused on identifying outcomes from various lethal and sublethal concentrations on these larvae (e.g. Connor, 1972; Martin et al., 1981; Pavičić et al., 1994; Rouchon & Phillips, 2017a, 2017b). However, larvae are highly dispersive and therefore unlikely to remain in the same area as their parents. Species with benthic development encapsulate embryos within a physical structure until they emerge as crawl-away juveniles. These capsules are not impervious to pollutants but may enable embryos to withstand higher concentrations of toxic substances than pelagic larvae (Averbuj et al., 2018; Untersee, 2007).

If adults, particularly females, have been exposed to pollutants during gametogenesis then offspring may inherit a portion of accumulated pollutants. For example, encapsulation reduces embryonic exposure to tributyltin (TBT) in the water column (Averbuj et al., 2018). There is evidence to suggest that TBT can be maternally inherited, with higher concentrations found within capsules than the surrounding water (Goldberg et al., 2004). Offspring may also inherit protective mechanisms, such as the protein metallothionein (MT) which binds to heavy metals to prevent cellular damage. Copper (Cu) polluted *Magallana hongkongensis* adults produce offspring with elevated Cu tissue concentrations, and synthesized MT (Weng & Wang, 2014, 2017).

Studies examining the development of freshwater pulmonates report significantly increased mortality at or above 20 µg Copper (Cu) L^{-1} (Das & Khangarot, 2011; Gao et al., 2017; Khangarot & Das, 2010). For example, *Stagnicola elodes* embryos exposed to 20 µg L^{-1}

cadmium (Cd) stalled at the gastrula stage before continuing development to the veliger stage, though none survived to hatch (Pietrock et al., 2008). Similarly, only 15% and 20% of *Lymnaea stagnalis* embryos exposed to 25 or 50 µg Cd L^{-1} survived to hatch, with most embryos dying during the earliest development stages (Gomot, 1998). Those that hatched experienced developmental delays, hatching at least 5 days later than their non-polluted counterparts.

Stress can cause slower embryonic growth rates, resulting in a smaller juvenile size than non-stressed conspecifics (Chaparro et al., 2014; Salas-Yanquin et al., 2021). The size at hatching is an important and commonly studied trait, as larger hatchlings are considered more likely to survive (Carrasco & Phillips, 2012; Carrasco et al., 2012; Marshall & Keough, 2008; Moran & Emlet, 2001; Rawlings, 1994; Spight, 1976a). For example, *Cominella virgata* hatch at ~3mm shell length (SL) and are less vulnerable to predation by medium sized smooth shore crabs (*Cyclograspus lavauxi)*, and had outgrown the grasp size of large crabs after 2 months (Carrasco & Phillips, 2012). Post hatching growth rates are similarly important to initial hatching size in juvenile survival and can be disrupted by stress. *Cominella virgata* hatchlings from starved mothers were similarly sized to those of well-fed mothers at hatching but did not grow as rapidly over the first month post-hatching (van der Sman et al., 2009).

Latent effects can arise when stress experienced during development causes changes in later life stages that aren't seen in non-stressed conspecifics (Pechenik, 2006). For example, *Acanthina monodon* embryos that experience over-crowding during their encapsulated development show lower rates of growth, oxygen consumption and ingestion post-hatching (Salas-Yanquin et al., 2021). Alternatively, exposure of *Physella pomilia* embryos to Cd resulted in a low reproductive output with few of the offspring surviving to hatch (Kimberly & Salice, 2014). Understanding how stressors impact developmental stages – in terms of both progress through the stages and growth rates throughout – can indicate how much of a challenge the stressor constitutes.

In some cases, embryonic stress exposure can improve juvenile tolerance to future stress. For example, juvenile *Physella pomilia* exposed to Cd during development showed greater survival when they experienced subsequent pollution (Plautz & Salice, 2013). This is likely due to pre-existing metal-binding proteins (metallothioneins (MT)) and other cellular

responses to heavy metal stress. Survival (or the inverse, mortality) rates are one of the simplest indicators of stress tolerance and are commonly used in pollution studies. Juveniles are usually more tolerant than early developmental stages, but less resilient than adults (Kimberly & Salice, 2013).

In Chapter 4, I found that adult *Cominella glandiformis* lay fewer capsules following exposure to high, though realistic, concentrations of copper (Cu) and zinc (Zn). When kept under the same pollution scheme, 3.5% of capsules survived to hatch from the highest pollution treatment (50 µg Cu/ + 100 µg Zn L^{-1}), and only 58.6% survived exposure to a lower treatment (20 µg Cu L^{-1} + 50 µg Zn L^{-1}). This chapter examines the effects of heavy metals on the development and post-hatching growth and survival of *Cominella glandiformis*. Specifically, I ask:

1) Does heavy metal exposure a) increase intracapsular mortality and b) reduce the size of surviving embryos?
2) Does exposure to heavy metal reduce a) survival or b) growth in recently hatched, pollution-naive juveniles?

5.2 Methodology

5.2.1 Study system

I focus on the early life stages of the whelk *Cominella glandiformis*, an iteroparous species which lays capsules containing multiple eggs over late spring to early summer (October to January; Ch 2). Lacking nurse eggs, all embryos are expected to hatch within 50 days. The context for my experiments (and the source location for adult whelks) is the Porirua Harbour, New Zealand (Figure 1.2(C)). Whelks are found throughout this harbour, particularly at Paremata Station (41°06'16.9"S 174°51'59.0"E).

5.2.2 Experimental overview

I address the effects of exposure to Cu or Cu + Zn at environmentally realistic concentrations (Table 4.1) on development, growth, and survival of early *Cominella glandiformis* life stages in two laboratory experiments. I exposed adult whelks to these treatments from early September 2020 (approximately one month before the laying season) until late November (when the last capsules were collected). I chose these pollution regimes based on prior experiments with this species (Ch 4) and a small pilot study. These concentrations could occur episodically after heavy rain in New Zealand (Cu Med, Cu+Zn), or reflect higher concentrations of pollution measured overseas (Cu High). I collected capsules laid by females in November and either followed their development on a weekly basis (Aim 1, all treatments), or set them aside to hatch for use in a post-hatching pollution experiment (Aim 2, Control only).

In August 2020, I collected adult *C. glandiformis* from Paremata Station in the Porirua Harbour and transported them to the Victoria University Coastal Ecology Lab. I kept whelks in flowing, filtered (≤1 μm) seawater (FSW) prior to experiment. As in Chapter 4, I tagged all females (identified by absence of penis) using bee tags affixed with super glue and measured their shell lengths (shell apex to siphonal notch) to the nearest 0.01mm using Vernier callipers. I fed the whelks with cockle meat twice a week.

I maintained adult whelks in pollution treatments (Table 1) from the 4th of September to the 30th of November 2020. Whelks were fed a diet of cockles (*Austrovenus stutchburyi*) twice weekly. Cockles were collected from Paremata Station and reared under the same water

treatments as the whelks that consumed them (see Chapters 3 and 4 for details). I applied water treatments to 10L trays (n=3 trays per treatment), filling each tray with ~5L filtered (≤1 µm) seawater (plus 20mL treatment aliquots; Table 1). Within each tray, I placed four containers containing female whelks. Three of these containers held a single female (plus a male; 'solo' containers), while the fourth contained four females (plus two males; 'aggregated' container). The capsules from 'solo' females were used to follow development. The capsules of 'aggregated' females from all treatments were intended to be used for the hatchling experiment, but none survived from polluted treatments. As in Chapter 4, I did not tag the males, and moved them between females within the tray after each feeding. I used an air-stone fed by an air pump (Resun LP-100 Airpump) to aerate the water in each tray. The temperature of the Control trays was taken around midday 5 days a week throughout the experiments (4th of September 2020 to 28th of January 2021). I recorded the number of capsules laid by females each week from the end of September to mid-November.

