# AQUACULTURE OF THE NEW ZEALAND GEODUCK CLAM, *Panopea zelandica*

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ABSTRACT

The geoduck *Panopea zelandica* has had significant interest from aquaculture New Zealand. To pave the way for the establishment of geoduck aquaculture, information on all areas of geoduck production needs to be determined. The aim of this thesis was to identify conditions and evaluate potential bottlenecks within hatcheries, during transport, and growout.

Within hatcheries, much needed information on the successful broodstock conditioning and subsequent larval development was obtained. Investigations into diet ratios of *Tisochrysis lutea* (ISO) and *Chaetoceros muelleri* (CM) during the conditioning period revealed important carry over effects. Broodstock that were conditioned on similar proportions of ISO:CM produced embryos that had the highest survival, highest transition into D – veligers, and had the lowest incidence of abnormalities. Fertilization success and embryonic development under different thermal conditions was also explored. Highest rates of fertilization were achieved at the highest temperature (23.7°C) tested. However, there appears to be a thermal maximum for the optimal development of *P. zelandica*, and embryos were greatly hindered at temperatures >18.5°C due to an increase in cellular blebbing of the developing embryosarising from uneven cell division. Once *P. zelandica* embryos have transitioned into D – veligers, they remain within the water column for up to 19 days prior to settlement. Therefore, it is no surprise that food availability on its own is a common stressor for the developing larvae. *P. zelandica* larvae of different ages were subjected to different diet ratios and subjected to acute thermal stress. This study showed

that susceptibility of larvae to thermal stress was reduces with increasing larval age, suggesting that older larvae are better able to acquire and store exogenic resources.

To establish the crucial link between hatcheries and growout sites, the behavioural and physiological responses during transport and recovery of juveniles was investigated for the first time. The duration of emersion had a clear effect on overall response and recovery. Following 3h of transport, smaller changes within haemolymph osmolality, haemocyte parameters and metabolism were experienced compared to 8h of transport. Also, animal behaviour returned to normal faster within the 3h transport group. Even after 5 days of recovery in standard conditions, the effects of emersion were still evident within the 8h transport group with the use of succinate to fuel the respiratory chain. However, it does appear that given the right conditions and enough food resources, the animals are able to successfully restore internal energy reserves.

The final study investigated the potential threat of marine heatwaves to juvenile geoducks within growout sites. This novel study focused on characterising animal behaviour, haemocyte subpopulations, and heat shock protein (hsp79 and hsp90). Geoducks appear to be highly sensitive to the effects of temperature, with animals actively borrowing out of the sediment when temperatures reached 25°C. This was also evident in the haemocyte populations, as there was an increase in the abundance of granulocytes and haemocyte aggregations indicating poor health conditions. However, an increase in the expression of hsp70 does suggest that the animals were actively trying to counter the effect of cellular damage incurred due the thermal strain.

In summary, this thesis provides important information on selected factors crucial for the development of aquaculture of *P. zelandica* in New Zealand.

# TABLE OF CONTENTS

**ABSTRACT** .......... **I**

**TABLE OF CONTENTS** .......... **IV**

**LIST OF TABLES** .......... **VII**

**LIST OF FIGURES** .......... **VIII**

**ATTESTATION OF AUTHORSHIP** .......... **XII**

**CO-AUTHOR CONTRIBUTIONS** .......... **XIII**

**ACKNOWLEDGMENTS** .......... **XVII**

**CHAPTER 1 – INTRODUCTION AND THESIS FRAMEWORK** .......... **1**

1.1 General Introduction .......... 2
1.2 Main research areas .......... 4
*1.2.1 Broodstock conditioning and Larval rearing* .......... *4*
*1.2.2 Transport of juveniles* .......... *6*
*1.2.3 Geoduck field growout* .......... *9*
1.3 Thesis Aims .......... 12
1.4 Thesis structure .......... 12

**SECTION 1: BROODSTOCK CONDITIONING AND LARVAL REARING** .......... **14**

**CHAPTER 2 – CARRY - OVER EFFECTS OF BROODSTOCK CONDITIONING ON THE SALINITY TOLERANCE OF GEODUCK CLAM (*PANOPEA ZELANDICA*) EMBRYOS** .......... **15**

Abstract .......... 16
2.1 Introduction .......... 17
2.2 Methods and Materials .......... 20
*2.2.1 Parental broodstock and source of gametes* .......... *20*
*2.2.2 Spawning and fertilization* .......... *20*
*2.2.3 Embryo challenge setup and procedure* .......... *21*
*2.2.4 Sampling procedure* .......... *24*
*2.2.5 Scanning electron microscopy (SEM)* .......... *25*
*2.2.6 Statistical analysis* .......... *26*
2.3 Results .......... 27
*2.3.1 Egg size* .......... *27*
*2.3.2 Embryo and larval survival* .......... *28*
*2.3.3 Development to D - veligers* .......... *29*
*2.3.4 Incidence of abnormal D-veliger development* .......... *30*
*2.3.5 Size of D-Veligers* .......... *33*
2. 4 Discussion .......... 35

**CHAPTER 3 - EFFECT OF TEMPERATURE ON EARLY DEVELOPMENT OF THE NEW ZEALAND GEODUCK *PANOPEA ZELANDICA* (QUOY & GAIMARD, 1835)** .....40

ABSTRACT..... 41
3.1 INTRODUCTION..... 42
3.2 METHODS AND MATERIALS..... 45
*3.2.1 Broodstock and spawning*..... 45
*3.2.2 Experimental setup*..... 45
*3.2.3 Sampling procedure*..... 46
*3.2.4 Sample analysis*..... 47
*3.2.5 Fertilization and development*..... 49
*3.2.6 Statistical analysis*..... 50
3.3 RESULTS..... 51
*3.3.1 Sampling procedure*..... 51
*3.3.2 Fertilization and development*..... 51
*3.3.3 Index of early cleavage*..... 54
*3.3.4 Normal development*..... 55
3.4 DISCUSSION..... 58

**CHAPTER 4 - CHANGES IN THERMAL TOLERANCE AND GROWTH DUE TO FOOD AVAILABILITY DURING LARVAL DEVELOPMENT OF THE NEW ZEALAND GEODUCK (*PANOPEA ZELANDICA*)**.....62

ABSTRACT..... 63
4.1 INTRODUCTION..... 64
4.2 METHODS AND MATERIALS..... 67
*4.2.1 Acquisition of larvae*..... 67
*4.2.2 Experimental procedure*..... 68
*4.2.3 Survival analysis*..... 71
*4.2.4 Statistical analysis*..... 71
4.3 RESULTS..... 72
*4.3.1 2 & 3 days post fertilization (unfed baseline)*..... 72
*4.3.2 2 – 5days post fertilization*..... 73
*4.3.3 5 – 8 days post fertilization*..... 74
*4.3.4 9 – 12 days post fertilization*..... 77
4.4 DISCUSSION..... 79

**SECTION 2: TRANSPORT OF JUVENILES** .....83

**CHAPTER 5 - PHYSIOLOGICAL RESPONSES OF JUVENILE NEW ZEALAND GEODUCK (*PANOPEA ZELANDICA*) FOLLOWING EMERSION AND RECOVERY**.....84

ABSTRACT..... 85
5.1 INTRODUCTION..... 86
*5.2.1 Animal husbandry*..... 89
*5.2.2 Experimental approach*..... 89
*5.2.3 Behavioural observations after emersion*..... 92
*5.2.4 Sample collection and analyses*..... 92
*5.2.5 Flow cytometry*..... 93
*5.2.6 Osmolality*..... 95
*5.2.7 GC-MS metabolomics*..... 95
*5.2.8 Statistical analyses*..... 99

5.3 Results ... 101
*5.3.1 Animal size* ... 101
*5.3.2 Behavioural observations* ... 101
*5.3.3 Physiological response to emersion* ... 102
*5.3.4 Physiological response of recovery phase 1 (R1: 1-day)* ... 105
*5.3.5 Physiological response of recovery phase 1 (R2: 5-day)* ... 107
5.4 Discussion ... 110
*5.4.1 Emersion response* ... 110
*5.4.2 Recovery phase 1 response (R1, 1-day)* ... 114
*5.4.3 Recovery phase 2 response (R2, 5-days)* ... 117
*5.4.4 Conclusions* ... 120
Supplementary data ... 122

**SECTION 3: GEODUCK FIELD GROWOUT ... 129**

**CHAPTER 6 - CHARACTERIZING SELECTED RESPONSE MECHANISMS OF JUVENILE GEODUCK (*PANOPEA ZELANDICA*) FOLLOWING ACUTE THERMAL SHOCK ... 130**

Abstract ... 131
6.1 Introduction ... 132
6.2 Methods and materials ... 136
*6.2.1 Animal acquisition and acclimation* ... 136
*6.2.2 Water parameters* ... 137
*6.2.3 Thermal exposure* ... 137
*6.2.4 Behavioural observations* ... 138
*6.2.5 Sample collections* ... 138
*6.2.6 Hemocyte preparations and assessment* ... 139
*6.2.7 Heat Shock Protein (HSP) analyses* ... 140
*6.2.8 Statistical analyses* ... 142
6.3 Results ... 144
*6.3.1 Experimental parameters* ... 144
*6.3.2 Behavioural observations* ... 145
*6.3.3 Hemocyte characterization* ... 146
*6.3.4 HSP 70 and 90 expressions* ... 150
6.4 Discussion ... 152

**CHAPTER 7 - DISCUSSION AND CONCLUSIONS ... 158**

7.1 Thesis background ... 159
7.2 Broodstock conditioning and larval rearing ... 161
7.3 Transport of juveniles ... 164
7.4 Field Growout ... 166
7.5 Future Recommendations ... 169
7.6 Conclusions ... 171

**REFERENCES ... 172**

## List of Tables

**Table 2.1** Salinity calculations used in the experiment to rear *P. zelandica* embryos……………………………………………………...………………………….….…22

**Table 3.1** The approximate post-fertilization developmental times of geoduck incubated at different temperatures (*P. zelandica* and *P. japonica*).……………………………………53

**Table 3.2** Summary of statistical analysis performed on the percentage of normally developing individuals of *P. zelandica* ……………………………………………...……..56

**Table 4.1** Concentrations of microalgal cells given to the larvae during the different diet ratio treatments…………………………………………………………………………….88

**Table 5.1** Wet weight and shell length data of juvenile geoducks from different treatment groups……………………………………………………..…………………………100

**Table 5.1S** Metabolites found in *Panopea zelandica* juveniles following emersion…....125

**Table 5.2S** Metabolites found in *Panopea zelandica* juveniles following 1-day of recovery after emersion…………………………………………………………………...…..……..126

**Table 5.3S** Metabolites found in *Panopea zelandica* juveniles following 5-days of recovery after emersion……………………………………………………..…………………...127

**Table 5.4S** Data (mean, STDEV, SE) obtained via flow cytometry for emersion, and recovery groups…………………………………………...……………………………128

**Table 6.1** qPCR Primers for HSP 70, HSP 90 and B-Actin………..……….…………..…..141

# List of Figures

**Figure 1.0** *Panopea zelandica* juvenile at 6 months old ……………………..………..……….1

**Figure section 1** Hatchery culture of *Panopea zelandica*……….……..……..…………...14

**Figure 2.0** Graphical abstract chapter 2……………………………………..…………15

**Figure 2.1** Experimental design and methods...…………………………..…......…23

**Figure 2.2** Egg diameters obtained from spawned females in feeding treatments……………………………………………………………………...………..27

**Figure 2.3** Survival of embryos reared at different salinities after 48h post fertilization……………………………………………………………………...……..29

**Figure 2.4** Proportion of sample population showing normal development of embryos to D-veligers following rearing in different salinities for 48h……………..………………………...30

**Figure 2.5** Abnormalities in D-veligers developed from embryos reared at salinities 35 and 32ppt……………………………………….…………………….…………………....…32

**Figure 2.6** Percentage of D-veliger larvae showing abnormalities within different salinities after 48h…………………………………………………………………………..…...…33

**Figure 2.7** Size boxplots of normally developing D-veligers, reared at different salinities for 48h after fertilization for each broodstock feeding ratio treatment………….………..……34

**Figure 3.0** Graphical abstract chapter 3……………………………………..…..……..39

**Figure 3.1** Setup of the aluminium temperature block showing the temperature gradient formed through the circulation of hot and cold water………………………………..……45

**Figure 3.2** Developmental stages observed for *P. zelandica* embryos...........................47

**Figure 3.3** Effect of temperature on early development of *P. zelandica*…………………....52

**Figure 3.4** Mean and maximum number of cleavages at 2-hours post-fertilization at different temperatures of *P. zelandica* embryos……………………………………….……54

**Figure 3.5** Mean percentage of normal early development of *P. zelandica* at different temperatures…………………………………………..…………………………...…55

**Figure 4.0** Graphical abstract chapter 4……….…………………………………...…61

**Figure 4.1** Experimental setup and procedure during the pelagic phase of *P. zelandica*…………………………………………………………………………………67

**Figure 4.2** The concentration of microalgal cells provided in the CUDL's per larva over during the pelagic growth stage…………………………………….………..……..68

**Figure 4.3** Survival of larvae post recovery on 3dpf (left) and shell hell lengths of larvae (right).…………………………....................................................................................71

**Figure 4.4** Survival of larvae post recovery on 5dpf (top). Shell lengths of larvae (bottom)………………………………………………………………...……………..73

**Figure 4.5** Survival of larvae post recovery on 8dpf (top). Shell lengths of larvae (bottom)…………………………..…………………………………………………...…75

**Figure 4.6** Survival of larvae post recovery on 12dpf (top). Shell lengths of larvae (bottom)……………………………………………………………………………...……..77

**Figure section 2** Potential transport route and modes from hatchery……….......……..82

**Figure 5.0** Graphical abstract chapter 5………………………………….……………...83

**Figure 5.1** Experimental design and approach…………………………………….….……90

**Figure 5.2** Percentage of juvenile geoduck siphons visible in holding tanks during recovery in seawater following emersion……………………….…………..……………………..101

**Figure 5.3** The haemocyte, metabolic and osmolality response of juvenile *Panopea zelandica* following emersion…………………………………………………………..…….103

**Figure 5.4** The haemocyte, metabolic and osmolality response of juvenile *Panopea zelandica* following first recovery phase………………………..…………..……………106

**Figure 5.5** The haemocyte, metabolic and osmolality response of juvenile *Panopea zelandica* following the second recovery phase………………………..……………..109

**Figure 5.1S** Plot showing the relationship between wet weight and shell length of juvenile geoducks sampled from the different experimental groups…………………..………122

**Figure 5.2S** Percentage of juvenile geoducks with fully extended siphons during recovery after emersion for each experimental group……………………………….…..…….123

**Figure 5.3S** Percentage geoduck juveniles with siphons visibly opened during recovery after emersion for each experimental group……………………………………………126

**Figure section 3** Outline of growout phase of *P. zelandica*…………………..…..……129

**Figure 6.0** Graphical abstract chapter 6………………………………….……..……….130

**Figure 6.1** Experimental design and outline………………………………..…..………..141

**Figure 6.2** Temperature monitoring during acute thermal stress experiment…………..143

**Figure 6.3** Geoduck behaviour with regards to their position within the sediment……..144

**Figure 6.4** Haemocyte cell types and aggregations observed in the haemolymph of *P. zelandica* stained with Giemsa stain…………………..………………………….…….......145

**Figure 6.5** Percentage of individual haemocyte subpopulation types recorded in *P. zelandica* at different temperatures……………………………..…………………………...…146

**Figure 6.6** Number (top) and size of haemocyte aggregations (bottom) recorded in *Panopea zelandica* at different experimental temperatures…………………..……..……148

**Figure 6.7** The expression of heat shock proteins in *P. zelandica* exposed to 17, 20 and 25°C. ……………………………………………………………………………………..…….150

**Figure 7.0** Linking the components of the thesis together………………..…………..…....157

# Attestation of Authorship

“I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgments), nor material which to substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of learning.”