5.2.3 Heavy Metal Exposure During Development

I identified 'solo' whelks from all four treatments that had consistently laid several capsules each week between late September and early November. Between the 16th and 27th of November, I examined the number and stage of eggs within capsules laid by these females. I had intended to use one whelk from each tray, but some whelks did not lay during this period. Instead, I used the capsules of three females from at least two trays, except for the Cu-Med treatment. Only two whelks from the same tray laid capsules during this period.

Capsules with four to nine eggs at either the one or two-cell stage were used in this experiment. Only nine capsules were collected from the Cu-Med whelks, compared to the 17-19 from the other three treatments. Embryos within capsules were photographed using a Canon EOS 70D (w) camera mounted on a dissecting microscope (Olympus S261) at 4.5x magnification, then individually sealed within a small mesh (400µm) bag. I kept capsules in FSW throughout this process. I placed these bags in the same aqueous pollutants as their parents, with two replicate 10L buckets (filled with ~5L FSW + 20mL treatment aliquots; Table 1) per treatment. I used an air-stone fed by an air pump (Resun LP-100 Airpump) to aerate the water in each bucket. To reduce algal overgrowth, I kept buckets under two layers of shade cloth, with black plastic sheets blocking direct light from windows. I changed

the water in the buckets twice a week, checking all surfaces and bags for signs of algal growth.

I photographed capsules once a week at 4.5x magnification, until each either hatched or died. While most capsules hatched on their own, I considered capsules "hatched" if they had a thinned plug and contained embryos with dark shells. I would then dissect the capsules with a scalpel to release the hatchlings. I recorded a capsule as dead if (1) it did not develop further in the subsequent two weeks, (2) its embryos were surrounded by detritus in the albumen matrix, or (in later developmental stages) (3) its embryos were not moving. Embryos from three of the four treatments were photographed at the same 'pre-veliger' stage (Figure 5.1). Using imageJ (Schneider et al., 2012), I measured the diameter of 1-cell embryos and maximum length (ML) of pre-veligers for comparisons across treatments.

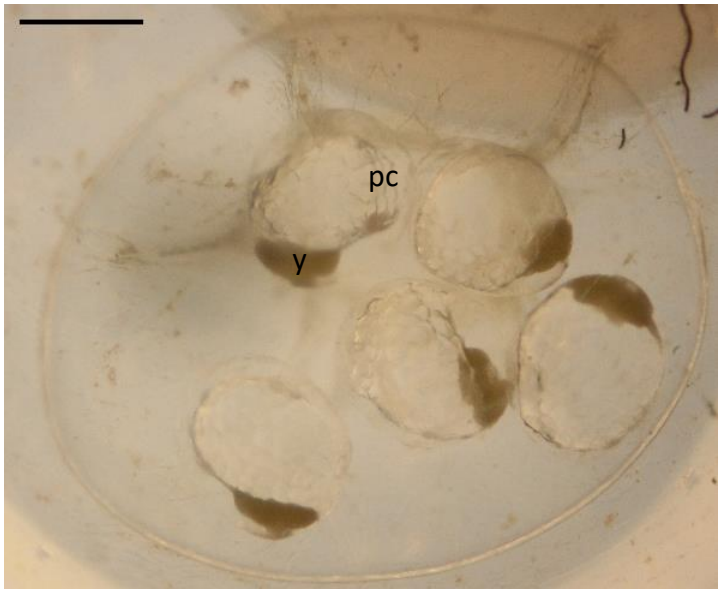

Figure 5.1: Five pre-veliger embryos viewed through the capsule base. y = yolk; pc = prismatic cells; Scale = 1 mm

5.2.4 Hatchling Experiment

I collected additional capsules from Control females, sampled from containers that included a single whelk, or groups of 4 whelks, between the 16th and 27th of November. As above, these capsules were sealed within mesh bags, and kept in additional (unpolluted) buckets. These capsules were not photographed during development but were monitored for algal growth, and signs of hatching or capsule death.

Many capsules were hatching or ready to hatch on 22 December, so I haphazardly allocated no more than three hatchlings from a capsule to one of 14 jars. Each jar contained 12 hatchlings. I placed the jars in ~5L of calcein (200 mg L^{-1}) for 26 hours. Calcein is a fluorescent dye that binds to calcium in the water column, and is thus incorporated in to new shell growth, creating a fluorescent band (Moran, 2000). This band indicates the shell length at the start of the experiment. I presented hatchlings with cockle tissue for the first two hours of exposure. After 26 hours, I rinsed the jars (and hatchlings within) with FSW to remove excess calcein. Using a plastic pipette, I then removed hatchlings to a petri dish, rinsed them again with seawater, then transferred them to labelled petri dish with more FSW. I examined hatchlings under a compound microscope with fluorescence (Leica DM 2500 LED) to confirm a calcein band. I selected the seven hatchlings with the clearest markings from each jar for the experiment.

I allocated three jars to each of three replicate 10L trays for both Control and Cu+Zn treatments. I filled each tray with ~5L of FSW (+20mL aliquot, Table 1), with an air-stone fed by an air pump. I presented hatchlings in both treatments with unpolluted cockle tissue for 2-4 hours twice a week, after which I changed the water. Once a week, I photographed each hatchling at 5x magnification under the compound microscope with fluorescence, removing any dead hatchlings. I ended the experiment after five weeks. I used imageJ to measure the length of new growth in the hatchlings remaining after five weeks.

5.2.5 Statistical Methodology

I conducted all statistical tests in R (V. 4.0.3) using RStudio (V 1.3.1093; RStudio Team, 2020). I visually checked data for normality, homogeneity and outliers using the autoplot function (package: ggplot (Kassambara, 2020)) which produces Fitted vs. Residual, QQ, Scale-Location and Constant Leverages plots. I used the glm() and lm() functions of the stats package (R Core Team, 2020) to perform the following tests.

I modelled the effects of Treatment and Week on the proportion of capsules that were dead or ‘not dead’ (hatched or alive) each week using a generalised linear model with binomial distribution. I used a similar model to examine the effects of Treatment and Week on the proportion of hatchlings remaining at Weeks 1 and 5. I used a linear model to examine the effect of Treatment (with nested random effect of Capsule ID) on the sizes of 1-cell embryos

and pre-veligers. Similarly, I modelled the effect of Treatment (with nested effect of replicate) on the growth of hatchlings after 5 weeks. These models used the glm() and lm() functions from the stats package (R Core Team, 2020).

5.3 Results

5.3.1 Development

Intracapsular mortality was influenced by treatment (GLM with binomial distribution: χ^2 = 287.97, 3 df, $p < 0.0001$), week (χ^2 = 333.14, 1 df, $p < 0.0001$), and the interaction of the two (χ^2 = 8.04, 3 df, p = 0.0451). All polluted capsules had died by week 6, compared to two (11%) in the Control treatment, the remainder having successfully hatched (Figure 5.2). The Cu-High capsules died the soonest, with over 50% dead by week 2, the remainder dying within the following week (Figure 5.2). None of these embryos progressed beyond an initial multicellular stage. In capsules exposed to Cu-Med and Cu+Zn treatments, 22% and 65% of capsules (respectively) survived to the pre-veliger stage. They did not progress further, with all but one of the capsules dying within a week of reaching this stage (Figure 5.2).