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## Co-author Contributions

The co-authored literature in this thesis have been broken down into five key components, each of which have been given a weighting to produce the completed output:

Experimental design = 5%
Sample collection and analysis = 10%
Data analysis and interpretation = 20%
Writing = 60%
Reviewing and editing = 5%

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**Chapter 4:** Changes in thermal tolerance and growth due to food availability during larval development of the New Zealand geoduck (*Panopea zelandica*) (Sharma et al., In review)

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| L. N. Zamora | Experimental design<br>Review/edit | 33%<br>25% | 2% | |

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“If I have seen further, it is only by **standing on shoulders of giants**”

Sir Isaac Newton 1676

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# CHAPTER 1 – INTRODUCTION AND THESIS FRAMEWORK

**Figure 1.0.** *Panopea zelandica* juvenile at 6 months old

## 1.1 General Introduction

The New Zealand geoduck (*Panopea zelandica*; Quoy & Gaimard, 1835) is a large sessile clam found in both the North and the South Islands of New Zealand. Geoducks (*Panopea spp.*) dig deep into the seabed, extending their siphons to the surface for feeding and respiration (Oliveira et al., 2011). Their siphons can extend up to four times their body length, which can be up to 80 – 100cm (Goodwin & Peace, 1989). Unlike other bivalves, geoducks have poorly developed abductor and foot muscles. Therefore, geoducks need to maintain their bodies buried to keep their shells closed and are unlikely to rebury if removed from the sediment. Geoducks are also long-lived with annual bands in polished shells showing that *P. zelandica* can live up to 85 years (Gribben & Creese, 2005). A similar species (*P. abrupta*) has been recorded to live up to 168 years (Orensanz et al., 2004). *P. zelandica* is known to occur throughout New Zealand's three main islands (North, South, and Stewart Island) (Powell, 1979) in subtidal sand and mud habitats (Gribben, 2003; Campbell et al., 2004). Ecological studies of known populations indicate that *P. zelandica* occurs mainly in shallow waters (5 – 25m) (Gribben, 2003, Gribben et al., 2004).

From a commercial perspective, geoducks are highly prized in Asian markets. For example, a high quality individual of 8 – 16 cm shell length and a total weight of 300 – 800g can fetch a price of between USD 220 – 330 $kg^{-1}$ in China (Shamshak & King, 2015). Therefore, in recent years this species has received considerable attention from the New Zealand aquaculture industry (Alfaro et al., 2014; Gribben & Heasman, 2015), with a significant projection to contribute to an overall aquaculture export sector of NZ$3 billion by 2030 (MPI 2030).

While this species has excellent potential to achieve a high commercial value, there are several obstacles that need to be overcome before reliable production and markets can

be established. Unlike other aquaculture bivalve species in New Zealand (e.g., Pacific oyster [*Crassostrea gigas*], and Greenshell™ mussel [*Perna canaliculus*]), geoduck spat (seed) cannot currently be sourced from the wild (Gribben & Heasman, 2015). Thus, development of a geoduck aquaculture industry would necessitate a reliable source of suitable quantities of high-quality hatchery-produced spat (Gribben & Heasman, 2015). A challenge associated with spat production of *P. Zelandica*, is that adults are difficult to obtain from the wild and maintain in captivity. In addition, current hatchery protocols and practices, which are based on other *Panopea* species, are not ideal for *P. zelandica*. Therefore, entirely new protocols need to be developed to establish an effective and efficient aquaculture industry for *P. zelandica*.

So far, considerable advances in hatchery production have been made with *P. zelandica* as a target species. These have been primarily focused on broodstock conditioning (Viet et al., 2014, Le et al., 2017), spawning (Le et al., 2018), and larval rearing (Le et al., 2017). In addition to the successes already achieved to produce spat, the development of culture methods that comply with NZ legislation is ongoing, requiring the production of sufficient spat numbers to allow evaluation of potential grow-out methods (King, 2010)

## 1.2 Main research areas

### 1.2.1 Broodstock conditioning and Larval rearing

In hatcheries, there are two main areas of interest when trying to acquire high quality spat. Firstly, for broadcast spawning bivalves, such as geoduck, parental investment is a crucial determinant of larval success in terms of development, growth, and survival (Utting & Millican, 1997). Previous studies have shown that bivalve fecundity is species-specific and can be influenced by diet (i.e., microalgal quantity and quality) (Lubet, 1976; Utting & Millican, 1997). In hatcheries, cultured microalgae are the main source of polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) for cultured bivalves (Viet et al., 2014). With the high diversity in nutritional composition (e.g., levels of lipids and PUFAs) among microalgal species, there is no single species of microalgae that can provide all the nutrients needed for broodstock conditioning during gametogenesis (Gouda et al., 2006; Hemaiswarya et al., 2011). For practical purposes, broodstock conditioning of bivalve species has tended to use microalgal species that are both readily available and have shown some success in supporting gonad development and spawning. One such diet combination is that of *Chaetoceros sp.* (rich in DHA) and *Isochrysis sp.* (rich in EPA), which has been used successfully with geoducks *P. generosa* (Marshall, 2012) and *P. zelandica* (Le et al., 2014) previously.

Secondly, optimizing the conditions during larval development is of crucial importance. Several ecological studies have clearly demonstrated that environmental factors, such as temperature, salinity, and carbon dioxide ($pCO_2$) play an important role in development, growth, and survival of geoducks and other bivalves (Hofmann & Somero,

1995; Doroudi et al., 1999; O'Connor & Lawler, 2004; Parker et al., 2009; Talmage & Gobler, 2011; Tang et al., 2012; Wang & Li, 2018). However, temperature is specifically important since it can directly influence immune responses and physiological processes, which in turn can reduces the overall incubation time of larvae (Hofmann & Somero, 1995; Desrosiers et al., 1996; Parker et al., 2009; Huo et al., 2017). For example, faster developmental rates (27 h instead of 3 days) were observed when *P. japonica* embryos were incubated at 19°C instead of 11°C (Lee & Rho, 1997). Similarly, for *P. zelandica*, Gribben et al. (2003) showed that a faster developmental rate was achieved at 17°C compared to embryos incubated at 15°C by Le et al. (2016). While it may seem beneficial to increase the temperature to maximize developmental rates, there appears to be a thermal threshold for survival and normal development, which is often species-specific. Exceeding temperature thresholds has been reported to cause physiological stress or death in other clam species (Kocovsky & Carline, 2001; Huo et al., 2017). Indeed Huo et al. (2017) found that larvae of *P. japonica* have an upper thermal threshold of 22°C, and larvae are not able to survive above this temperature. In addition, comparisons of thermal scope of activity for *P. zelandica* show that four-month-old juveniles have a narrower thermal optimum (15-19°C) than three-year-old young adults (11-19°C) (Le et al., 2017).

However, subjecting larvae to increased temperatures can have an adverse effect on the overall larval performance and mortality. In response to thermal and other stressors, organisms deploy a suit of mechanisms to repair and protect cells from further damage (Lesser, 2006; Oksala et al., 2014). The upregulation of these stress response mechanisms can be energetically costly (Jeno & Brokordt, 2014). Therefore, food availability also plays a crucial role in the ability for an organism to cope with stressors (Jeno & Brokordt, 2014, Delorme et al., 2020).

Food availability on its own is a common stressor for marine organisms and can have an important effect on the overall larval growth and development. Larvae need a constant supply of exogenous nutrients to undergo metamorphosis. Indeed, reduction in microalgal concentrations have been attributed to delayed metamorphosis and an extended larval stage on other bivalves, such as *Teredo navalis* and *Bankia gouldi* (Mann & Gallager, 1985). Reducing the supply of microalgae (food) can also cause stunted growth and mortalities (De Costa et al., 2012; Bassim et al., 2015). Indeed, increasing the number of microalgal cells in the diet has also been shown to increase larval survival and daily growth rate (shell length) in *P. abrupta* (Marshall et al., 2010) and other bivalve larvae, such as mussels (Pettersen et al., 2010), oysters (Rico-Villaet al., 2006), and scallops (Cerón-Ortiz et al., 2009).

Thus, limitations in nutrition can consequently impact their response to other environmental stressors, such as temperature and pathogen loads (Wagner et al., 2015). Indeed, when subjected to increasing temperatures, the larvae of *Mercenaria mercenaria*, *Crassostrea virginica*, and *Argopecten irradians* have showed decreased growth, and development and increased mortalities (Talmage & Gobler., 2011). In addition, when acute thermal stress was compounded with bacterial stress on *C. gigas* larvae, there was a decrease in shell growth and an increase in mortalities (Liu et al., 2017). Therefore, it is important to understand how stress factors affect the organism in tandem to fully evaluate the overall performance of an organism.

### 1.2.2 Transport of juveniles

The next phase in aquaculture involves transporting spat from hatcheries to potential growout farms. To minimise stress to spat, most hatcheries are located onsite to minimise the time needed to transport spat to growout sites. This does not appear to

be the case with a potential *P. zelandica* hatchery, since at the moment there are no established geoduck hatcheries or known plans to establish one. However, a multi-species hatchery at the Cawthron based in Nelson, New Zealand could provide the initial seed for a new aquaculture venture for this species. An additional production step to consider is the transport of seed or juveniles to grow-out destination, which would involve the animals being removed from the sediment and exposed to air for the duration of the transfer period. Considering that *P. zelandica* is restricted to subtidal habitats and rely on the surrounding sediment to keep their valves closed (due to the absence of a well-developed adductor muscle and disproportionately large tissue-to-shell ratio), it is important to minimise the duration of emersion and disinterment. This potential stress needs to be eliminated or minimised since it is well established that this species does not thrive living out of the sediment (Feldman et al., 2004; Gribben and Heasman, 2015). The use of hatchery-reared spat, however, necessitates the removal of animals from their sediment, warranting a deeper look into the stress associated with transport of *P. zelandica* spat to potential grow-out areas.

Stress associated with transport of other bivalves, such as mussels (Chandurvelan et al., 2013; Zamora et al., 2019; South et al., 2020), oysters (Dharmaraj et al., 1991) and scallops (Christophersen, 2000) has been well documented, with transport stress ultimately being detrimental to the survival of the animal. For example, simulated transport of great scallop (*Pecten maximus*) spat, showed reduced survival with increasing periods of aerial exposure (Christophersen, 2000). Even though adult geoducks (*P. globosa,* and *P. generosa*) are transported live without seawater to markets (local and overseas) (Shamshak & King, 2015), there is lack of information regarding the effects of transport on recovery of juveniles. Apart from stressors experienced during transport, the physiological mechanisms implemented by geoduck

to survive the subsequent replanting process are unknown. Typically stress responses can be assessed by considering molecular, cellular, biochemical, physiological and/or behavioural responses (Waller & Cope, 2019). For instance, the presence of siphon apertures in geoducks indicates active respiration and feeding; this behavioural observation is currently used as an indicator of general stress (Davenport and Wong, 1986). In addition, other tools, such as flow cytometry can be used to provide useful information regarding the overall haemocyte health parameters and immunological state (Rolton & Ragg, 2020). Flow cytometry assays, such as haemocyte cell count, cell viability and oxidative stress assays, have been used to determine cellular stress responses in other molluscan species (Van Nguyen & Alfaro, 2019; Delorme et al., 2021). To date, flow cytometry has been scarcely applied to geoducks, with the exception of haemolymph sampling previously used in *P. globosa* to characterise haemocyte cell types (Hernández-Méndez et al., 2020). Haemolymph can also be used to track osmolality (electrolyte–water balance), resulting in an indication of the geoducks' capacity to cope with changes in the external environment (Byrne et al., 1989; McFarland et al., 2013). In addition to haemolymph assessments, different geoduck tissues have also been used to investigate physiological changes, such as sex identification through specific enzyme-linked immunosorbent assays (Kim et al., 2018), transcriptomic analyses (Juárez et al., 2018), proteomics analyses (Timmins-Schiffman et al., 2020) and now metabolomics analyses. By assessing metabolites, valuable insights into the biochemistry of physiological processes are likely to be gained (Want et al., 2013), as seen in scallops (*P. maximus*) where carbohydrate and glycogen levels were decreased with an increase in emersion time (Fleury et al., 1996; Maguire et al., 1999) and increases in octopine content were documented under oxygen limited conditions (MacDonald et al., 2006).

### 1.2.3 Geoduck field growout

The grow-out phase of the geoduck farming cycle typically starts when clams are out planted into sediments and spans until the animals reach market size (Vanblaricom et al., 2015). One of the major challenges faced in bivalve aquaculture is the recurrence of mass mortalities, which reduce production and cause high economic costs (Soon & Ransagan, 2019). In general, mortalities can be the result of both physicochemical and abiotic factors, such as big waves (Thórarindsóttir et al., 2009), high silt load (Mouthon, 2001), fluctuations in salinity (Levinton et al., 2011; Pollack et al., 2011; Munroe et al., 2013), temperature fluctuations (Brown et al., 2008; Rodrigues et al., 2015; Ortega et al., 2016; Rolton et al., 2022), oxygen depletion (Ilarri et al., 2011), food availability (Soon et al., 2016), predation (McKindsey et al., 2007), and toxic compounds (Ramli et al., 2013; Simoes et al., 2015). All the previously mentioned variables would potentially need to be evaluated as the aquaculture for *P. zelandica* gets established.

However, the aquaculture industry could face its biggest challenge due to the current global climate change crisis. Reports have confirmed that oceans around some regions in New Zealand are warming, and that climate extremes, such as marine heatwaves are expected to become more intense and longer compared to the present day (Behrens et al., 2022; Stevens et al., 2022). Events, such as marine heatwaves have significant implications for aquaculture and fisheries in New Zealand (Burgh-Day et al., 2022). Increased temperatures and temperature anomalies affect the metabolic and physiological responses of commercially important fish and shellfish species (Rolton et al., 2022). However, the effects of sudden temperature fluctuations on the endemic geoduck clam *P. zelandica*, are largely unknown.

Due to the current increase in global sea temperatures and the increased chances of marine heatwaves continuing in the future, organismal responses to temperature changes remain an important factor for research consideration. Indeed, burrowing bivalves exposed to temperatures above their optimal ranges tend to exhibit lower burrowing capacity, decreased feeding and growth rates, and increased rates of mortality (Macho et al., 2016). A common behavioural response to thermal stress in bivalves includes valve closure and/or increased borrowing (Sakurai et al., 1996, Domínguez et al., 2021). Even though valve closure provides an escape from temperature extremes as seen in mussels, *Limnoperna fortune* (Andrade et al., 2018), valve closure is not an option for geoducks as these bivalves are unable to fully close their shells. Considering that sediment at depth is cooler than the surface layers, moving deeper within the sediment may provide animals an escape from temperature stress (Macho et al., 2016). Consequently, Macho et al. (2016) documented the change in subsurface burrowing frequency and siphon activity as a useful measure of stress in clams.

As pointed out by Hernández-Méndez et al. (2020), haemocyte assessment allows one to detect the impact of environmental changes on animal health, which can be used as a valuable measure of physiological response to stressors. Elevated temperatures have also been shown to affect clam haemocyte parameters, such as cell viability, adhesive capacity, and cell types (Monari et al., 2007; Pérez–Velasco et al., 2022). Haemocytes constitute the cellular component of haemolymph, which moves through the circulatory system, migrating to locations, such as connective tissue and epithelia (Hine, 1999). Apart from their main function in host defense mechanisms, bivalve haemocytes partake

in physiological functions, such as nutrient digestion, transportation, and distribution, wound healing, detoxification, shell mineralisation and excretion (de la Ballina et al., 2022). Bivalve haemocytes vary in size and abundance but are generally made up of three main cell types, which include small hyalinocytes, large hyalinocytes, and granulocytes, as demonstrated in *P. globosa* (Hernández-Méndez et al., 2020).

At the molecular level, cells react to heat stress by upregulating some specific genes, such as chaperone proteins and those involved in cell stress defence mechanisms (Lindquist & Craig, 1988; Feder & Hofmann, 1999). Typically, heat shock proteins (HSPs) play on important role in protein folding and biosynthesis, with HSP70 controlling cell homeostasis, proliferation, differentiation, and cell death, while HSP90 is involved in immune regulation, signal transduction, and cell cycle regulation (Masanja et al., 2022; Xu et al., 2022). In a previous study on *P. generosa*, heat shock proteins were increased in larvae following exposure to ciliates as a typical stress response (Timmins-Schiffman et al., 2020). Targeted proteomics were used to investigate pH variations in *P. generosa*, providing a peptide database for quantifying multiple proteins simultaneously (Spencer et al., 2019). Ultimately, HSPs play a key role in controlling protein homeostasis and are among the main indicators of stress-induced protein damage (Tomanek, 2008).

Assessing the effects of elevated temperatures on biological parameters, such as health, growth, and nutrition is important for understanding and predicting resilience (Ewere et al., 2021). Temperature has been the focus of several geoduck studies. For example, *P. globosa*, had increased metabolic rates to temperatures of 29°C (Juarez et al., 2018). Whereas in *P. generosa*, 19°C was deemed as a temperature resulting in significant growth rates (Arney et al., 2015). On the other hand, at 19°C, both *P.*

*zelandica* (Le et al., 2017) and *P. japonica* (Nam et al., 2015) had reduced aerobic scope and increased mortalities, respectively. However, acute temperature stress responses in geoduck are poorly described. Covering this knowledge gap could have important implications on the establishment *P. zelandica* growout farms.

## 1.3 Thesis Aims

The overall aim of this thesis is to identify conditions and practices to establish *P. zelandica* in the New Zealand aquaculture industry. The main areas of focus were based around potential bottlenecks from hatchery culture to growout farms of *P. zelandica*. These findings provide insights into growth and development of *P. zelandica* over its lifecycle. Overall, the thesis looks at three main areas of geoduck growth and development:

1. Broodstock conditioning, embryonic, and larval development of *P. zelandica* in hatcheries.
2. Transport of *P. zelandica* to potential growout locations.
3. Effects of heatwaves in growout fields.