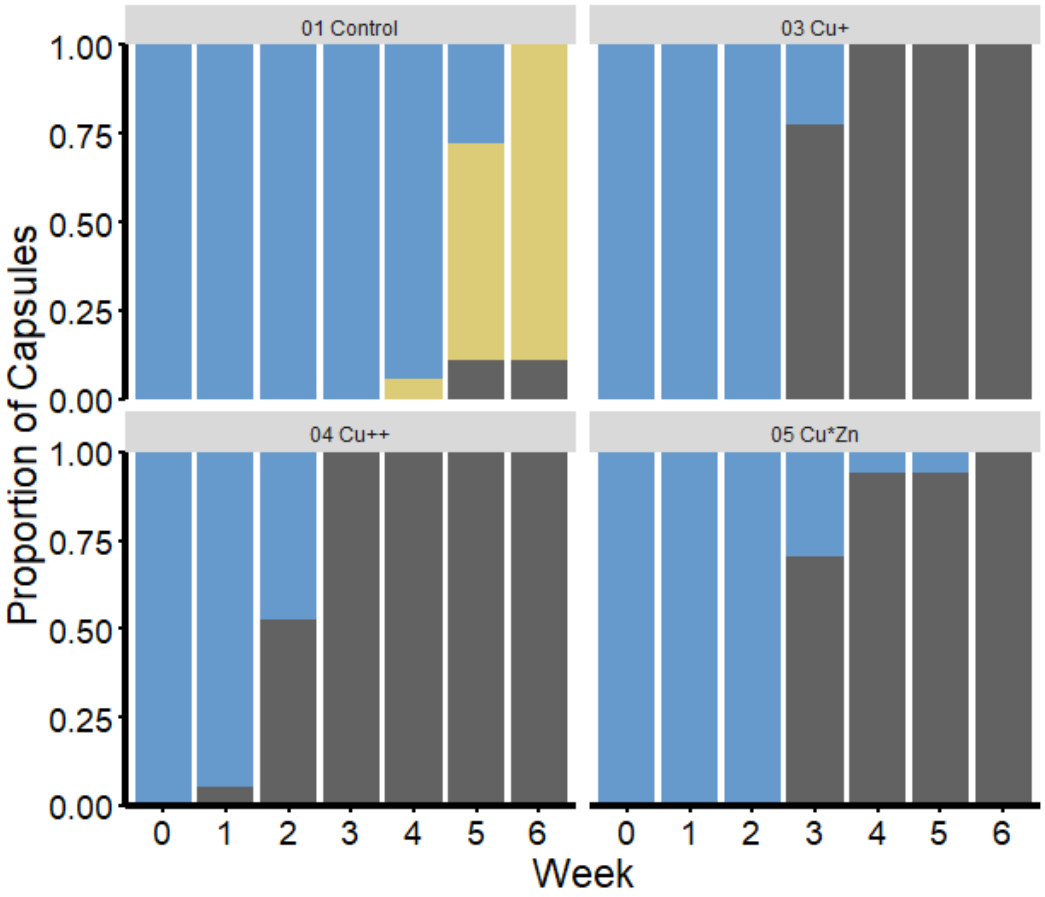

Figure 5.2: Cumulative proportion of capsules alive (blue), hatched (yellow), and dead (grey) from the four treatments across six weeks. n = 18 capsules in Control, 9 capsules in Cu-Med, 18 capsules in Cu-High, and 17 capsules in Cu+Zn.

Capsules contained similarly sized eggs across treatments (ANOVA: $F_{3, 58} = 1.48$; $p = 0.2296$), with an average diameter of 0.28 mm (+/- 0.017 std dev). Polluted embryos that reached the pre-veliger stage were much smaller than their Control counterparts (one way ANOVA: $F_{2, 100} = 233.04$; $p < 0.0001$; Tukey test: $p < 0.0001$; Figure 5.3). Where the average pre-veliger from the Control treatment was 0.83 mm (+/- 0.07mm stdev.), those from Cu-Med and Cu+Zn were 42% and 17% smaller (Tukey: $p < 0.0001$; Figure 5.3). Interestingly, pre-veligers exposed to Cu+Zn 30% larger than those of Cu Med (Tukey: $p < 0.0001$; Figure 5.3). Hatchlings from the Control treatment averaged 1.03 (+/- 0.09) mm shell length (SL).

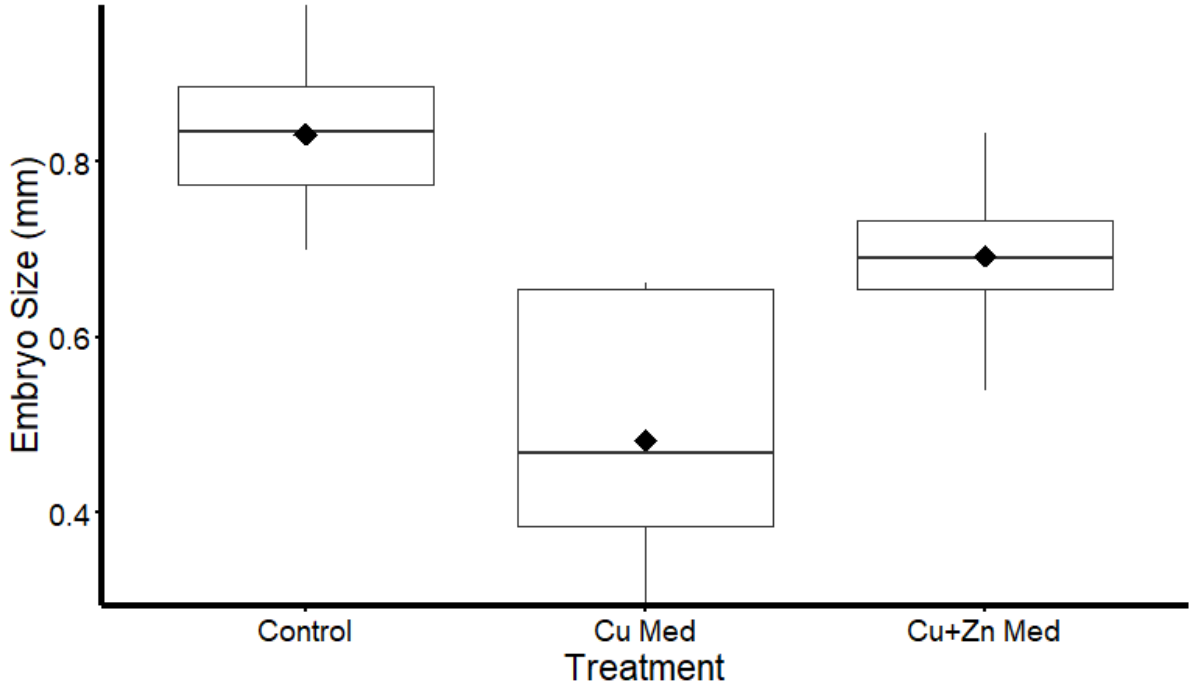

Figure 5.3: Average size of pre-veliger embryos from three treatments. The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR (interquartile range) of the box. Mean represented by black diamond. n = 84 embryos across 18 bags from Control, 9 embryos across 2 bags from Cu Med, and 39 embryos across 11 bags from Cu+Zn Med

5.3.2 Hatchling Experiment

Exposure to Cu+Zn did not reduce survival over the first 5 weeks post-hatching (GLM with binomial distribution: $\chi^2 = 1.25$, 1 df, $p = 0.2636$). 71% and 65% of hatchlings from Control and Cu+Zn treatments, respectively, remained at the end of the experiment (Figure 5.4). Across treatments, however, there was a significant reduction in the number remaining 1 week post-hatching (91%) and after five weeks (53%) (GLM: $\chi^2 = 14.83$, 1 df, $p = 0.0001$; Figure 5.4). There was no interaction between treatment and week ($\chi^2 = 0.53$, 1 df, $p = 0.4684$).