## 1.4 Thesis structure

To achieve these objectives of this thesis, 5 separate experiments conducted throughout the lifecycle of *P. zelandica*. Chapters 2 to 6 were written as original papers. These case studies, in combination with the introduction and discussion comprise 7 chapters of this thesis.

The general introduction sets the context and rationale for this thesis, and it provides a general literature review laid out (chapter 1). Chapter 2 & 3 focuses research on the first 48h of embryonic development. With respect to transgenerational effects broodstock conditioning on different diet ratios, chapter 2 takes a closer look at the development of embryos exposed to varying degrees of salinity. Chapter 3 focuses on optimizing the speed of development by rearing embryos at a range of temperatures. Thermal tolerance and growth of pelagic larvae of *P. zelandica* under varying degrees of food availability was investigated in chapter 4. Stressors encountered during transport from hatcheries and recovery in potential growout farms was simulated in chapter 5. The metabolic profiles in the gills and tissues were studied in detail during transport period and recovery. The next experimental chapter (chapter 6) explores the effect of thermal stress as experienced through marine heatwaves on *P. zelandica* and provides potential considerations for growout fields. Chapter 6 also takes a closer look at the overall behavioral responses and the expression of heat shock proteins (HSP70 & HSP90) within the gill tissue and haemolymph in order to understand the effects of sudden temperature increases. The general discussion (chapter 7) brings the thesis research together and provides an in-depth analysis of the implications of the findings as well as the potential development and future directions for geoduck aquaculture in New Zealand.

# SECTION 1: BROODSTOCK CONDITIONING AND LARVAL REARING

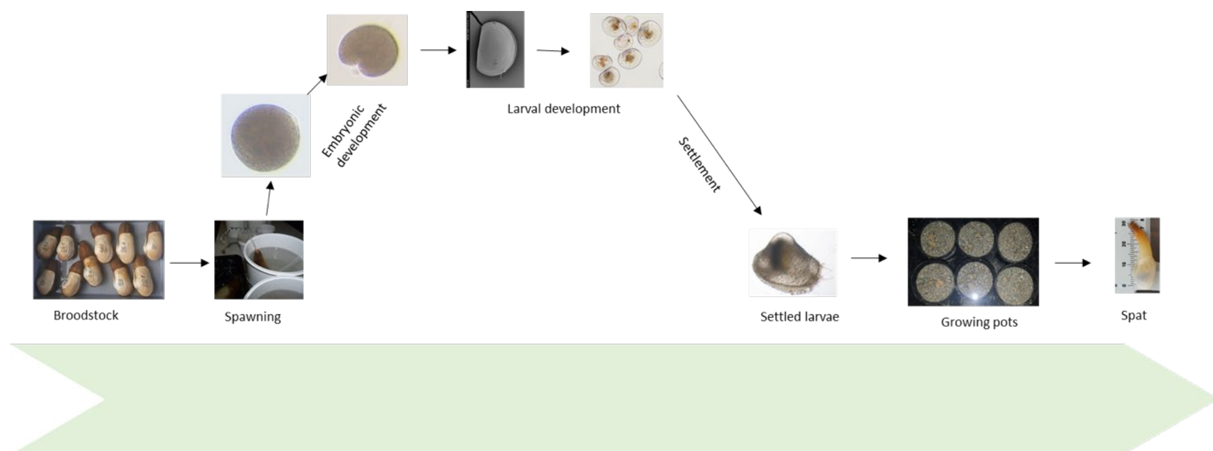


**Figure section 1.** Hatchery culture of *Panopea zelandica*.

# CHAPTER 2 – CARRY - OVER EFFECTS OF BROODSTOCK CONDITIONING ON THE SALINITY TOLERANCE OF GEODUCK CLAM (*PANOPEA ZELANDICA*) EMBRYOS

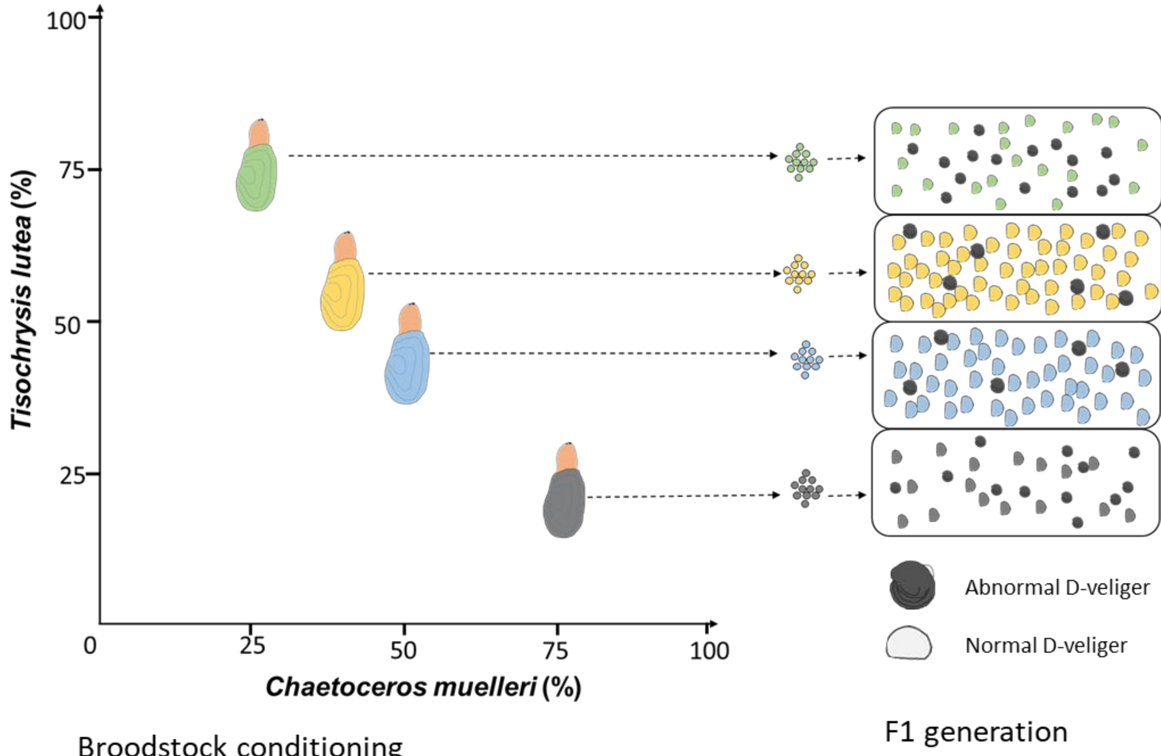


**Figure 2.0** Graphical abstract

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This chapter has been accepted as:

Sharma, S. S., Alfaro, A. C., Ragg, N. L., & Zamora, L. N. (accepted). Carry-over effects of broodstock conditioning on the salinity tolerance of the New Zealand geoduck (*Panopea zelandica*). *Aquaculture Research*.

# Abstract

The New Zealand geoduck (*Panopea zelandica*) has seen considerable interest from the NZ aquaculture industry. A major bottleneck in culturing *P. zelandica* is early life stages mortality (e.g., embryo). Therefore, in this study, we investigated the embryonic performance and their transition to the first feeding larval stage (D-veliger) under different salinities (26, 30, 32, and 35ppt) of four different offspring groups generated from broodstock being fed different ratios (25:75, 50:50, 60:40, and 75:25) of the haptophyte *Tisochrysis lutea* (formerly *Isochrysis galbana*) [ISO] and the diatom *Chaetoceros muelleri* [CM] during gametogenesis. Broodstock within all diet ratio treatments successfully conditioned, producing viable embryos. Average egg size ranged between 75 - 80µm and were not affected by the diet ratios of the broodstock. Survival 48h post fertilization, D-veliger larvae yield, and incidence of abnormalities depended on both the embryo rearing salinity and broodstock feeding ratios. The combined salinity of 32-35ppt and a feeding ratio of 50:50 and 60:40 (ISO:CM) had the highest survival of embryos (56.0 – 77.5%), highest production of D-veliger larvae (> 65%), and lowest incidence of abnormalities within D-Veliger (< 47%). The size of the larvae decreased with decreasing salinities, with the largest found at 35ppt (101.22 ± 0.49µm in shell length). Embryos and larvae did not survive at salinity 26ppt. These results suggest that diet during gametogenesis can play a role on the offspring ability to cope with environmental stressors at least during the critical first few days after fertilization. These findings provide important information on transgenerational effects due to broodstock diet especially during the early life stages.

## 2.1 Introduction

The New Zealand geoduck (*Panopea zelandica*; Quoy & Gaimard, 1835) is a large sessile clam found in both the North and the South Islands of New Zealand. This species has received considerable attention from the New Zealand aquaculture industry (Alfaro, Jeffs, & King, 2014; Gribben & Heasman, 2015), with a significant projection to contribute to an overall aquaculture export sector of NZ$3 billion by 2035 (Stenton-Dozey, Heath, Ren, & Zamora, 2021).

While this species has excellent potential to achieve a high commercial value, there are several obstacles that need to be overcome before reliable production and markets can be established. Unlike other aquaculture bivalve species in New Zealand (e.g., Pacific oyster [*Crassostrea gigas*], Greenshell™ mussel [*Perna canaliculus*]), geoduck spat (seed) cannot currently be sourced from the wild (Gribben & Heasman, 2015). Thus, development of a geoduck aquaculture industry would necessitate a reliable source of suitable quantities of high-quality hatchery-produced spat (Gribben & Heasman, 2015). A challenge associated with spat production of *P. zelandica*, is that adults are difficult to obtain from the wild and maintain in captivity. In addition, current hatchery protocols and practices which are based on other *Panopea* species, are not ideal for *P. zelandica*. Therefore, new protocols are being developed to establish an effective and efficient aquaculture industry for *P. zelandica*.

In general, for broadcast spawning bivalves, such as geoduck, parental investment (i.e., gamete quality) is a crucial determinant of larval success in terms of development, growth, and survival. Previous studies have shown that bivalve fecundity is species-specific and can be influenced by diet (i.e., microalgal quantity and quality) (Lubet, 1976; Utting & Millican, 1997). In hatcheries, cultured microalgae are the main source of

polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) for cultured bivalves (Li et al., 2014). With the high diversity in nutritional composition (e.g., levels of lipids and PUFAs) among microalgal species, there is no single species of microalgae that can provide all the nutrients needed for broodstock conditioning during gametogenesis (Gouda, Kenchington, Hatcher, & Vercaemer, 2006; Hemaiswarya, Raja, Ravi Kumar, Ganesan, & Anbazhagan, 2011). For practical purposes, broodstock conditioning of bivalve species has tended to use microalgal species that are both readily available and have shown some success in supporting gonad development and spawning. One such diet combination is that of *Chaetoceros sp.* (rich in DHA) and *Isochrysis sp.* (rich in EPA), which has been used successfully with geoducks *P. generosa* (Marshall 2012) and *P. zelandica* (Le et al., 2014) previously.

However, even when broodstock successfully spawn, the development of embryos and larvae might not be "optimal". Indeed, the energy required during early embryonic development is fully dependant on the resources provided for in the egg (Waters, Lindsay, & Costello, 2016). Also, the development of embryos into the first feeding larvae (i.e., D-veliger) is a process of intense cellular activity during which any impairment within series of biochemical and physiological mechanisms can result in malformed larvae (Leverett & Thain, 2013). Thus, broodstock history tends to have a significant effect beyond gametogenesis and into embryonic development (Waters, Lindsay, & Costello, 2016). During development, embryos are more sensitive to environmental stressors compared to the juvenile and adult stages, especially with regards to salinity and temperature (Bayne & Bayne, 1976; Chaparro, Cubillos, Montiel, Paschke, & Pechenik, 2008). Indeed, even minor deviations from optimal temperature ranges can cause reduced developmental rates and increased abnormalities of

embryos and larvae of *P. zelandica* (Sharma et al., 2020). As for salinity, pacific geoduck (*P. japonica*) embryos have a low salinity tolerance with both embryos and larvae unable to survive at salinities below 26ppt (Huo et al., 2017). High salinity susceptibility was also seen in scallop (*Argopecten irradians irradians*) embryos, which have a narrow salinity range for normal development (Tettelbach & Rhodes, 1981). There is little to no information about the optimal salinity range for development of *P. zelandica* embryos. In addition, it is likely that *P. zelandica* may have a distinct salinity tolerance range since, unlike other *Panopea* species which have a broader depth distribution range extending into the intertidal zone, for known populations of *P. zelandica* is found subtidally from 5 to atleast 25m (Gribben, 2004).

The present study was therefore designed to assess the efficacy of a range of practical microalgal mix ratios in the conditioning of geoduck broodstock through carry-over effects of their offspring (i.e., embryos and larvae), in terms of survival, D-veliger metamorphosis, incidence of abnormalities, and size of D-veligers after exposure to four different salinity regimes.

## 2.2 Methods and Materials

### 2.2.1 Parental broodstock and source of gametes

Broodstock *Panopea zelandica* (105 – 130mm shell length, 500-800g wet weight) were collected from Golden Bay (South Island, New Zealand) and were conditioned at the Cawthron Institute's Aquaculture Park (Nelson, New Zealand) as a part of an ongoing geoduck research program. Briefly, four groups of 10 geoducks (total of 40 animals) were housed in 100L tanks connected to a flow through seawater system (Figure 1a blue lines). Each broodstock group was fed one of four different microalgal diet ratios, mixing the haptophyte *Tisochrysis lutea* (formerly *Isochrysis galbana*; "ISO") and the diatom *Chaetoceros muelleri* ("CM") (i.e., 25%:75% = treatment code BSFR1, 50%:50% = BSFR2, 60%:40% = BSFR3, 75%:25% = BSFR4, ISO:CM proportions; Figure 1a green lines). The concentration of microalgal cells in the feeding tanks was kept constant by keeping the outflow cell concentration at 40cells/µl. All geoducks were fed for a period of 90 days, at which point all animals were induced to spawn, providing the gametes necessary to evaluate salinity tolerance in their offspring during embryonic development (i.e., 48h after fertilization).

### 2.2.2 Spawning and fertilization

All broodstock were induced to spawn by adding an excess of microalgae directly into the holding tanks following current best practice available for this species. Once the animals began to spawn, they were separated and placed in individual 4L containers

(0.3x0.3x0.45m). This was done to ensure that premature fertilization did not occur. The number of spawned females and males in each feeding treatment were: 1 female and 3 males BSFR1; 3 females and 2 males BSFR2; 2 females and 3 males BSFR3; and 2 females and 2 males BSFR4. The collected eggs were checked for shape and size, whereas sperm were checked for concentration and motility. All the gametes from all the individuals appeared normal and none were rejected. All gametes were then stored at 4°C. The females that spawned within each feeding treatment had their eggs pooled together after being checked. The sperm from all males across feeding treatments were pooled to standardise paternal effects. The eggs from individual treatments were suspended in 1,000mL beakers of filtered seawater and fertilized with sperm at a concentration of 500:1 (sperm:egg ratio), following previous observations of high fertilization ratios and low polyspermy (Le et al., 2016). Eggs and sperm were left to fertilize for 30min, at which point the excess of sperm was washed away through a 43µm mesh with FSW (Figure 2.1b). The fertilized eggs were concentrated in 1L beakers and adjusted to get a final concentration of 2,000 fertilized eggs $mL^{-1}$. Fertilization success was measured by observing the formation of polar bodies under an Olympus Omax compound microscope at 20x magnification.

### 2.2.3 Embryo challenge setup and procedure

The challenge experiment was designed to evaluate embryonic development (obtained from the four different broodstock groups) under four different salinities in tissue culture dishes (TCD's). The salinities selected were 26, 30, 32, 35ppt and were created by adding different volumes of reverse osmosis (RO) filtered freshwater to 1µm filtered UV treated seawater (Table 2.1) at 15°C. Each broodstock group and salinity combination

(4 x 4) had six 4mL TCD wells, resulting in a total of 96 wells used for evaluation (Figure 2.1c).

Each TCD well had 3.9mL of the salinity solution to which 0.1mL of the concentrated fertilized eggs from each broodstock group was added, giving a final volume of 4mL with 200 embryos in each well (Figure 2.1d). Embryos were then incubated at 17°C in a humidity-controlled environment for 48h.

**Table 2.1**. Salinity treatment preparation

| Seawater (mL) 35ppt | | RO Freshwater (mL) 0ppt | Final volume (mL) and salinity |
|---|---|---|---|
| 1000 | + | 0 | 1000 (35ppt) |
| 906.25 | + | 93.75 | 1000 (32ppt) |
| 833.33 | + | 166.67 | 1000 (30ppt) |
| 653.85 | + | 346.15 | 1000 (26ppt) |

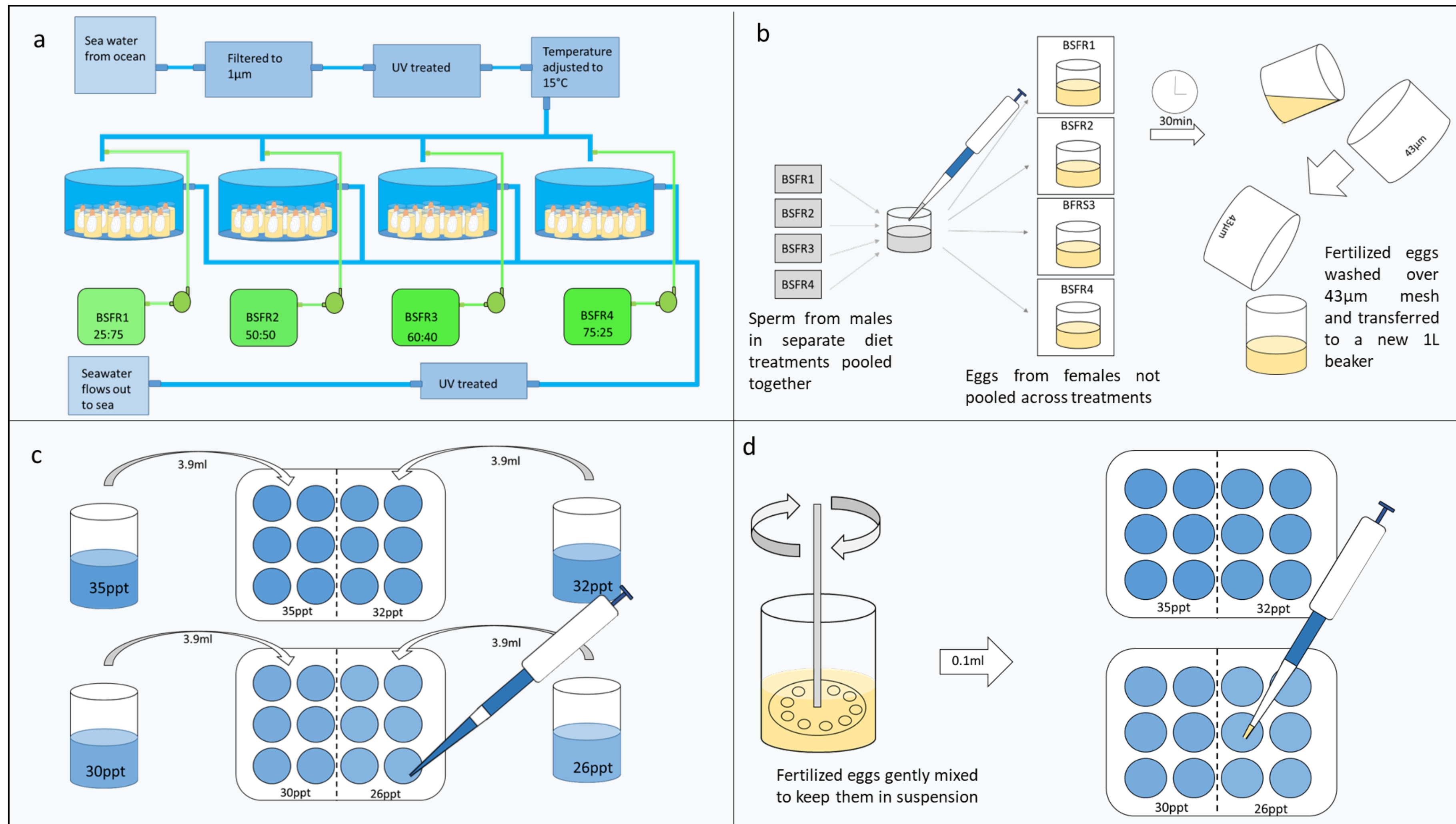


**Figure 2.1.** Experimental design and methods. (a). Flow-through system (blue) connected to the geoduck broodstock housing tanks showing the pathway of seawater. Each housing tank connected to its own algal tank with a pneumatic pump (green lines) that pumped pre-mixed concentrations into the housing tanks. (b). Spawning and fertilization procedure. (c). Making of the salinities and setting up the TCD for the fertilized eggs. (d). Transfer of embryos into TCDs (**Note:** this was repeated for all broodstock feeding ratio treatments).

### 2.2.4 Sampling procedure

After 48h, 0.5mL 4% formalin was added to each TCD well to fix the embryos and larvae, which then progressively sank to the bottom of each TCD well. After 10min, the embryos and/or larvae were observed under an Olympus Omax compound microscope at 10x magnification. All the samples were observed/processed within 24hrs and preserved in 70% ethanol for analysis under a scanning electron microscope. There were four different parameters evaluated after 48h including: 1 – survival; 2 – total proportion of D-larvae present (abnormal and normal); 3 - proportion of abnormally developing D-larvae; and 4 – size of normally developing D-veligers.

Survival, which included embryos, trochophores, and D-veligers was measured by counting the individuals that were visible, regardless of developmental stage and abnormalities, in each TCD well for each feeding ratio and salinity treatment. Different early development stages were identified by descriptions provided by Le et al., (2016) and by Sharma et al. (2020). Survival percentages were calculated using the formula given below.

$$Survival\ \% = \frac{total\ number\ of\ embryos\ and\ larvae\ visible}{200\ (total\ number\ of\ fertilized\ eggs\ initially\ added)}\ X\ 100$$

The D-veliger stage was chosen as an endpoint as it is a transition from a non-feeding phase to a feeding larval state. The proportion of normally developed D-veliger larvae was estimated as follows:

$$D - veliger\ \% = \frac{Number\ of\ normally\ developed\ D\ larvae}{200(total\ number\ of\ fertilized\ eggs\ initially\ added)}$$

Incidence of abnormalities in D-veligers and the fine-scale nature of these abnormalities were determined under a scanning electron microscope. Abnormalities were recorded

as any deformity in the shell structure of the D-veligers (Figure 5). The percent incidence of abnormality at this stage was then calculated using the formula given below.

$$Abnormality\ \% = \frac{number\ of\ abnormally\ developed\ D{-}veligers}{Total\ number\ of\ D{-}veligers}\ X\ 100$$

Size of D-veligers was recorded during observations under the SEM. This was done by taking the average measurement for 50 normally developing D-veligers for each larval salinity treatment combination. The D-veligers were measured along the longest axis of the shell.

### 2.2.5 Scanning electron microscopy (SEM)

Preserved D - veligers were prepared following method described by Let et al., (2017), washed with phosphate buffer (138 mM NaCl, 2.7 nM KCL, 10 nM$Na_2HPO_4$, 1.8 mM $KH_2PO_4$; pH = 7.4) for 5min, then rinsed with 1min with deionised water. D – veligers were then dehydrated through an ascending series of analytical grade ethanol 50, 60, 70, 80 90, and 100% for 15min each. After dehydration, samples were soaked in 98% chloroform for 30 seconds, and then dried for 12h in a desiccator. Dried samples were placed on adhesive carbon disks and mounted on aluminium stubs. Samples were then sputter coated with carbon for 40 seconds using an ion sputter coater (Hitachi E-1045) and then imaged via SEM at 5.0kV.

Abnormalities in D-veligers were characterised based on observations by His et al., 1997; Saidov & Kosevich (2021) and Lasota et al., (2018). It is important to note that not all abnormally developing D-veligers had a singular abnormality and thus, during characterisation, larvae were grouped based on the most prominent abnormality type.

### 2.2.6 Statistical analysis

Egg sizes were analyzed using One-Way ANOVA, comparing the effects of Broodstock feeding ratio as a factor (four levels: BSFR1, BSFR2, BSFR3, and BSFR4), and egg diameter (µm) as the dependent variable.

Survival, proportion of population attaining D- stage, incidence of abnormalities, and size of D-veligers were analyzed using Two-Way ANOVAs, with Broodstock feeding ratio (four levels: BSFR1, BSFR2, BSFR3, and BSFR4) and survival, proportion of embryos metamorphosing on to D - veligers, incidence of abnormalities within D – veligers, and size of normally developing D-veligers reared in different salinities (four levels: 26, 30, 32, and 35ppt) as dependent variables. All data were checked for normality and homoscedasticity using the Shapiro-Wilk and Levene's tests, respectively (Queen et al., 2002). Survival, proportion of population attaining D- stage, incidence of abnormalities, and size of D-veligers were square root transformed to meet assumptions of normaility and homogeneity of variances.

For all analyses, Tukey pairwise comparisons were used to examine the significant differences among the factor levels. All statistical analyses were conducted using the R Studio software R-4.1.0; and the significance was taken at $\alpha = 0.05$.

# 2.3 Results

## 2.3.1 Egg size

Egg sizes did not differ (ANOVA, $p$ = 0.89) among broodstock feeding treatments (Figure 2.2). All the eggs obtained ranged between 75-80μm in diameter, which was within the normal variation described by Le et al., (2016).

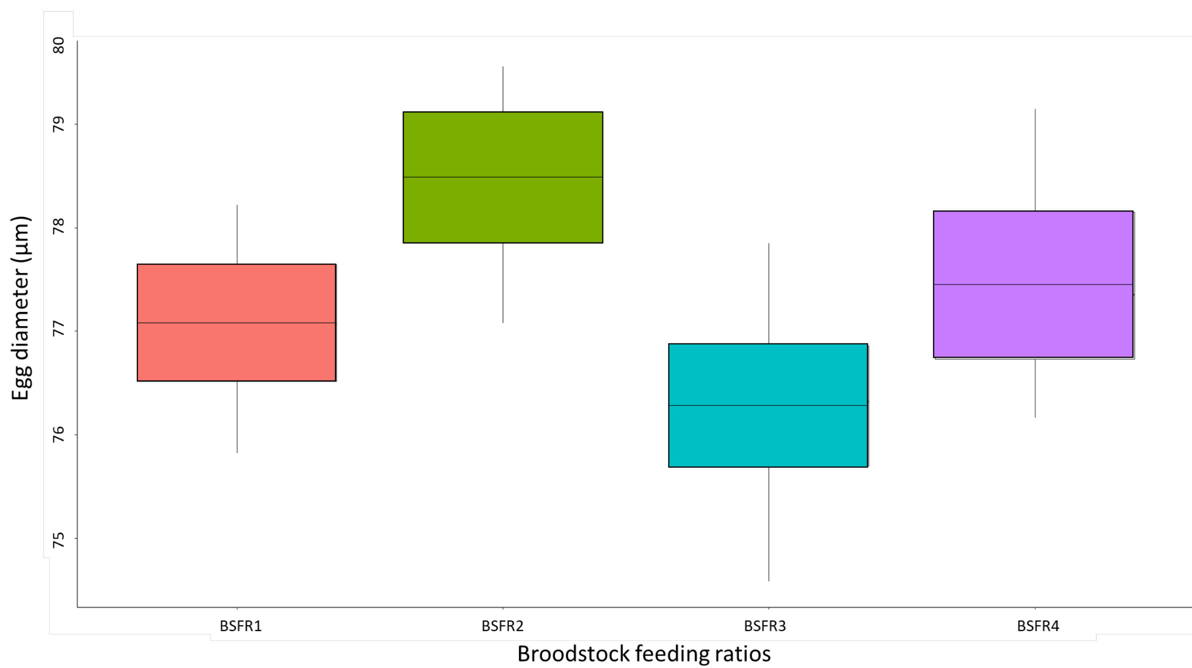


**Figure 2.2** Egg diameter boxplots obtained from spawned females in feeding treatments, including *Tisochrysis lutea* and *Chaetoceros muelleri* at cell ratios of 25:75 (BSFR1), 50:50 (BSFR2), 60:40 (BSFR3) and 75:25 (BSFR4) treatment; (n=50 eggs for each group).

### 2.3.2 Embryo and larval survival

Survival 48h after fertilization was high in all broodstock conditioning treatments when embryos were raised at 32 and 35ppt salinities (56.0 - 75.7%; Figure 2.3), but overall survival at 30ppt was below 30% regardless of broodstock feeding ratio. There were no survivors at salinity 26 ppt.

Significant differences in survival were based on both broodstock feeding ratios (two-way ANOVA, $F_{(3,80)} = 22.80$, $p < 0.001$) and rearing salinities (two-way ANOVA, $F_{(3,80)} = 1050$, $p < 0.001$). There was also a significant (two-way ANOVA, $F_{(9,80)} = 6.26$, $p < 0.001$) interaction between broodstock feeding ratio and rearing salinity.

Overall, mean survival in different salinities showed no significant differences between 32 and 35ppt salinity exposures. The survival from different broodstock feeding treatments did, however, differ significantly within salinity exposure, with BSFR2 and BSFR3 treatments tending to support better survival (Figure 2.2) in salinities 30ppt and over.

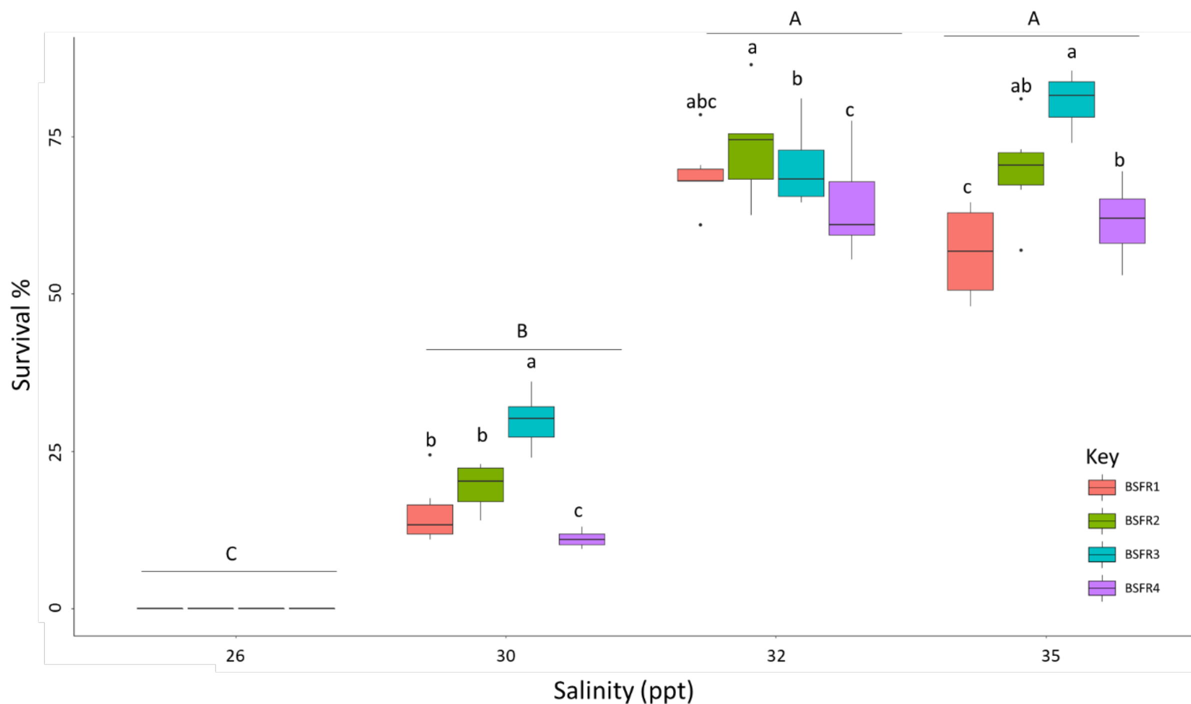


**Figure 2.3.** Survival boxplots at different salinities 48h post-fertilization for each broodstock feeding ratio treatment. **Note**: letters above boxplots (uppercase – salinity; lowercase – individual diet treatments) denote significant differences (two-way ANOVA, Tukey $p > 0.05$). Broodstock feeding treatments, including *Tisochrysis lutea* and *Chaetoceros muelleri* at cell ratios of 25:75 (BSFR1), 50:50 (BSFR2), 60:40 (BSFR3) and 75:25 (BSFR4).

## 2.3.3 Development to D - veligers

After 48h post fertilization, more than 65% of the surviving embryos in salinities 35 and 32ppt had transitioned into D-veligers (Figure 2.4). The transition of D-veligers in the 30ppt salinity was below 30% with no D – veligers observed at 26ppt.

The percentage of individuals transitioning to D-veligers was significantly different among broodstock feeding treatments (two-way ANOVA, $F_{(3,80)} = 11.1$, $p = 0.001$). There was also a significant difference in the transition to D – veligers among embryos reared in different salinities (two-way ANOVA, $F_{(3,80)} = 7366.6$, $p = 0.001$), and a significant interaction between both broodstock feeding and embryo rearing salinities (two-way ANOVA, $F_{(9,80)} = 36.3$, $p = 0.001$).

Overall, embryos obtained from BSFR2 treatment had a higher percentage of transition into D-veligers when compared to larvae obtained from the other treatments (Figure 3).