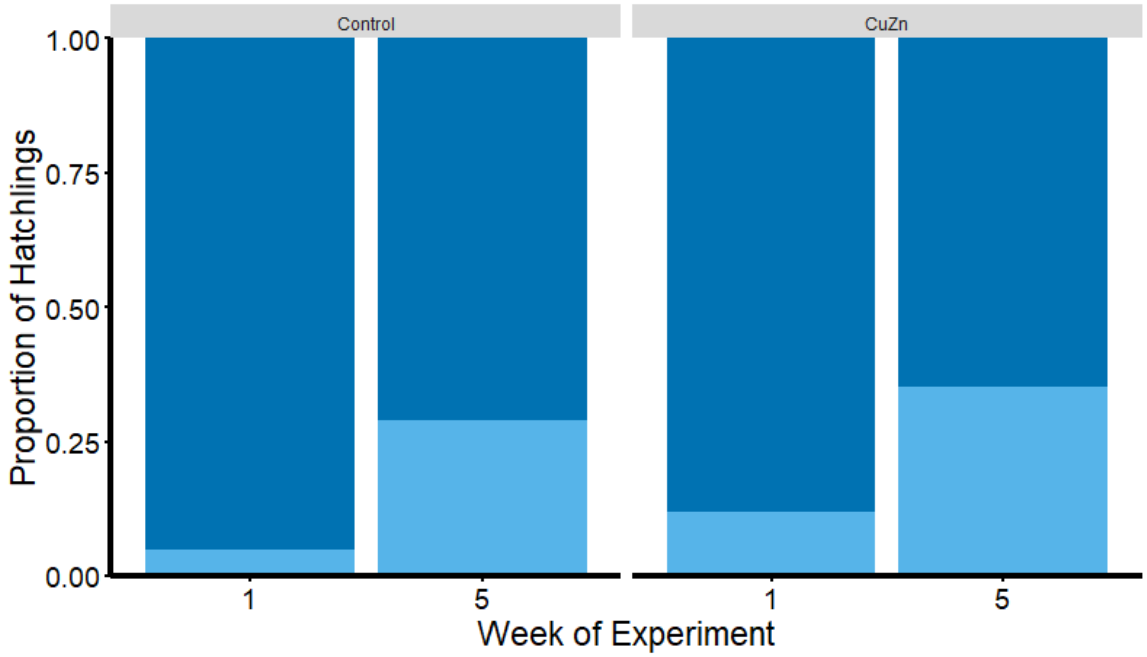

Figure 5.4: Proportion of hatchlings alive (dark blue) or dead (light blue) in Control (left) and Cu+Zn (right) replicates after 1 and 5 weeks of treatment exposure. n = 6 jars per treatment.

Overall, hatchlings surviving to Week 5 showed similar growth across both treatments (Linear model, nested random effect of replicate: $F_{1, 42} = 0.7676$, $p = 0.3860$), the average hatchling growing by 1.43 mm (+/- 0.90 mm stdev) (Figure 5.5). There were four major outliers in the data – three large hatchlings from the Control treatment (3.02 to 9.35 mm), and one from the Cu+Zn which did not grow. I considered these to be biologically meaningful and kept them in the model.

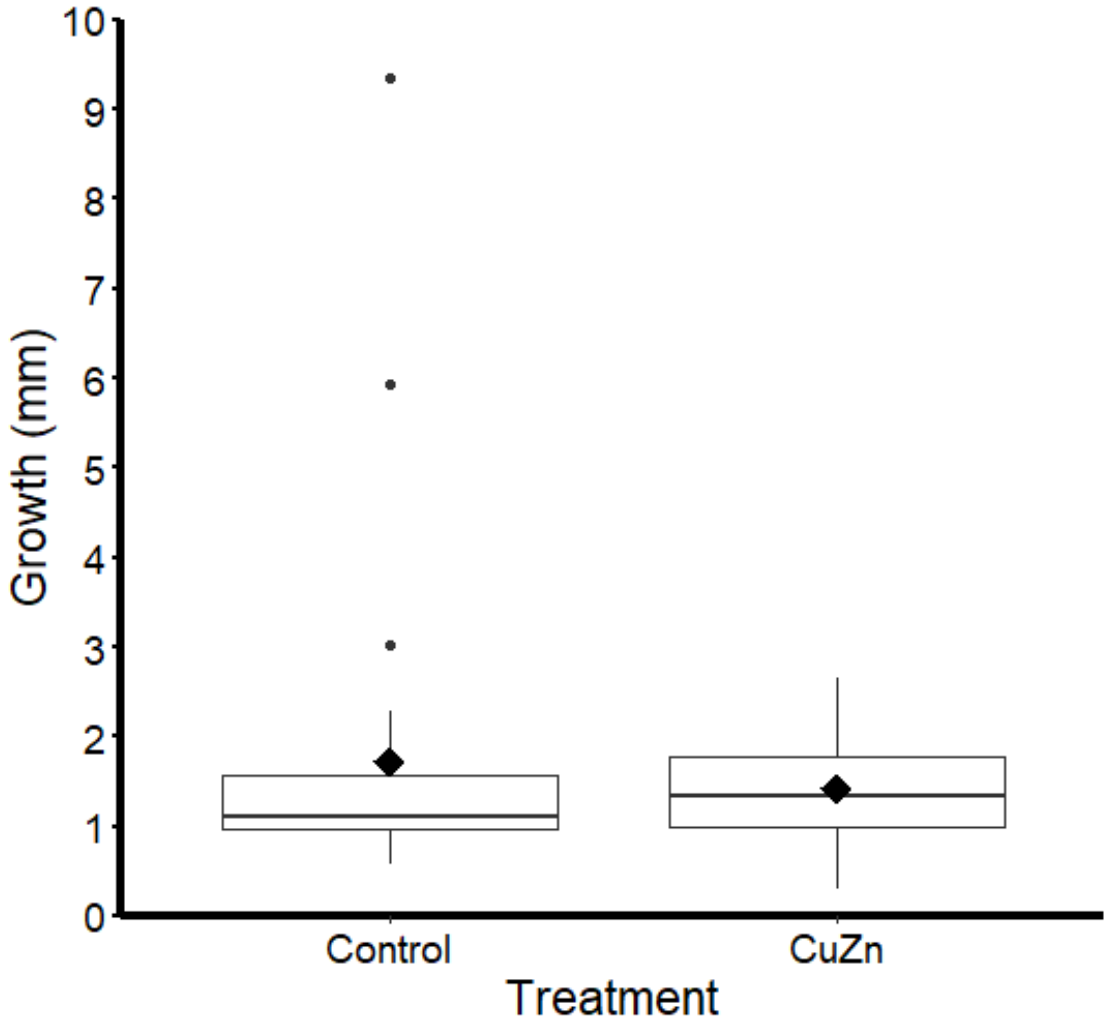

Figure 5.5: Average growth (mm) of hatchlings in each of 6 replicate jars after five-week exposure to A) Control (unpolluted) and B) aqueous Cu+Zn pollution. The box denotes the 25 to 75 percentile range, with median represented by the centre line within the box. Whiskers indicate the smallest and largest values within 1.5*IQR (interquartile range) of the box. Mean represented by black diamond. n = 26 hatchlings from Control and 27 hatchlings from Cu+Zn treatments.