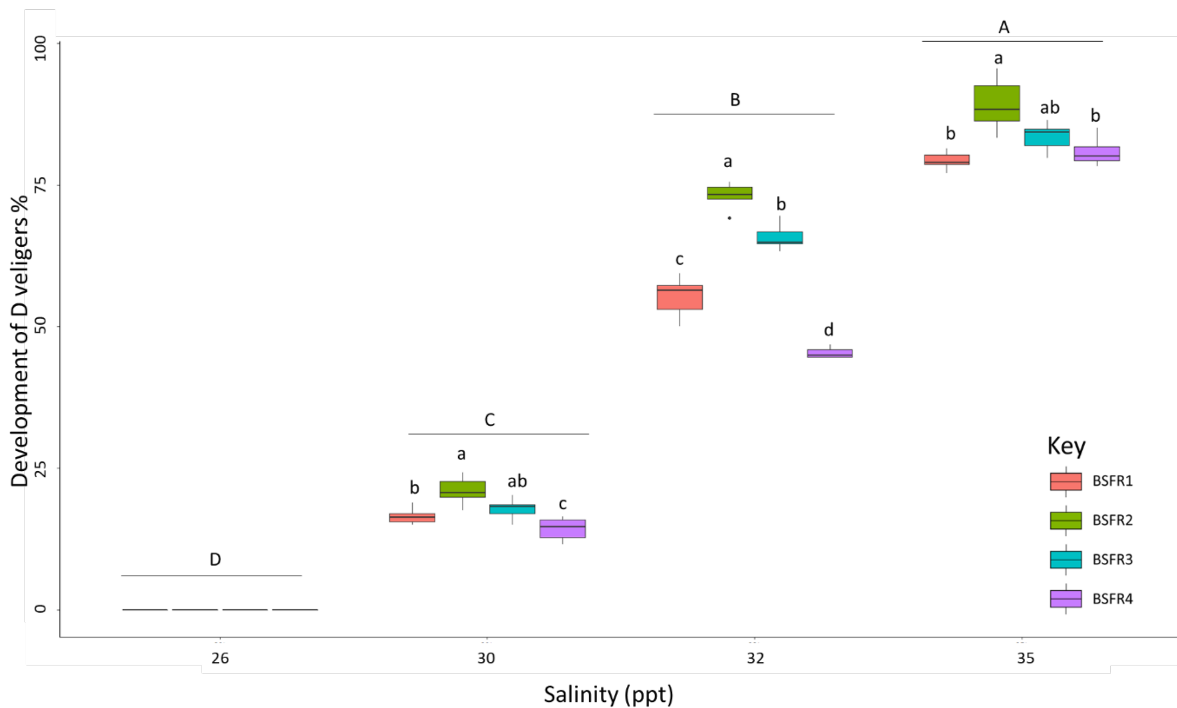


**Figure 2.4.** Proportion boxplots of normally developed D-veligers reared at different salinities for 48h after fertilization for each broodstock feeding ratio treatment. Note: Boxplots with different letters denote significant differences (two-way ANOVA, Tukey $p < 0.05$) (uppercase – between salinity treatments; lowercase – within each salinity treatment), n=200 individuals for each treatment. Broodstock feeding treatments, including *Tisochrysis lutea* and *Chaetoceros muelleri* at cell ratios of 25:75 (BSFR1), 50:50 (BSFR2), 60:40 (BSFR3) and 75:25 (BSFR4).

## 2.3.4 Incidence of abnormal D-veliger development

The overall morphological abnormalities ranged from minor deformations on the D-veliger to total distortion of the shell (Figure 2.5). Minor abnormalities included deformities on the D-veliger shell, such as indentations and minor ripples on the surface of the shell (Figure 2.5b), in and around shell edge/margins (Figure 2.5c), and hinges causing concavity of the shell hinge (Figure 2.5d). Major deformities included apparent helical whirling of the D-veliger shell (Figure 2.5e) and eversion of the soft tissues.

Incidence of abnormally developing D-veligers was significantly affected by both broodstock diet (two-way ANOVA, $F_{(3,80)} = 46.85$, $p < 0.001$) and embryo rearing salinity (two-way ANOVA, $F_{(3,80)} = 1631.28$, $p < 0.001$). There was also a significant interaction between broodstock feeding and rearing salinity of the embryos (two-way ANOVA, $F_{(9,80)} = 10.38$, $p < 0.001$).

The incidence of D-veliger abnormalities increased with decreasing salinity exposure. The percentage of abnormally developing D-veligers was between 4 – 11% at 35ppt, 15 – 47% at 32ppt, and 37 – 55% at 30ppt (Figure 2.6). There were no larvae observed at the lowest salinity of 26ppt.

Overall, regardless of the rearing salinity, the incidence of abnormalities within the D-veligers was lower in the BSFR2 (50:50) and BSFR3 (60:40) treatments (Figure 2.6).

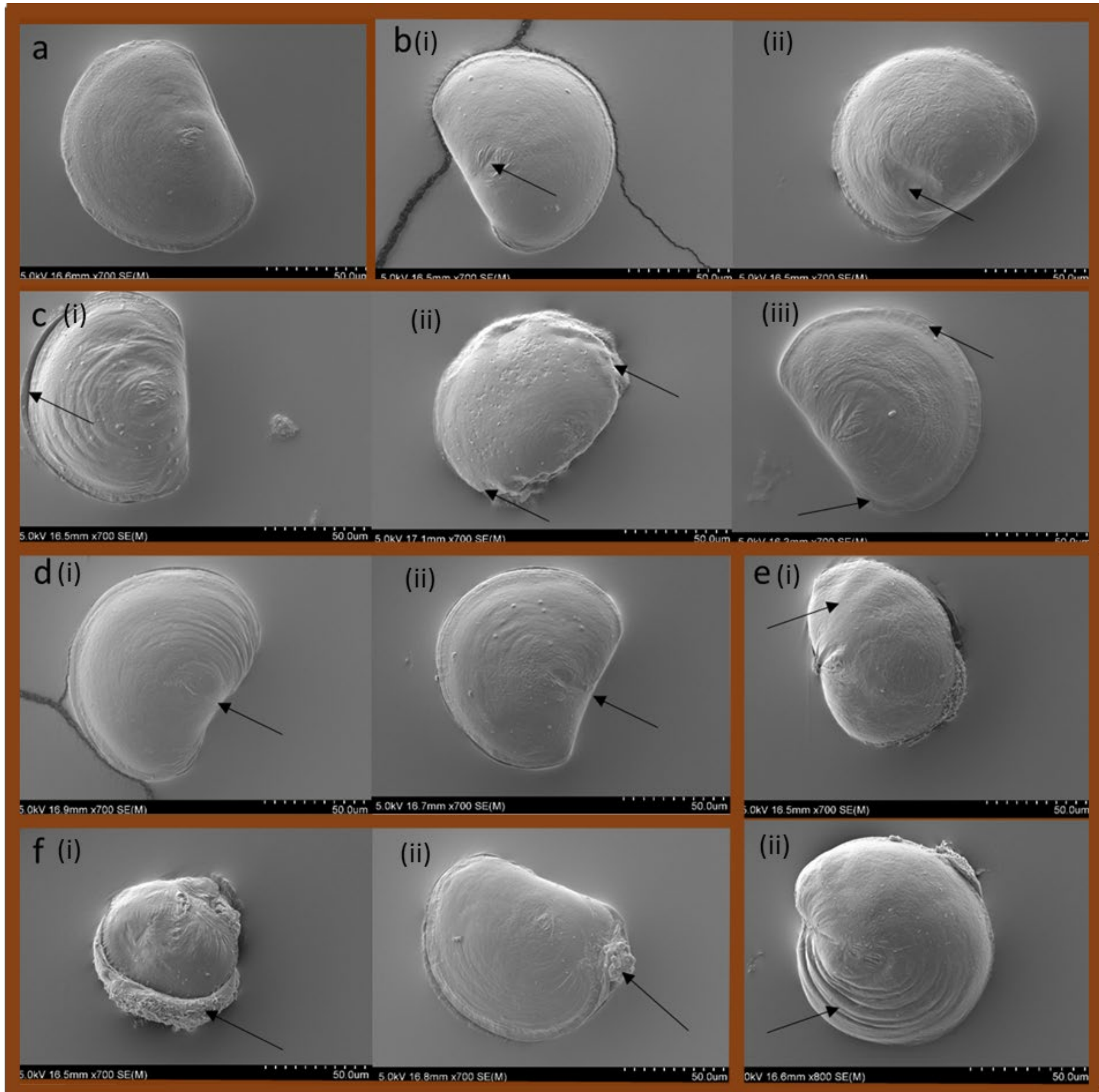


**Figure 2.6**. Abnormalities identified in D-veligers developed from embryos reared at 35 and 32ppt salinity. (**a)** Normally developing D-veliger for reference. Abnormalities included (**b) (i-ii)** Indentation of the shell—exemplified in two individuals, (**c) (i-iii)** developmental issues around the shell margins – exemplified in three individuals, (**d) (i-ii)** concaving of the D-veliger shell hinge —exemplified in two individuals, (**e) (i-ii)**helical deformation of the shell —exemplified in two individuals, and (**f) (i-ii)** eversion of soft tissues — exemplified in two individuals. Black arrows indicate the locations of shell distortions.

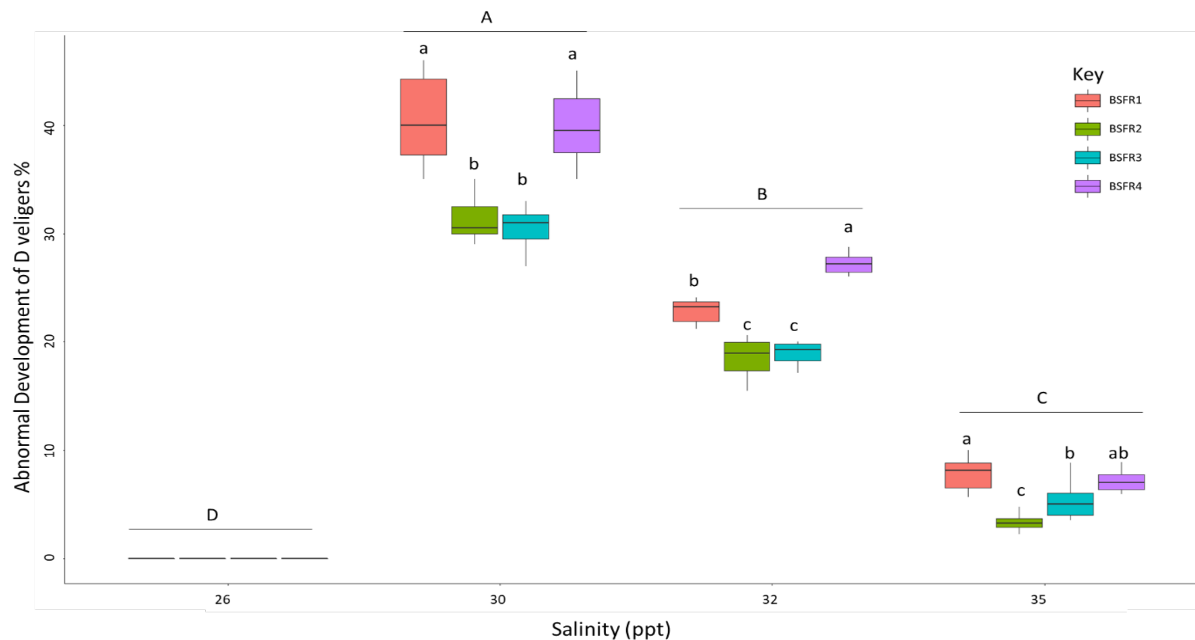


**Figure 2.6.** Percentage boxplots of D-veliger larvae showing abnormalities, reared at different salinities for 48h after fertilization for each broodstock feeding ratio treatment. Note: Boxplots with different letters denote significant differences (two-way ANOVA, Tukey $p$ < 0.05) (uppercase – between salinity treatments; lowercase – within each salinity treatment), n = 200. Broodstock feeding treatments, including *Tisochrysis lutea* and *Chaetoceros muelleri* at cell ratios of 25:75 (BSFR1), 50:50 (BSFR2), 60:40 (BSFR3) and 75:25 (BSFR4).

### 2.3.5 Size of D-Veligers

The size (shell length) analysis of D-veligers was restricted to normally developing larvae at 35, 32, and 30ppt salinities. There were no surviving individuals at salinity 26ppt.

There were significant effects of both broodstock feeding ratio (two-way ANOVA, $F_{(3,704)}$ = 0.5, $p < 0.001$) and embryo rearing salinity (two-way ANOVA, $F_{(3,704)}$ = 0.03, $p < 0.001$) on the size of the newly formed D-veligers. The interaction between salinity and feeding ratio was also significant (two-way ANOVA, $F_{(9,6704)}$ = 0.002, $p < 0.001$).

The size of D-veligers decreased with decreasing salinities. The average sizes of the D-veligers were 101.22 ± 0.49µm at 35ppt, 99.90 ± 0.57µm at 32ppt, and 99.57± 0.54 at 30ppt (Figure 2.7). Even though there were differences seen within the larvae from

different broodstock feeding ratios the overall effect was not as pronounced as seen with the rearing salinity.

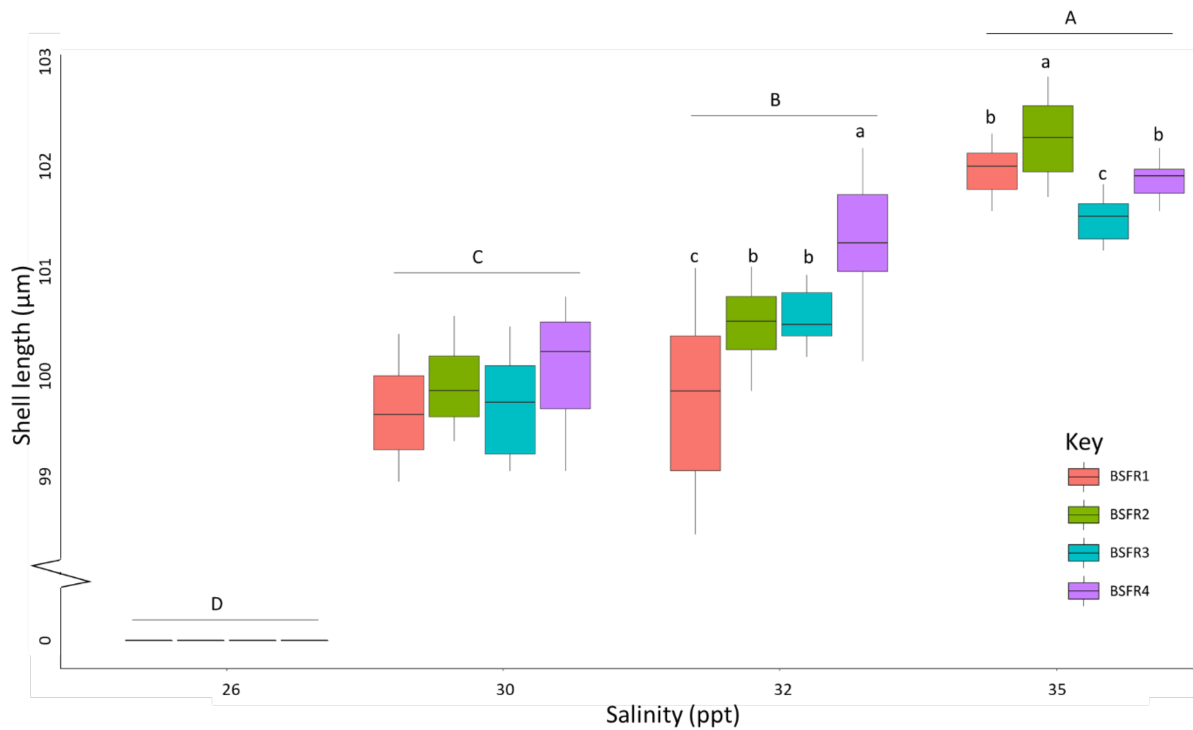


**Figure 2.7.** Size boxplots of normally developing D-veligers, reared at different salinities for 48h after fertilization for each broodstock feeding ratio treatment. Note: Boxplots with different letters denote significant differences (two-way ANOVA, Tukey $p < 0.05$) between salinity treatments. No veligers survived at 26ppt salinity. Broodstock feeding treatments, including *Tisochrysis lutea* and *Chaetoceros muelleri* at cell ratios of 25:75 (BSFR1), 50:50 (BSFR2), 60:40 (BSFR3) and 75:25 (BSFR4).

## 2.4 Discussion

In the present study, broodstock from all microalgal feeding treatments completed gonad maturation and spawned eggs and sperm. Differences in the microalgal ratios during conditioning (i.e., gametogenesis), however, had important carry-over implications on embryo survival, and transition into normally developed D-veligers when being reared at different salinities. The lower limit for the survival of *P. zelandica* embryos appears to be between 26 – 30ppt salinity; as no fertilized eggs survived at 26ppt, and only <30% survivors were recorded after 48h. However, the embryos obtained from broodstock that were conditioned with similar proportions of *Tisochrysis lutea* and *Chaetoceros muelleri* (ISO:CM 50:50 or 60:40) had an overall higher survival, greater number on larvae transitioning into D-veligers, and reduced incidence of abnormalities within the D-veligers at lower salinities, potentially indicating a wider salinity tolerance.

The influence of diet on the reproductive development of the females was not immediately obvious, with females in all feeding ratio treatments successfully spawning and producing viable embryos. This might be in part due to the extended conditioning period of 90 days provided as previous broodstock conditioning data on *P. zelandica* suggests that 73 days is sufficient in gonad maturation (Le et al., 2014) the extended conditioning period would have given the broodstock the opportunity to accumulate the necessary lipids and proteins for gonad maturation. Indeed, the ability for broodstock to acquire the necessary resources due to extended conditioning period was also seen in *P. generosa* (Marshall et al., 2014). Marshall et al., (2014) suggested that a minimum amount of time for broodstock conditioning is crucial to acquire enough resources for gonad maturation when *P. generosa* is fed with different levels of *Isochrysis* sp. and CM

(at 1:1 ratio) for different amounts of time. Similarly, García-Esquivel et al. (2013) found that gonad development in *P. globosa* could be achieved on a single species diet of *Tisochrysis lutea* if sufficient time was provided. However, in both of the previous studies (Marshall et al., 2014 & García-Esquivel et al., 2013) the quality of the gametes produced were not studied in detail.

The egg diameters of *P. zelandica* from all feeding ratio treatments were well within the range reported by Le et al., (2014) and Gribben & Hay (2003). The low variability in egg diameters within *P. zelandica* could be attributed to the species-specific nature of eggs in some bivalve species, e.g., mussels (Fearman et al., 2009). Even though the egg sizes were similar among treatments, it is possible that the biochemical composition (e.g., lipids, proteins) could have differed among treatments. For example, when mussels (*M. galloprovincialis*) broodstock were conditioned on different ratios of *Chaetoceros calcitrans* and *Pavlova lutheri,* spawned individuals produced similar-sized eggs, but the composition within the eggs and the viability of the developing larvae greatly reflected the nutritional content of the algal diet (Fearman et al., 2009).

Viability of *P. zelandica* embryos was clearly affected by the broodstock conditioning. In the present study, equal amounts of CM (rich in DHA) and ISO (rich in EPA) in the diet may have provided the best nutritional balance for the subsequent viability of embryos and larvae. Indeed, diet quality of broodstock and viability of offspring have been studied for the oysters *Ostrea chilensis* (Wilson et al., 1996), and *O. edulis* (Gonzalez Araya, Mingant, Petton, & Robert, 2012), the scallops *Argopecten purpuratus* (Martínez-Pita, Sánchez-Lazo, & García, 2016), and *A. nucleus* (Velasco & Barros, 2007), and the clam *Ruditapes decussatus* (Abbas et al., 2018; Utting & Millican, 1997). For example, when the diet of the scallop *Nodipecten nodous* is enriched with DHA and EPA during the conditioning period, the resultant veliger larvae have a greater survival (Sühnel et al.,

2012). This agrees with a study on mussels, *M. galloprovincialis*, which also found that diet composition of the broodstock had a significant effect on larval viability. This was attributed to the inability for the broodstock to successfully deposit glycogen (simple sugars) and sterols during the conditioning period (Pettersen et al., 2010). In the present study, since the nutritional content of microalgae provided have very different amounts of EPA and DHA; feeding ratios either lacking in DHA or EPA tended to produce *P. zelandica* embryos and/or larvae with reduced viability under low salinity stress. Similarly, Huo et al. (2017) found that lower salinities caused a reduction in the viability of *P. japonica* embryos. This was suggested to be the result of gradual swelling and final rupture of the embryos. However, in the present study, embryos obtained from females fed ratios with equal amounts of ISO:CM were more resilient to the changes in salinity. Based on these results, it is likely that providing broodstock with a balanced ISO:CM ratio may result in embryos with better osmotic regulation and improved resilience to osmotic stress.

A decrease in rearing salinity of the embryos greatly reduces the D-veliger yield. This negative effect of low salinity on larval yield has been well-documented in a number of marine bivalves, such as the oysters *Crassostrea belcheri* (S. Tan & Wong, 1996), *C. iredalei* (Fang et al., 2016), and *C. rhizophorae* (Dos Santos & Nascimento, 1985); the mussels *Perna viridis* (S.-H. Tan, 1996*), Mytilus edulis*, and *M. trossulus* (Qiu, Tremblay, & Bourget, 2002); the scallop *Pinctada imbricata* (O'Connor & Lawler, 2004), and the clams *Katelysia rhytiphora*, and *Anadara trapezia* (Nell, O'Connor, Heasman, & Goard, 1994), including the geoduck *P. japonica* (Huo et al., 2017). However, broodstock conditioned on microalgal ratios with similar proportions of ISO:CM overall tended to have a higher proportion of individuals transitioning into D-veligers, regardless of the rearing salinities.

There was a clear effect of both broodstock feeding rations and embryonic salinity exposure on the incidence of abnormal development of the D-veligers, which increased with decreasing salinities. This is in agreement with other studies on oysters (*C. rhizophorae*; Dos Santos & Nascimento, 1985 and *C. iredalei*; Fang et al., 2016), and clams (*P. japonica*; Huo et al., 2017). For *P. japonica*, the authors suggested that abnormalities, in the case of irregular cleaveages in the D-veligers, were attributed to lower salinities which resulted in osmotic changes during early embryonic development. In contrast, broodstock that were provided with similar feeding ratios of ISO:CM had on average less incidence of abnormal development within the D-veligers at lower salinities and also a reduced baseline abnormal development in ambient seawater (35ppt).

The shell length of the D-veligers measured at 48h post fertilization was affected more by the rearing salinity of the embryos than the feeding ratio of the broodstock. There was a decrease in overall size of the D-veligers with decreasing salinities. Indeed, Tan & Wong (1996) also found that in *Crassostrea belchri*, a decrease in salinity negatively affected D-veliger size. The overall decrease in D-veliger size was suggested to be influenced by the salinity at which the broodstock was conditioned (O'Connor & Lawler, 2004; Nowland et al., 2019; Yaroslavtseva & Sergeeva, 2010). Broodstock used in this study which were conditioned at 35ppt salinity and the largest-sized D-veligers were also recorded at salinity 35ppt compared to other salinities. Further investigations are needed to determine whether the salinity tolerance of *P. zelandica* embryos and larvae is an after effect of broodstock conditioning salinity.

In conclusion, the results of the present study indicate that *P. zelandica* broodstock can be successfully conditioned with different microalgal ratios of ISO and CM, producing viable gametes. Embryos of *P. zelandica* were observed to be highly sensitive to changes in salinity. Rearing embryos in decreasing salinity from 35ppt had a negative

impact on embryonic survival, D-veliger yield, an increase in incidence of abnormalities in the D-veligers, and an overall reduction of the size of D-veligers. The lower limit for survival of *P. zelandica* embryos is between 26 – 30ppt. The *P. zelandica* embryos obtained from broodstock that were kept on similar proportions of ISO and CM (50:50 and 60:40) tended to have a higher baseline survival, D-veliger yield and a decrease in abnormalities of the D-veligers. This suggested that having similar proportions ISO and CM during the conditioning period produces embryos that are more tolerant to the changes in salinity.

# CHAPTER 3 - EFFECT OF TEMPERATURE ON EARLY DEVELOPMENT OF THE NEW ZEALAND GEODUCK *PANOPEA ZELANDICA* (QUOY & GAIMARD, 1835)

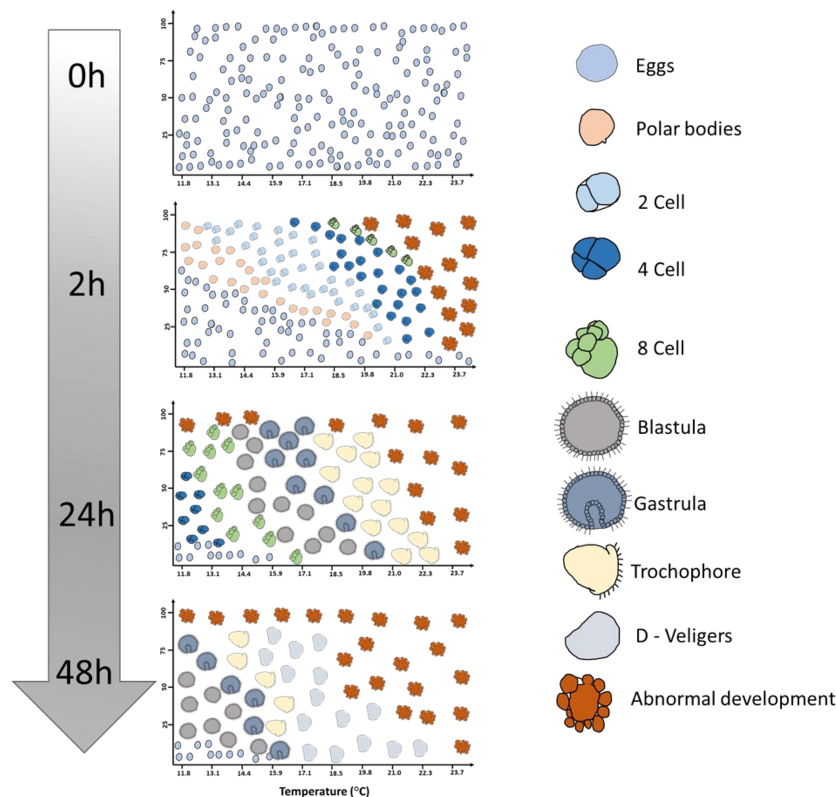


**Figure 3.0** Graphical abstract

This chapter has been published as:

Sharma, S. S., Alfaro, A. C., Ragg, N. L., & Zamora, L. N. (2020). Effects of temperature on early development of the New Zealand geoduck Panopea zelandica (Quoy & Gaimard, 1835). *Aquaculture Research*, *51*(2), 751-760.

# Abstract

Ambient seawater temperature is an important factor during the early life stages of marine invertebrates. Temperature is often manipulated in hatcheries to shorten the incubation period before the larval rearing phase. In this study the effect of temperature on the early development of the geoduck *Panopea zelandica* was investigated over a 48h period to identify the optimum temperature for fertilization and development in a controlled environment. Eggs and sperm collected from broodstock were exposed to ten temperatures ranging between 11.8 – 23.7°C, and fertilization and subsequent development was monitored over 48 hours. Highest rates of fertilization were achieved at 23.7°C, which was the highest temperature tested in this study. However, the development of *P. zelandica* embryos was greatly hindered at temperatures > 18.5°C due to a range of abnormalities arising from uneven cell division and cellular blebbing. All larvae died at the highest temperature of 23.7°C within 48h of exposure. The combined fertilization success and successful development data indicate that 18.5°C is the optimal temperature for incubating *P. zelandica* embryos under hatchery conditions.

## 3.1 Introduction

The New Zealand geoduck *Panopea zelandica* (Quoy & Gaimard, 1835) is a large, infaunal, sessile clam inhabiting subtidal areas around New Zealand. Geoduck populations in New Zealand are estimated to be relatively small with patchy distributions, based on washed up shells on local beaches (Gribben, 2003; Campbell et al., 2004). Ecological studies on known populations indicate that *P. zelandica* occurs mainly in shallow waters (5 – 25m) in sandy and muddy habitats (Gribben, 2003). Annual bands in polished shells show that *P. zelandica* can live up to 85 years (Gribben & Creese, 2005).

From a commercial perspective, geoducks are highly prized in Asian markets. For example, a high quality individual of 8 – 16 cm shell length and a total weight of 300 – 800g can fetch a price of between USD 220 - 330 $kg^{-1}$ in China (Shamshak & King, 2015) . Thus, the potential for cultivating *P. zelandica* has received considerable attention and is expected to add value and diversification for the New Zealand aquaculture industry (Alfaro et al., 2014). To date the New Zealand aquaculture sector has been focused on three main export species: Chinook salmon (*Oncorhynchus tshawytscha*), Pacific oyster (*Crassostrea gigas*) and Greenshell™ mussels (*Perna canaliculus*). Thus, the addition of *P. zelandica* to the New Zealand aquaculture sector is likely to assist in the aim to achieve NZ$1 billion in net exports by 2025 (Carter, 2012). To develop a geoduck industry in New Zealand, a reliable source of seed production needs to be ensured as it is highly unlikely that there will a known source of wild seed supply for *P. zelandica* (Gribben & Heasman, 2015). The culture of hatchery reared seed also offers better management of supply and quality control (Gribben & Heasman, 2015). Presently, there is very little scientific information available regarding the

cultivation conditions for *P. zelandica* (Le et al., 2016). In addition, species-specific protocols for hatchery spat production will need to be developed. Initial cultivation trials conducted at the Cawthron Institute, Nelson, were based on existing protocols for the North American species *P. generosa* and were found to not be ideal for *P. zelandica*. Since then, there have been substantial improvements made in broodstock conditioning and spawning for *P. zelandica* (Le et al. 2016, 2017). However, there still is a large bottleneck around larval growth and development.

Previous ecological studies have clearly demonstrated that environmental factors, such as temperature, salinity, and carbon dioxide ($pCO_2$) play an important role in fertilization success, development, growth and survival of geoducks and other bivalves (Hofmann & Somero, 1995; Doroudi et al., 1999; O'Connor & Lawler, 2004; Parker et al., 2009; Talmage & Gobler, 2011; Tang et al., 2012; Wang & Li, 2018). However, temperature is specifically important since it can directly influence immune responses, physiological processes, embryonic development, growth and survival (Hofmann & Somero, 1995; Desrosiers et al., 1996; Parker et al., 2009; Huo et al., 2017). Le et al. (2014) found that *P. zelandica* adults consumed more food and matured faster at 7-12°C, when compared to higher temperatures (16-17°C).

Increased ambient temperatures have also been observed to enhance fertilization rates in bivalves, such as oysters (Parker et al., 2009), scallops (Desrosiers et al., 1996) and surf clams (Clotteau & Dubé, 1993). In contrast Talmage & Gobler (2011) found that for two species of oysters (*Saccostera glomerata* and *Crassostera gigas*), fertilization success decreased with increasing temperatures. Such discrepancies suggest that the effect of temperature on fertilization success is species-specific.

In hatcheries, temperature is often manipulated to reduce the incubation time to develop larvae from embryos (see review by Helm, 2004). For example, faster developmental

rates (27 h instead of 3 days) were observed when *P. japonica* embryos were incubated at 19°C instead of 11°C (Lee & Rho, 1997). Similarly, for *P. zelandica*, Gribben et al. (2003) showed that a faster developmental rate was achieved when embryos were incubated at 17°C compared to the 15°C used by Le et al. (2016). While it may seem beneficial to increase the temperature to maximize developmental rates, there appears to be a thermal threshold for survival and normal development, which is often species-specific. Exceeding temperature thresholds has been reported to cause physiological stress or death in other clam species (Kocovsky & Carline, 2001; Huo et al., 2017). Indeed Huo et al. (2017) found that larvae of *P. japonica* have an upper thermal threshold of 22°C and larvae are not able to survive above this temperature. In addition, comparisons of thermal scope of activity for *P. zelandica* have shown that four-month old juveniles have a narrower thermal optimum (15-19°C) than three-year old young adults at temperatures between 11-19°C (Le et al., 2017).

This study investigates the effect of temperature on fertilization and subsequent embryonic development of *P. zelandica* to determine optimum hatchery rearing conditions. It is envisaged that this information will support the development of an emerging geoduck aquaculture industry in New Zealand.

## 3.2 Methods and materials

### 3.2.1 Broodstock and spawning

*P. zelandica* broodstock were collected from Golden Bay, South Island, New Zealand in 2016 and were kept at the Cawthron institute Aquaculture Park, Nelson, as a part of an ongoing geoduck research program. Individual geoducks were conditioned in 100 L tanks for 6 weeks (flow through system with 1µm filtered seawater [FSW] at 15°C supplemented with *Chaetoceros muelleri* and *Tisochrysis lutea* microalgae) prior to spawning induction, as described by Le et al. (2014).

Geoducks were induced to spawn individually by raising the ambient temperature of the flow through system as described by Le et al., (2014), The number of spawned individuals included six females and 7 males. Collected gametes were checked (shape of the eggs, sperm motility), cleaned and counted before fertilization.