5.4 Discussion

Intracapsular development was highly sensitive to heavy metal pollution, with no embryos surviving from capsules in pollution treatments. The development of embryos exposed to 50 µg Cu L^{-1} stalled early on, and all died within three weeks. Embryos exposed to 20 µg Cu L^{-1} (with or without 50 µg Zn L^{-1}) had slower growth than conspecifics in the Control treatment, though none survived beyond a pre-veliger stage. Recently hatched juveniles were more resilient than embryos to Cu+Zn pollution, showing similar survival and growth rates to conspecifics in the Control treatment.

5.4.1 Embryonic Development

The present study appears to be the first to report the effects of heavy metal pollutants on the encapsulated embryos of a marine gastropod. The bulk of studies examining the effects of pollution on encapsulated development use freshwater pulmonate snails. These snails often have a more rapid developmental rate than *C. glandiformis*, hatching within a month (e.g. 14 days for *Racesina luteola* (Das & Khangarot, 2010) and *Lymnaea stagnalis* (Bandow & Weltje, 2012)), and are common model species for comparing anthropogenic pollutant effects. They may also be more sensitive to heavy metal pollution, with 2.5 µg Cu L^{-1} capable of stalling *Physella acuta* development in the final stages of development (Gao et al., 2017).

Pooled across both 20 µg Cu L^{-1} treatments (i.e., with and without Zn), 50% of embryos reached the pre-veliger stage within the same week as those in Control conditions. Similarly, development of *Racesina luteola* often stalled at the trochophore stage in embryos exposed to $\geq$18 µg Cu L^{-1} (Khangarot & Das, 2010). Those surviving to hatch experienced delayed development and morphological abnormalities. While no developmental abnormalities are described in the present study, research on freshwater pulmonate snails has found malformed eyes and tentacles at concentrations as low as 12.5 µg Cu L^{-1} (*Physella acuta,* Gao et al., 2017). A pilot study conducted over the 2019/2020 summer, which included a lower Cu-dose treatment (5 µg L^{-1}), saw no effect on embryonic development.

The developmental stages seen in non-polluted *C. glandiformis* were similar to those reported for *C. maculosa* and *C. virgata* (Carrasco & Phillips, 2014). Like *C. glandiformis*, *C. maculosa* encapsulates multiple embryos (7.7 +/- 2 (Carrasco & Phillips, 2014)) which hatch at 1.6 (+/-0.02) mm SL. *Cominella virgata* encapsulates only one embryo, which hatches at

2.7 (+/- 0.01) mm SL. *C. glandiformis* hatchlings are the smallest of the three, averaging 1.03 (+/- 0.09) mm SL. The developmental time was longer than in the present study, taking nine to ten weeks compared to the present study's five to six. Ambient water temperatures were slightly higher in the present study (17.9°C (±1.6 stdev), Appendix-Table 5.1) vs. 15.4°C (±1.6)), which may account for this. Some polluted capsules contained multiple developmental stages, but all embryos died within a week.

Previous studies have also reported slower growth rates in stressed embryos (Chaparro et al., 2018; Chaparro et al., 2014; Coeurdassier et al., 2003). Unpolluted pre-veliger embryos were at least 17% the size of those exposed to 20 µg Cu L^{-1} (with or without Zn). Copper pollution can reduce metabolic rates (Brown et al., 2004; MacInnes & Thurberg, 1973; Osterauer et al., 2009) and cause cellular stress that is energetically expensive to withstand (Brown et al., 2004 and citations therein). Two potential explanations for the smaller polluted embryos of the present study depend on whether embryos can regulate their growth. If embryo growth is proportional to their energy supply (i.e. embryos cannot control it), then the metabolic costs of withstanding pollution will sap energy, resulting in slower growth. If embryos can 'choose' their growth rate, they may trade-off slower growth for storing more energy for subsequent needs. For example, Chaparro and Paschke (1990) concluded that larger *Crepipatella dilatata* embryos relied on resources stored during earlier developmental stages to attain their larger size.

Previous research has found evidence of capsule walls thinning during development. The timing of this has been linked to increased metabolic demands from later-stage embryos (Brante, 2006; Segura et al., 2010) and may also provide additional nutrition (Buchner-Miranda et al., 2018; Ojeda & Chaparro, 2004). For example, capsules containing 'late' stage (early to calcified veligers) *Fusitriton oregonensis* had walls 50% thinner than capsules containing 'early' stage (egg to trochophore) embryos (Brante, 2006). A similar trend was also observed in *Crepipatella dilatata* capsules (Segura et al., 2010). Though not quantified, I observed capsule walls becoming less robust by the time they hatched, suggesting that they had thinned. Thinning could allow more rapid diffusion of pollutants across the capsule wall, exposing embryos to increasing Cu concentrations as development progresses. Future research on how Cu concentrations vary between capsules and treatments (similar to Goldberg et al. (2004) for TBT) would help to elucidate the role of wall thinning in Cu

toxicity. Other explanations for embryo death include chronically depleted energy stores, or toxic poisoning which prevented cellular functions.

5.4.2 Post-hatching survival and growth

Due to the high mortality in polluted capsules, the responses of juveniles with polluted vs. non-polluted experiences could not be compared. Pollution-naïve hatchlings showed similar survival and growth rates, irrespective of the conditions experienced. This suggests that, once hatched, juveniles have a similar Cu and Zn tolerance as adults. Previous studies have found evidence of impaired growth at much lower concentrations (e.g. 5 or 12.5 µg Cu L^{-1} in *P. acuta* (Gao et al., 2017)), highlighting the importance of understanding responses and tolerances across a wide range of taxa.

Post-hatching growth is important in reducing sources of mortality (Carrasco & Phillips, 2012; Carrasco et al., 2012; Marshall & Keough, 2008; Moran & Emlet, 2001; Rawlings, 1994; Spight, 1976a). *Cominella glandiformis* surviving to five weeks grew 1.43 (+/- 0.9) mm SL, more than double the average hatching size in this species (1.12 (+/- 0.09) mm (Chapter 4). However, the growth and survival rates do not preclude negative effects of Cu or Zn on the juveniles. For example, Cu can interfere with chemosensation (Boyd, 2010; Kwan et al., 2015; Lurling & Scheffer, 2007), and (though at higher concentrations) reduced the foraging efficiency of adult *C. glandiformis* (Chapter 3). Juveniles in the present study were placed on or beside the cockle tissue, but juveniles in the field would have to locate food. Juvenile ramshorn snails (*Marisa cornuarietis*) exposed to 10 µg Cu L^{-1} for only 7 days showed damage in the hepatopancreas, gills and epidermis (Sawasdee et al., 2011). I exposed *C. glandiformis* juveniles to twice this concentration for five-times as long, so similar damage seems likely to have occurred.

5.4.3 Conclusion

This is one of few studies that examine encapsulated development of a marine gastropod under polluted conditions. Like many species, the early developmental stages of *C. glandiformis* are sensitive to heavy metal stress. Polluted embryos grew more slowly than unpolluted conspecifics and died before hatching. Once hatched, juveniles may be as tolerant as adults to realistic though high Cu and Zn concentrations. The slower growth rates

in pollutant-exposed embryos suggests that juvenile performance could be impaired by prior exposure if they did survive.

Chapter 6 – Overall Discussion.