### 3.2.2 Experimental setup

The experimental setup followed the protocol described by Delorme & Sewell (2013). Briefly, 20ml scintillation vials were used to observe early development for the first 48 h after addition of the sperm to the eggs at different temperatures. These vials were held in an aluminum temperature block containing sixty chambers (six possible replicates at ten different temperatures) (Figure 3.1). A temperature gradient was established and maintained by two recirculating heating/cooling water baths at each end of the temperature block. The ten temperatures in the thermal gradient ranged from 11.8 to 23.7°C were chosen based on Lee & Rho (1997) assessment of early development of

*P. japonica*. For each temperature, there were three experimental vials. These vials were filled with UV treated FSW and kept in the temperature block for 1 h to allow each vial to reach their specific temperature. The water temperature was measured after an hour with a hand-held thermometer positioned in the middle of each vial to ensure thermal stability.

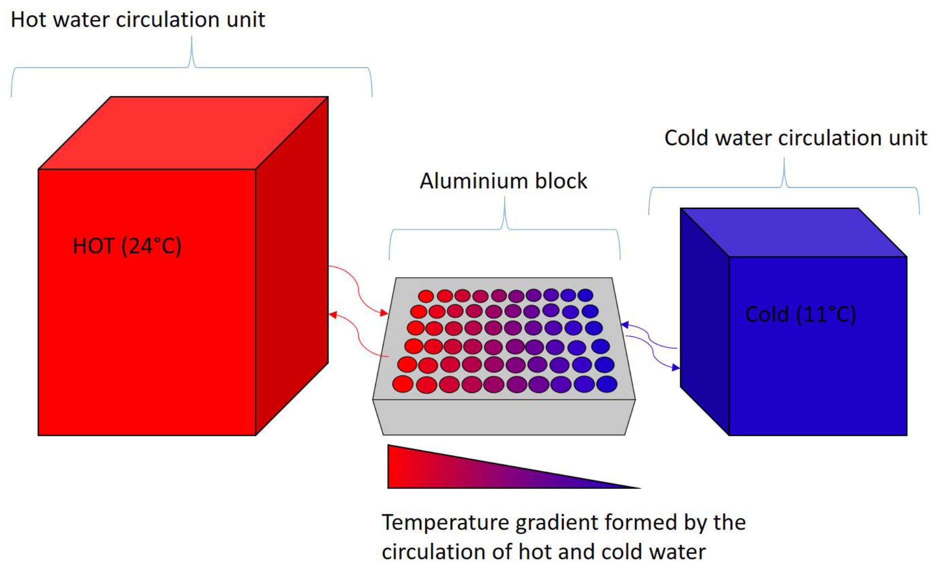


**Figure 3.1**. Setup of the aluminum temperature block showing the temperature gradient formed through the circulation of hot and cold water.

Around 3,000 eggs were placed into each 20-ml scintillation vial using a pipette to give a final egg concentration of approximately 150 eggs $ml^{-1}$. The eggs were acclimated for 30 mins at each specific temperature. To fertilize the eggs a concentration of approximately 450 sperm : 1 egg was used, following previous observations of high fertilization ratios and low polyspermy (Le et al., 2016).

### 3.2.3 Sampling procedure

The development of *P. zelandica* was assessed over a 48-hour period after addition of sperm. The addition of sperm is also regarded as time zero and the point of fertilization.

Samples of 100 - 1000μl of eggs were taken from each replicate temperature at each time point post-fertilization (at 20 minutes, 1, 2, 4, 8, 24, and 48-hours post-fertilization). For the first 4 hrs, 100μl egg samples were taken from the bottom of each vial. For samples taken at 8 h and after, the vials were gently agitated and 1000μl were extracted from the water column. This was done to ensure that the swimming life stages were incorporated in each sample. Samples were stored in 1.5ml Eppendorf tubes fixed with 100μl of formalin (4%) at 4°C. After 48 hours, the formalin was removed and replaced with 70% ethanol. The samples were assessed within a week to determine the developmental stage of the larvae.

### 3.2.4 Sample analysis

The developmental stages (larval composition and proportion) for each time point and temperature were determined by observation under an Olympus Omax compound microscope within a week of samples being taken. During the observation of each sample, the first 100 individuals that were encountered were considered for analysis. Each developmental stage was quantified when 50% of the embryos were observed at that particular stage. The developmental stages at each sampling time were scored as follows: eggs, formation of the first polar body, 2-cell, 4-cell , 8-cell, greater than 8 cells, morula , blastula, gastrula, trochophore, late trochophore, D-stage larvae and abnormal embryos (Figure 3.2), as described by Le et al. (2016). Abnormal development was defined as any abnormal cell division, deformed embryos/larvae and the appearances of blebs around the eggs.

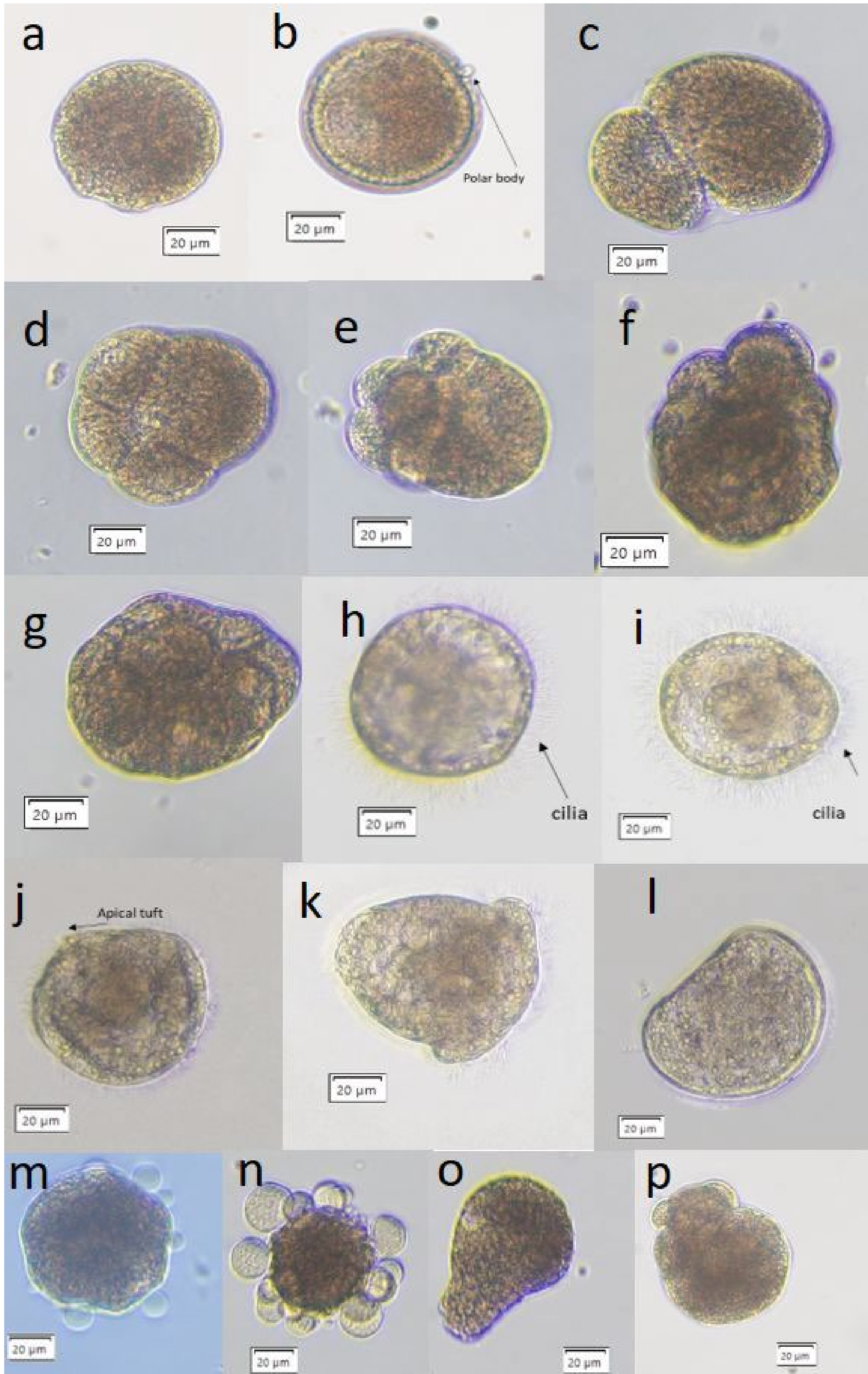


**Figure 3.2.** Developmental stages observed for *P. zelandica*. Eggs (a), formation of the polar body (b), 2-cell (c), 4-cell (d), 8-cell (e), greater than 8 cells (f), morula (g), blastula (h), gastrula (i), trochophore (j), late trochophore (k), D-stage larvae (l) and abnormal embryos (m-p).

### 3.2.5 Fertilization and development

Unfertilized eggs were identified as eggs without a fertilization membrane. Therefore, fertilization success was determined by observing the proportion of unfertilized eggs within the first 100 eggs/or embryos encountered.

Developmental assessment of the embryos was conducted by calculating the percentage of eggs and embryos at each developmental stage. The percentage of normal development was calculated as the proportion of abnormally developing embryos within the first 100 eggs/or embryos encountered. The percentage of successful D-stage larvae were quantified after 48-hours post-fertilization. This was carried out after removing any unfertilized eggs and/or abnormally developing embryos from the sample, and calculating the percentage of D-veliger larvae in the sample of normally developing embryos.

*Index of early cleavage*

An index of early cleavage was calculated from data obtained at 2-hours post-fertilization for each temperature (Delorme & Sewell, 2013; Sewell & Young, 1999). This index is simply the mean number of cleavages present in each sample at a certain time after fertilization. The 2-hours post-fertilization sampling period was chosen as there were sufficient cleavages observed in all the samples, and the number of abnormalities was very low at this time. The maximum cleavage observed in each sample was also recorded. The number of cleavages for each individual present in each sample was determined based on their respective number of cells (i.e. 0 = eggs and polar bodies, 1 division = 2-cells, 2 divisions = 4-cells, 3 divisions = 8-cells, 4 divisions = >8-cells).

### 3.2.6 Statistical analysis

Statistical analyses were performed on the percentage of normally developing individuals, ratio of successful D-stage larvae, and the index of early cleavage. All data were checked for normality and homoscedasticity using the Shapiro-Wilk and Levene's tests, respectively (Quinn & Keough, 2002).

For the percentage of larvae with normal development, the data were arcsine-transformed and analyzed separately for each time period with temperature as the factor and the percentage of normally developed larvae as the dependant variable. As the data did not meet the assumptions of normality and homoscedasticity, a Kruskal-Wallis test was performed, followed by Kruskal-Wallis Dunn test (Pohlert, 2014).

Data on the index of early cleavage and the percentages of D-stage larvae were analysed untransformed as both met the assumptions of normality and homoscedasticity. A One-Way Analysis of Variance (ANOVA) was used for these analyses, and differences among temperatures were identified using a post-hoc Tukey test (Quinn & Keough, 2002). All statistical analyses were performed using the R® Software.

# 3.3 Results

## 3.3.1 Sampling procedure

Temperatures in the scintillation vials were consistent among the three replicates with a standard deviation of 0.1°C for all the temperatures. Temperatures were recorded at 0, 18, and 48-hours post-fertilization. Mean temperatures in the study consisted of 11.8, 13.1, 14.4, 15.9, 17.1, 18.5, 19.8, 21.0, 22.3, and 23.7°C. In addition, dissolved oxygen and pH were measured at the end of the experiment with a Hach, IntelliCAL™ LDO101 probe and a pH tester with spear electrode, respectively. There was no difference in $O_2$ concentration and pH between experimental vials and control vials (without eggs/embryos).

## 3.3.2 Fertilization and development

The rate of fertilization was higher at higher temperatures. Within 20 minutes of adding sperm to the scintillation vials, more than 50% of the eggs had a visible fertilization membrane around them or had formed visible polar bodies at temperatures ≥ 17.1°C (Figure 3.3).

The development rate of *P. zelandica* embryos increased with an increase in seawater temperature (Figure 3.3 and Table 3.1). Within 20 minutes post-fertilization, the polar bodies were evident in temperatures between 11.8 - 14.4°C, whereas at temperatures ≥ 15.9°C, a small proportion of eggs (3 – 10%) had already gone through the first cleavage (2-cell) stage (Figure 3.3 and Table 3.1). The percentage of unfertilized eggs recorded at 20 mins decreased from 90% at 11.8°C to 40% at 23.7°C. At temperatures

≥ 15.9°C, 2 - 5% of the embryos underwent second cleavage within 1 hour post-fertilization, and this was not observed in temperatures ≤ 14.4°C until 4 hours post-fertilization. After 4 hours, the fertilization rate of *P. zelandica* eggs were also higher than 90% for temperatures ≥14.4°C (Fig. 3). The third cleavage was observed at 2 hours post-fertilization at temperatures ≥ 19.8°C, however the incidence was very low (< 5%). At 4-hours post-fertilization, the percentages of 8-cell embryos reached over 50% in temperatures 15.9 – 19.8°C with low percentages of 8+ cells (< 15%) also observed at these temperatures (Figure 3.3).

Blastulae were first observed at 8 hours post-fertilization at temperatures ≥ 14.4°C, and there were no successfully developing embryos at 23.7°C (see below). More than 50% of embryos were at the blastula stage at temperatures ≥ 15.9°C, with small percentages of gastrulae (<5%) also observed. At 24 hours post-fertilization, > 50% of embryos were at the gastrula stage at temperatures ≥15.9 - 21°C with low percentages (< 20%) of trochophores also observed at these temperatures (Figure 3.3 and Table 3.1). At 48-hours post-fertilization, D-stage larvae were observed in temperatures ≥ 14.4°C (Figure 3.3). There was a significant difference in net larval survival between the different temperatures and the proportion developing to D-stage larvae ($H$ = 27.4, d.f. = 9, $P$ < 0.005). The proportions of D-stage larvae were significantly higher at 17.1 and 18.5 °C, representing more than 50% of larvae observed at these temperatures (Figure 3.3).

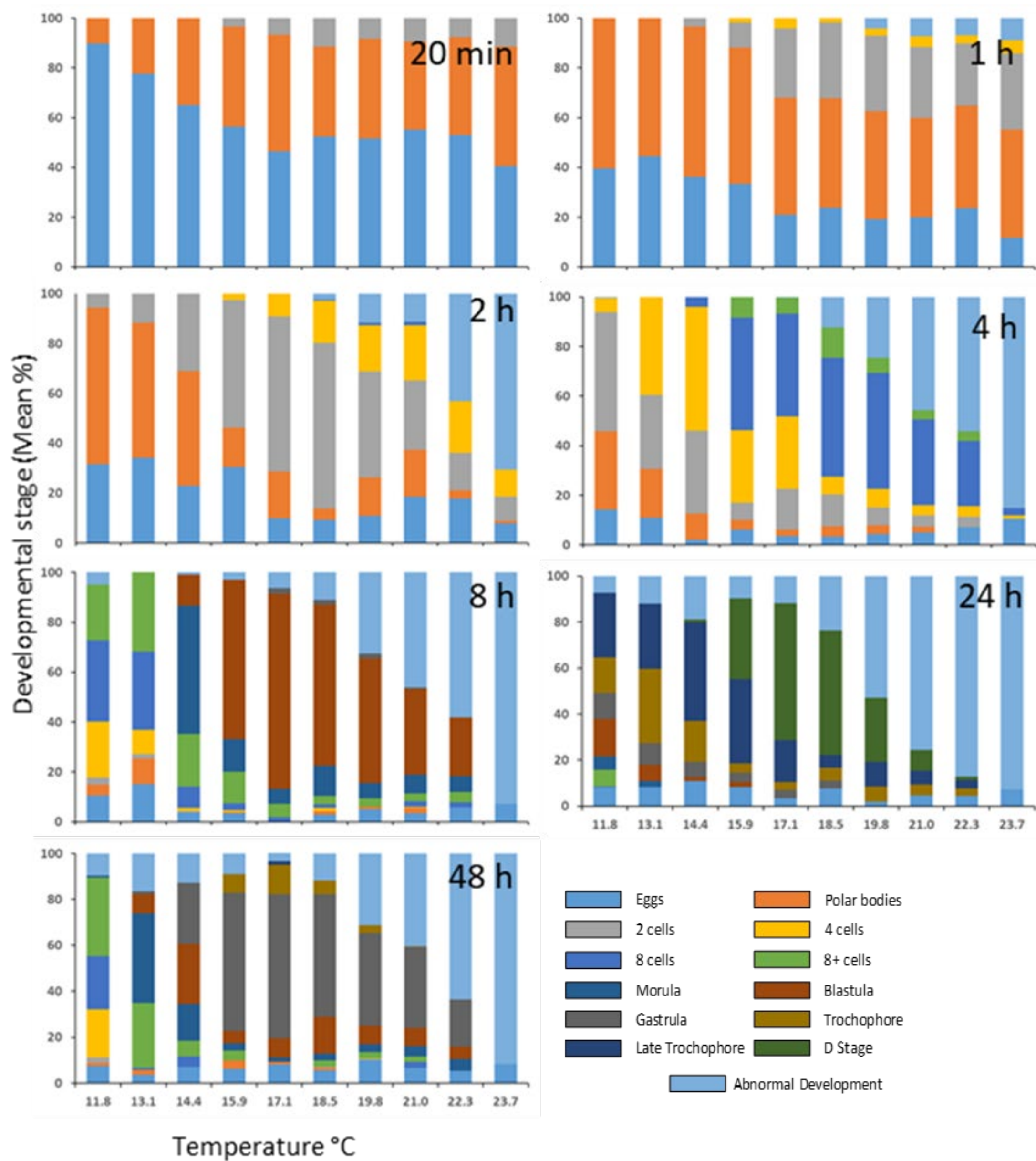


**Figure 3.3.** Effect of temperature on early development of *P. zelandica*. Data correspond to the mean percentage of each developmental stage identified (*n* = 3) at each temperature. Sampling time indicated on the right top corner of each panel represents time post-fertilization.