6.1 Summary of Major Findings

My thesis examined how adult *Cominella glandiformis* respond to heavy metal stress and how this affects their reproduction and offspring. This is the first detailed examination of reproduction in *C. glandiformis* and is complementary to previous studies on related rocky shore species (Carrasco & Phillips, 2014; van der Sman, 2007). Larger females laid a greater number of capsules, which were larger than those produced by smaller conspecifics. While this species encapsulates a consistent number and size of eggs to each capsule, slightly larger eggs occur in larger capsules (Chapter 2). Copper (Cu) pollution (with or without zinc (Zn)) affected every life stage of this species. In adults, one month exposure impaired their foraging abilities and reduced food consumption (Chapter 3). Further, polluted whelks may expose themselves to greater pollution by preferential consumption of contaminated food (Chapter 3). Concentrations at or above 20 µg Cu L^{-1} delayed reproduction, potentially reducing overall reproductive output (Chapter 4). In Chapter 4, polluted capsules that survived to hatch produced similar numbers and sizes of hatchlings compared to Controls. In a subsequent study that followed intracapsular development, no capsules survived to hatch from polluted treatments (Chapter 5). Development either stalled in early multicellular stages (50 µg Cu L^{-1}) or before reaching the veliger stage (20 µg Cu L^{-1}). Capsules contained similarly sized eggs across treatments, though polluted embryos also grew more slowly than their conspecifics in the Control treatment. Pollution-naïve juveniles appear to be similarly tolerant as adults, showing similar growth and survival after 5 weeks in conditions that killed all embryos (Chapter 5). This is one of few studies to examine the effects of pollutants on the encapsulated development of a marine species, and throughout adult and early stages of the lifecycle.

6.2 Pollution in Coastal Ecosystems

Marine organisms come in to contact with pollutants through their surrounding sediment, water column and diet. Studies examining physiological biomarkers (e.g. body burden, metallothionein (MT)) have concluded that whelks primarily accumulate heavy metals through their diet (Blackmore, 2000; Blackmore & Morton, 2002; Blackmore & Wang, 2004a, 2004b; Wang & Ke, 2002). These studies focus on the cellular responses surrounding

metal uptake via these pathways, such as the role of MT in regulating body burden (Blackmore & Wang, 2004a). Due to this focus, the studies necessarily used single-pathway exposure, which is not ecologically realistic – especially for dietary exposure. It is unlikely that an unpolluted whelk in an unpolluted environment will encounter a highly contaminated prey item. In Chapter 3, I used the more realistic exposure treatments of aqueous-only or aqueous+dietary pollution. Whelk responses did not differ between exposure pathways, suggesting that any effects of the intermittent dietary exposure did not exceed those of ongoing aqueous exposure. This suggests that cellular responses activated to withstand the aqueous exposure would be largely sufficient to mitigate dietary contamination. This is consistent with Wang and Rainbow's conclusion that the exposure pathway is often less important than the cellular responses in influencing accumulation (Wang & Rainbow, 2005).

Heavy metal pollution reduced food consumption and foraging ability in adult *C. glandiformis*, likely reducing body condition (Chapter 3). These are common results of heavy metal pollution and can have significant consequences for subsequent growth, reproduction, and survival (Amiard-Triquet, 2009; Cheung & Lam, 1999; Das & Khangarot, 2010; Gagné et al., 2002; van der Sman et al., 2009). Copper exposure can reduce metabolic rates (Brown et al., 2004; Marshall et al., 2004) and interfere with feeding regulation via neurotoxic damage (Rozsa and Salanki, 1990), ultimately reducing food consumption. At a behavioural level, this can be difficult to differentiate from active avoidance of dietary exposure. The whelk *Nassarius siquikorensis* consumed less tissue from heavily polluted oysters but consumed less polluted oysters at a normal rate (Guo et al., 2013), indicating that this was an active choice. Whelks fed the less-contaminated oysters exposed themselves to higher dietary pollution than conspecifics presented with highly contaminated prey.

Whelks rely on chemosensation to locate food (Braithwaite et al., 2017; Wilson & Weissburg, 2012; Yu et al., 2019), and likely to detect predators, conspecifics, and suitable egg-laying surfaces (Boyd, 2010; Rittschof et al., 2002; Seuront & Spilmont, 2015). As discussed by Wilson and Weissburg (2012), slower moving whelks are more likely to lose a scent trail, further reducing foraging efficiency and success. Whelks have been reported to move towards bait stations from 20 m away (Lapointe & Sainte-Marie, 1992) and, under

laboratory conditions, juvenile *Rapana venosa* could track prey from up to 4m away (Yu et al., 2019). Whelks with impaired foraging abilities could travel a significant distance before losing the scent (i.e., no energetic reward). Alternatively, they may take much longer to reach the food and face higher competition from other organisms that got there faster (i.e. reduced energetic reward).

The preferential consumption of polluted cockles by polluted whelks (Chapter 3) suggests that whelks may expend this energy on obtaining sub-optimal prey. Polluted prey are probably in poor condition themselves (e.g. Gagne et al., 2002) and therefore provide less nutrition to the whelks. Further, whelks can assimilate up to 90% of the metal in prey tissues when exposed to dietary-only pollution (Cheung & Wang, 2005), worsening their own body burden. While pollution-naïve hatchlings may be similarly metal-tolerant as adults (Chapter 5), they likely experience similar, if not worse, physiological effects. Copper concentrations have been reported to cause changes in the histopathology of juvenile (10 μg L^{-1}, Sawasdee et al., 2011) and adult (50 μg L^{-1}, Otludil & Ayaz, 2020) freshwater snails. In the experiments of Chapters 3 (adults) and 5 (juveniles), whelks were regularly fed and did not need to expend energy to obtain food. These results likely overestimate the performance of whelks in the wild, though offer an indication of tolerance in a more 'ideal' setting.

6.3 Zn regulation

Zinc is a common coastal pollutant (Ali et al., 2019; Li et al., 2012; Sorensen & Milne, 2009) and elicits varying responses in organisms, depending on the exposure route (e.g. Blackmore & Morton, 2002; Blackmore & Wang, 2003), concentration (Blackmore & Wang, 2002), or co-occurring metals (e.g. Rouchon & Phillips, 2017a). *Cominella glandiformis* appears to regulate Zn, with the only negative effects seen in the size of pre-veliger embryos (Chapter 5) and a slightly lower proportion of capsules surviving compared to the Control treatment (Chapter 4). While previous research suggests that whelks bioaccumulate Zn (and other metals) from their diet (Blackmore & Morton, 2002; Blackmore & Wang, 2004b; Cheung & Wang, 2005), their focus on cellular responses makes comparisons with my reproductive results (Chapter 3) difficult.

6.4 Reproduction

Chapter 2 established the natural levels of variation that could be expected in *C. glandiformis*, as no previous baseline existed. This species encapsulates a consistent number and size of eggs to each capsule (Chapter 2, Chapter 4). Larger females laid larger (and more) capsules, with slightly larger eggs occurring in larger capsules (Chapter 2). Though overall production was similar in whelks from each site, those from Browns Bay reached peak output three weeks earlier than Paremata Station whelks. This was likely due to site-specific experiences, such as prior nutrition (e.g. *C. maculosa* (van der Sman, 2007)). As discussed in Chapter 4, warmer temperatures speed development, but can also reduce hatching success (Gallardo & Cancino, 2009; Smith et al., 2013). The delayed laying seen in polluted females (Chapter 4) may therefore increase the likelihood of fewer offspring surviving to hatch, as most will be developing during warmer late-December/January. Since females from Paremata Station and Browns Bay laid capsules throughout the season, significant offspring mortality is less likely.

The average *C. glandiformis* capsule was 58% and 71% the size of *C. maculosa* or *C. virgata* capsules, respectively (Chapter 2; Carrasco & Phillips, 2014). Despite this smaller size, the diameter of *C. glandiformis* eggs was slightly larger than either of these species. Smaller objects have a greater surface area to volume ratio, which may be an advantage in an ecosystem likely prone to oxygen limitation. Further, research on salmon eggs suggests that larger egg sizes may be more resilient in low oxygen environments, and could be selected for in systems prone to this stressor (Einum et al., 2002). Taken together, the larger eggs in a smaller capsule suggest that *C. glandiformis* reproduction is well suited to low oxygen environments. The larger egg diameter also suggests that females may provision more lipids to each embryo than their rocky shore relatives.

Energy stored in various tissues during periods of food abundance is used to support the organism through scarcity, stress, and gonad maturation (Amiard-Triquet, 2009; Bi et al., 2016; Goodchild et al., 2019; Sokolova et al., 2012). Thus, females in poor condition have lower reproductive outputs than well-resourced conspecifics (Bae et al., 2016; Chatzinikolaou & Richardson, 2010; Gagné et al., 2002). This can extend to maternal investment, with smaller capsules (Chatzinikolaou & Richardson, 2010; van der Sman et al., 2009) reported, or poorer performance as juveniles (van der Sman et al., 2009). Starved

Tritia reticulata may prioritise surviving to reproduce in the future over investment in the present breeding season (Chatzinikolaou & Richardson, 2010). Similarly, and as discussed in Chapter 3, polluted *C. glandiformis* may have prioritised present growth over future energy stores in response to Cu pollution.

In Chapter 4, even the most polluted whelks showed consistent per-capsule investment, with a fixed number and sizes of eggs (and hatchlings). This suggests a strong selection for initial hatching size and, based on egg diameter, embryos having access to similar yolk supplies. A combination of differences in lipid content (e.g. proportion of Tag (Carrasco et al., 2016)) and metal exposure (maternally-inherited or direct exposure) likely explains the significant mortality in embryos (Chapters 4 and 5). This is one of few studies to examine the effects of pollution on benthic development in a marine species and, to my knowledge, the only one to examine heavy metal pollution. Confirming the body condition of whelks prior to the laying season, and whether this relates to the maternal transfer of lipids and metals to offspring, would help elucidate some of these trends.

6.5 Development

Heavy metals accumulate in the digestive gland of whelks, especially under dietary exposure (Leung & Furness, 1999; Wang & Ke, 2002)}. Stored lipids are transferred to the gonad during gametogenesis (Bi et al., 2016), likely transporting heavy metals as seen in the clam *Mya arenaria* (Gagné et al., 2002). Clams collected from a metal-polluted marina were in poorer condition than those of a reference site, and a higher percentage had inactive gonads (Gagné et al., 2002). Gonad homogenates from this polluted site had lower protein and lipid concentrations and elevated Zn, cadmium (Cd) and mercury (Hg) burdens. Similarly, while polluted females produced more vitellin (the main yolk protein) than reference females, it contained less protein and elevated metal concentrations – approximately 100 times higher than in the gonad homogenate. For encapsulated developers, capsule walls allow diffusion of molecules with a low-molecular weight (Pechenik, 1983; Rawlings, 1999), but may limit larger molecules such as tributyltin (TBT) (Averbuj et al., 2018) and Cu (Untersee, 2007). Goldberg et al. (2004) found concentrated TBT concentrations in volute (*Pachycymbiola brasiliana*) capsules, suggesting that maternal inheritance may be an important exposure route for embryos.

Polluted *C. glandiformis* embryos either died rapidly (50 µg Cu L^{-1}) or grew slowly and died before hatching (20 µg Cu L^{-1}, with and without 50 µg Zn L^{-1}) (Chapter 5). Time constraints prevented me from keeping capsules from polluted parents in unpolluted water, but this would be a simple method for examining the importance of maternally inherited pollution. Further, oyster (*Magallana hongkongensis*) larvae kept in clean seawater can rapidly eliminate maternally inherited tissue burdens (Weng & Wang, 2017). Future research should examine the relative importance of these exposure routes, including whether any initial burden is rapidly shed, and how this affects intracapsular concentrations.

The rapid death of embryos exposed to 50 µg Cu L^{-1} suggests that, even if pollution was patchy and they had some respite, these embryos are unlikely to survive to hatch. Those exposed to 20ug Cu L^{-1}, however, may successfully hatch if pollutant exposure was intermittent. Based on current management practices in New Zealand, it is unlikely that *C. glandiformis* is exposed to 20 µg Cu L^{-1} for prolonged periods, though these concentrations might occur briefly after heavy rain. Even brief stress-exposure during development can impact the organism's subsequent performance, (Pechenik, 2006; Rouchon & Phillips, 2017b). Future research should examine the effects of pulse-events on species with entirely benthic development, and how this impacts their subsequent growth and survival. Benthic development limits dispersal potential (Dohner, 2016), making recruitment from less-impacted areas unlikely.

It is unclear why >50% of capsules survived heavy metal exposure in Chapter 4, but none survived in Chapter 5. The most obvious reason is handling effects, as the Chapter 4 capsules were largely untouched between harvest and hatching, but Chapter 5 capsules were handled every week. The (arguably higher) survival of Control capsules suggests that this would have been a minor stress. Further, I had collected capsules for use in the hatchling experiment (kept in Cu+Zn med conditions) which also failed to develop, despite the lack of handling. The average temperature during Chapter 5 was 17.9 ^{o}C (+/- 1.6 std dev), compared to 17.8 ^{o}C (+/- 2.0) for Chapter 4. While this doesn't capture differences in daily extremes, embryos seem to have experienced similar temperatures during development in both experiments. Perhaps the experiences of adult whelks prior to collection influenced their offspring. For example, adult *Biomphalaria glabrata* (a freshwater

pulmonate) fed a more nutritious and calorific diet produced offspring that were more resilient to Cd pollution (Plautz, Funkhouser, et al., 2013).

6.6 Pollution and juvenile performance

Access to greater yolk supplies should result in a larger hatching size (Chaparro et al., 1999; Chaparro & Paschke, 1990; Lloyd & Gosselin, 2007; Rivest, 1983; Spight, 1976a), and size at hatching has been linked to the likelihood of survival (Carrasco et al., 2012; Marshall et al., 2006; Moran & Emlet, 2001; Spight, 1976a). Despite a larger egg diameter than its rocky shore relatives, *C. glandiformis* hatch at just 1.1 mm (Chapter 4) compared to 1.63 mm in *C. maculosa* (Carrasco & Phillips, 2014). Perhaps *C. glandiformis* hatchlings have plenty of stored lipids, which aid their growth and survival. The average hatchling doubled in size over five weeks, even when polluted (chapter 5). For comparison, the larger hatching *C. maculosa* and *C. virgata* had only 1.2- and 1.3-times longer shell lengths, respectively, after one month (Carrasco & Phillips, 2012). These comparisons must be considered with caution, however, as any number of variables could have contributed to this difference. Nonetheless, whether *C. glandiformis* hatch with large energy reserves, and whether this supports high growth rates under non-optimal conditions, warrants further investigation.

In Chapter 4, capsules exposed to 5 µg Cu L^{-1} showed similar, if not slightly higher, survival rates to Control capsules (Chapter 4). The subsequent performance of juveniles seems likely to be similar too. Thus, if embryos can develop during a period of minimal pollution ($\leq$5 µg Cu L^{-1}), juveniles may show similar growth and survival rates, though further experiments are needed to confirm this. The death of almost all embryos exposed to Cu+Zn pollution prevented my planned factorially-crossed experiment (polluted vs. unpolluted hatchlings in polluted vs. unpolluted conditions). Stressed mothers may produce offspring that are more tolerant to pollutants (e.g. Plautz, Guest, et al., 2013; Plautz & Salice, 2013; Weng & Wang, 2014), but poor maternal condition and/or maternally inherited tissue burdens can cause slower growth rates in juveniles (Bae et al., 2016; van der Sman et al., 2009; Weng & Wang, 2017). Stress experienced during early development can also increase sensitivity to future stressors (Kimberly & Salice, 2014b; Rouchon & Phillips, 2017b) and impact subsequent performance (Chaparro et al., 2018; Ellis et al., 2009; Salas-Yanquin et al., 2022). However, exposure to Cd during *Physella pomilia* development improved juvenile tolerance of subsequent Cd pollution and had a stronger effect than parental pollution experience

(Plautz & Salice, 2013). Future research should examine whether benthic developers with varied exposure histories – including short pulses during early or late development stages – respond differently to pollution as juveniles.

As Chapter 5 used juveniles from unpolluted parents, they may have greater energy reserves than would occur in previously polluted hatchlings. The fact that similar growth and survival was seen in polluted and unpolluted juveniles indicates that juvenile stages can survive in similar conditions as adults. Whether they experience other effects – such as lesions (Otludil & Ayaz, 2020), or the reduced foraging ability seen in adults (though at higher concentrations, Chapter 3) – remains to be seen. Two-week-old juvenile *Hemifusus tuba* (a buccinoid whelk) consume 92% of their tissue-weight per day in food, compared to ~4% by adults (Morton, 1986). If their prey is contaminated, juveniles could be exposed to high levels of dietary pollution. I fed juvenile *C. glandiformis* unpolluted cockle tissue to reduce any confounding effects of food avoidance on growth rates.

6.7 Conclusions

Understanding the sublethal effects of common pollutants informs management decisions and improves our general understanding of stress-tolerance and the associated consequences. Copper pollution appears to negatively affect every life stage of this species – from foraging efficiency and condition to reproductive output and embryonic survival. Despite their poor condition, females maintained per-capsule investment though their output was lower. Pollution exposure reduced offspring survival, further decreasing reproductive success. If embryos can develop during periods of reduced pollution, juveniles may be tolerant of heavy metal pollution. These effects are noticed from $\geq$1 month exposure in the lab. Natural exposure levels are likely to be lower in concentration, but potentially over the lifetime of the individual. These low-level, long-term effects should be further studied.

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Appendix

$$DO_{sat} = \exp[A_0 + (A_1 \times T_s) + (A_2 \times T_s^2) + (A_3 \times T_s^2) + (A_3 \times T_s^3) + (A_4 \times T_s^4) + (A_5 \times T_s^5) \times (B_0 + (B_1 \times T_s) + (B_2 \times T_s^2) + (B_3 \times T_s^3)) + (C_0 + S^2)\ ]$$ Appendix-Equation 3.1

where A_0, A_1, A_2, A_3, A_4, A_5 and B_0, B_1, B_2, B_3 and C_0 are constants calculated by (Benson & Krause, 1980); S is salinity; and T_S is a scaled temperature (in Kelvin) calculated as:

$$T_S = \ln\left[(298.15 - T) \times (273.15 + T)^{-1}\right]$$ Appendix-Equation 3.2

Where T represents temperature (°C).

To convert Appendix-Equation 3.1 to micomol O_2 L^{-1}, the density of water (kg/m^3) must first be calculated as follows (taken from Millero and Poisson (1981)):

$$\rho = \rho_o + A \times S + B \times S^{3/2} + C \times S$$ Appendix-Equation 3.3

Where A, B and C are constants calculated by (Millero & Poisson, 1981); S is salinity, and ρ_o is the density of water, calculated with Appendix-Equation 3.4

$$\rho_o = 999.842594 + 6.793952 \times 10^{-2} \times T - 9.09529 \times 10^{-3} \times T^2 + 1.001685 \times 10^{-4} \times T^3 - 1.120083 \times 10^{-6} \times T^4 + 6.536332 * 10^{-9} \times T^5$$ Appendix-Equation 3.4

The concentration of O_2 (118micromole L^{-1}) in the seawater is calculated

$$Conc_{seawater} = DO_{sat} \times (\frac{\rho}{1000})$$ Appendix-Equation 3.5

The percent O_2 measured in each initial and final sample was converted to a concentration (micromole O_2 L^{-1}) using the following equation:

$$Conc_{Sample} = percent \times (\frac{Conc_{seawater}}{100})$$ Appendix-Equation 3.6

Where percent is the percent O_2 measured in each sample.

Appendix-Table 3.1: Two-way ANOVA results examining effects of treatment and week number on food consumption using nested data (Consumption ~ Treatment (Replicate)*Week) (Chapter 3).

Source of Variation	df	Sum of Squares	F-value	p-value
Treatment	4	7.6038	9.8333	<0.0001
Week	3	1.3661	2.3555	0.0808
Treatment*Week	12	5.7673	2.4861	0.0103
Treatment(Replicate)*Week	60	7.7126	0.6649	0.9416
Residuals	60	11.5989		

Appendix-Table 4.1: Average temperatures (°C) of the Control replicates from the third week of females laying (end of October 2019) to the final week of hatchlings emerging (end of January 2020) (Chapter 4). During Weeks 3 to 8 temperatures are from three adult replicates, and one to three capsule replicates (i.e. for bagged capsules developing to hatch). Weeks nine to 16 are for 1 to three capsule replicates (I reduced the number of buckets as capsules hatched).

Week starting	Week	Average Temperature (°C) (+/- std. dev)
28/10/2019	3	13.72 (+/-0.7)
04/11/2019	4	16.41 (+/-0.5)
11/11/2019	5	15.64 (+/-1.1)
18/11/2019	6	16.08 (+/-1)
25/11/2019	7	18.68 (+/-1.8)
02/12/2019	8	19.79 (+/-0.6)
09/12/2019	9	18.43 (+/-0.9)
16/12/2019	10	18.43 (+/-2.4)
23/12/2019	11	17.31 (+/-0.9)
30/12/2019	12	19.33 (+/-0.5)
06/01/2020	13	18.55 (+/-1.2)
13/01/2020	14	17.75 (+/-0.5)
20/01/2020	15	18.67 (+/-0.7)
27/01/2020	16	21.34 (+/-0.9)

Appendix-Table 5.1: Average temperatures (oC) from all Control replicates during the development and hatchling experiments (Chapter 5). Capsules followed for development were collected over two weeks. Hatchlings emerged at the end of week 0, with the first growth/survival check in week 1.

Week starting	Week (Dev. Expt.)	Week (Hatchling Expt.)	Average Temperature (^{o}C) (+/- std. dev)
16/11/2020	0/1		17.62 (+/-1.6)
23/11/2020	1/2		17.02 (+/-1.3)
30/11/2020	2/3		16.41 (+/-1.6)
07/12/2020	3/4		18.98 (+/-0.7)
14/12/2020	4/5		17.25 (+/-0.9)
21/12/2020	5/6	0	18.56 (+/-0.6)
28/12/2020	6/7	1	17 (+/-2)
04/01/2021	7/8	2	19.57 (+/-0.8)
11/01/2021	8/9	3	20.34 (+/-1.1)
18/01/2021	n/a	4	17.92 (+/-1.2)
25/01/2021	n/a	5	20.72 (+/-0.4)