**Table 3.1**. The approximate post-fertilization developmental times of geoduck incubated at different temperatures (*P. zelandica* and *P. japonica*). Each stage is defined when 50% of the individuals have been observed at that stage. *P. zelandica* data are derived from the current study and from Le et al. (2015) at 15°C. *P. japonica* data were obtained from Lee & Rho (1997). *Le et al. 2015.

| Stage | ***P.zelandica* at** | | | | | | | | | | | ***P.japonica* at** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 11.8°C | 13.1°C | 14.4°C | **15°C*** | 15.9°C | 17.1°C | 18.5°C | 19.8°C | 21°C | 22.3°C | 23.7°C | 11°C | 14°C | 17°C |
| Polar body | 20 min | 20 min | 20 min | **15 - 20min** | | | | | | | | | | |
| 2 Cells | 1 h | 1 hr | 1 hr | **1.5 h** | 20 min | 20 min | 20 min | 20 min | 20 min | 20 min | 20 min | 2 h | | |
| 4 Cells | 4 h | 4 h | | **2.5 h** | 1 h | 1 h | 1 h | 1 h | 1 h | 1 h | 1 hr | 4 h | | |
| 8 Cells | | | | **4 h** | 2 h | 2 h | 2 h | 2 h | 2 h | 2 h | 2 h | 9 h | 5.4 h | 4.3 h |
| 8 + Cell | 8 h | 8 h | 8 h | **5 h** | 4 h | 4 h | 4 h | 4 h | 4 h | 4 h | 4 h | 15 h | | |
| Morula | 24 h | | | **6 h** | | | | | | | | | | |
| Blastula | | | | **8 h** | 8 h | | | | | 8 h | | 23 h | 18.7 h | 12.3 h |
| Gastrula | | 24 h | 24 h | **18 h** | | 8 h | 8 h | 8 h | 8 h | 24 h | | | | |
| Trochophore | | | | **35 h** | 24 h | 24 h | 24 h | 24 h | 24 h | | | 2 d | 33.8 h | 23.6 h |
| Late trochphore | 48 h | 48 h | | **39 h** | | | | | | | | | | |
| D-Veliger | | | | **62 h** | 48 h | 48 h | 48 h | 48 h | 48 h | 48 h | | 3 d | 62.4 h | 42.7 h |

## 3.3.3 Index of early cleavage

The rate of early cleavage was higher at higher temperatures ($H$ = 24.5, d.f. = 9, $P$ < 0.001) (Figure 3.4). The mean number of divisions in embryos maintained at temperatures between 15.9 – 22.3°C was significantly higher than those observed at temperatures between 11.8 – 14.4°C and 23.7°C. This trend was more evident in the number of maximum divisions, as the maximum number of cleavages occurred at the highest tested temperatures (Figure 3.4).

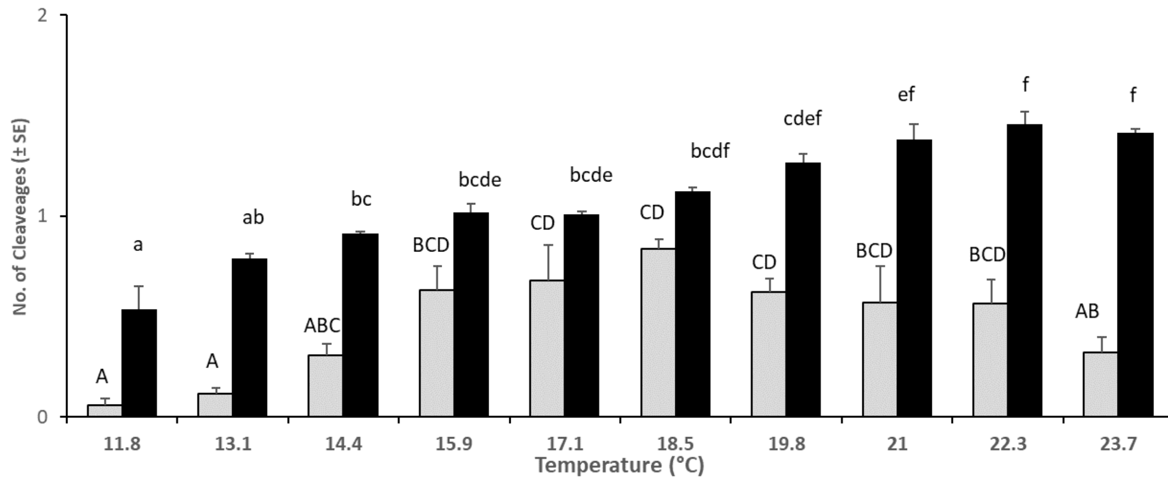


**Figure 3.4**. Mean (grey bar) and maximum (black bar) number of cleavages (± SE, $n$ = 3) at 2-hours post-fertilization (index of successful development) at different temperatures of *P. zelandica* embryos derived from early development shown in Fig 1. Significant differences among temperatures ($P$ < 0.05) are denoted by upper case (mean division) and lower case (maximum division) letters above each bar.

## 3.3.4 Normal development

The normal development of *P. zelandica* decreased with increasing time and seawater temperature (Figure 3.5). At 20-minutes post-fertilization, no abnormalities were detected across the entire temperature range ($H$ = 0, d.f. = 9, $P$ = 1). After 1 hour post-fertilization, the percentage of normally developing embryos significantly decreased with increasing temperatures ($H$ = 28.1, d.f. = 9, $P$ < 0.001). The highest proportion of abnormally developing embryos was observed at 23.7°C (Table 3.2). At 8-hours post-fertilization, the differences in development were even more pronounced ($H$ = 26.1, d.f. = 9, $P$ < 0.001), with no embryos surviving past the blastula stage at 23.8°C. The percentage of embryos developing at 22.3°C (the highest temperature that the embryos survived at) was significantly lower (41%) compared to normal development observed at 11.8, 13, 14.4, 15.9°C. At 48 hours post-fertilization, the percentage of normal development was significantly lower at higher temperatures ($H$ = 27.5, d.f. = 9, $P$ < 0.001) when compared with the percentage of normal development at lower

temperatures (Figure 3.5). The percentage of normal development 48 hours post-fertilization indicates a high proportion of normal development (> 75%) at temperatures lower than 18.4°C.

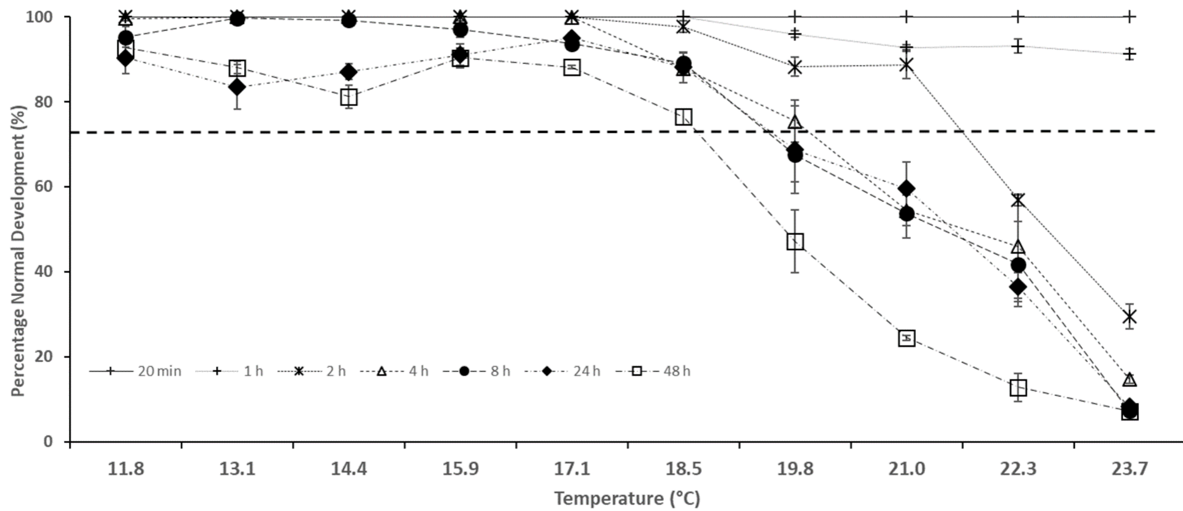


**Figure 3.5.** Mean percentage of normal early development (± SE, *n* = 3) of *P. zelandica* at different temperatures. Different lines represent different sampling time points from 20 mins to 48 hours post-fertilization. Dashed line represents 75% threshold for the normal development of *P. zelandica* embryos/larvae.

**Table 3.2** Summary of statistical analysis performed on the percentage of normally developing individuals of *P. zelandica* at the different sampling times post-fertilization. Kruskal-Wallis results H and P (Kruskal-Wallis statistic and p-value respectively) are shown for each time point using temperature as factor. Significant results ($p < 0.05$) given by post-hoc Kruskal Dunn pairwise tests are also shown.

| **Factor: Temperature (d.f = 9)** | | | |
|---|---|---|---|
| **post-fertilization** | ***H*** | ***P*** | **posthoc Kruskal Dunn test (P < 0.05)** |
| 20 min | 0 | 1 | - |
| 1 h | 28.1 | < 0.001 | 23.7°C ≠ 11.8, 13.1, 14.4, 15.9, 17.1, 18.5°C |
| 2 h | 27.8 | < 0.001 | 23.7°C ≠ 11.8, 13.1, 14.4, 15.9, 17.1, 18.5°C<br>22.3°C ≠ 11.8, 13.1, 14.4, 15.9, 17.1°C |
| 4 h | 27.5 | <0.001 | 23.7°C ≠ 11.8, 13.1, 14.4, 15.9, 17.1°C<br>22.3°C ≠ 11.8, 13.1, 14.4, 15.9, 17.1°C<br>21.0°C ≠ 11.8, 13.1, 14.4, 15.9, 17.1°C |
| 8 h | 26.1 | < 0.001 | 22.3°C ≠ 11.8, 13.1, 14.4, 15.9°C<br>21.0°C ≠ 13.1, 14.4, 15.9°C<br>19.8°C ≠ 13.1, 14.4°C |
| 24 h | 24.51 | < 0.001 | 22.3°C ≠ 11.8, 15.9, 17.1, 18.4°C<br>21.0°C ≠ 17.1°C<br>19.8°C ≠ 17.1°C |
| 48 h | 27.5 | < 0.001 | 22.3°C ≠ 11.8, 13.1, 14.4, 15.9, 17.1°C<br>21.0°C ≠ 11.8, 13.1, 15.9°C<br>19.8°C ≠ 11.8°C |

## 3.4 Discussion

Temperature is one of the most important factors influencing the performance of marine invertebrates, and it plays a fundamental role in a wide range of biological and physiological processes (Fujisawa, 1989; Parker et al., 2009; Huo et al., 2017). In this study, *P. zelandica* eggs/embryos/larvae were exposed to different thermal regimes to evaluate the effect of temperature on fertilization success and subsequent development over a 48-hour period. *P. zelandica* showed an increase in the rate of fertilization with increasing temperatures. The highest rates of fertilization were achieved at 23.7°C, which was the highest temperature tested in this study. However, the rate of development resembled a binomial distribution with the optimum rate of development achieved at 18.5°C. At lower temperatures, the rate of development was reduced, but due to the increase in incidences of abnormalities and cellular destabilization associated with increasing temperature, any positive gains in development were over-shadowed by the developmental issues observed at temperatures greater than 19°C. Results of fertilization success and development indicate that *P. zelandica* could be incubated at around 18°C for a high fertilization success and a high D-veliger yield.

The fact that the rate of fertilization for *P. zelandica* increased with an increase in temperature from 11.8 – 23.7°C is consistent with other studies in a variety of species, including oysters (Parker et al., 2009), scallops (Desrosiers et al., 1996), surf clams (Clotteau & Dubé, 1993) and sea urchins (Ho et al., 2013). Ho et al. (2013) suggested that the increased rate of fertilization is likely to be due to the stimulation of sperm and a reduction in water viscosity with increasing temperatures. The rate of fertilization in the present study (> 80% at 14.4°C, 4 hours post-fertilization) falls within the range of observations made by Le et al. (2016) (>80% at 15°C, 3 hours post-fertilization) on *P. zelandica*. However, at higher temperatures (≥17.1°C), the rate of fertilization (≥ 90%)

was higher than previous studies on this species (Gribben et al., 2014; Le et al., 2016). Gribben et al. (2014) fertilized *P. zelandica* eggs at 17°C and achieved a lower fertilization rate of 70%. The sperm:egg ratio used in this study was at a lower concentration (500:1) compared to Gribben et al. (2014) (10,000:1). Therefore, other factors, such as egg density and sperm:egg contact time could have largely contributed to the differences in fertilization observed. The rate of fertilization in bivalves has been shown to increase with an increase in egg density (Clotteau & Dubé, 1993; Desrosiers et al., 1996; Le et al., 2016). However, the optimal concentration of eggs $ml^{-1}$ appears to be species-specific. For example, Le et al. (2014) observed that for *P. zelandica*, the optimal concentration of eggs to achieve the highest rates of fertilization was around ~100 eggs $ml^{-1}$. In the present study, the density of eggs was similar to that suggested by Le et al. (2014), whereas Gribben et al. (2014) used a significantly lower concentration of 20 eggs $ml^{-1}$. In addition, the sperm-egg contact time should be considered. For example, Gribben et al. (2014) noted that for *P. zelandica*, the rate of fertilization increased with an increase in sperm-egg contact time even at lower sperm concentrations. In the present study, the sperm was kept in suspension with the eggs, whereas both Gribben et al. (2014) and Le et al. (2016) washed the sperm out after a defined contact time period (10 mins and 40 mins, respectively).

The developmental rate of *P. zelandica* in the present study also increased with increasing temperature. This is in agreement with several other studies on marine invertebrates that have shown that higher temperatures tend to enhance the rate of cleavage of the developing embryos up to a certain point (Dehnel & Kong, 1979; Dos Santos & Nascimento, 1985; Chen & Chen, 1992; Lee & Rho, 1997; Delorme & Sewell, 2013; Nam et al., 2015; Huo et al., 2017). The developmental rates at recorded temperatures in the present study are similar to those observed by Le et al. (2016) on

*P. zelandica* (at 15°C) and by Lee & Rho (1997) on *P. japonica* (at 11, 14, and 17°C). Nam et al. (2015) further shortened the incubation time of *P. japonica* to reach D-veliger larvae to 27 h by maintaining the embryos at 19°C. While it may be beneficial for hatcheries to maximize the developmental rate, the thermal threshold for normal development should not be exceeded (Dos Santos & Nascimento, 1985; Parker et al., 2009; Parker et al., 2010; Delorme & Sewell, 2013; Huo et al., 2017). As the rate of abnormalities greatly increases with increasing temperatures. In this study, after 48 hours post fertilization the incidence of abnormalities exceeded 25% for temperatures > 18.5°C. The normal ecological sea surface temperatures for the adult geoducks in this trial ranges between 14°C in winter with summer temperatures reaching as high as 21°C, with spawning occurring when the ambient sea temperatures reaches 15°C in spring and autumn (Gribben et al., 2004). Previous studies on *P. generosa* (Goodwin, 1973) and *P. japonica* (Huo et al., 2017) embryos showed that geoducks have a narrow temperature range which is more apparent in juveniles. With the projected increase in global sea temperatures in the near future there could be detrimental implications for the spawning and development of *P. zelandica.* Indeed, several invertebrate species exhibit abnormal development when the ambient temperature increases above the normal ecological levels (Kennedy et al., 1974; Parker et al., 2010; Gibson, 2011; Nam et al., 2015; Teaniniuraitemoana et al., 2016; Huo et al., 2017).

In conclusion, our study shows that there are benefits to using higher temperatures than those encountered by the embryos in the wild to maximize the fertilization success and the development of *P. zelandica.* However, there appears to be a thermal maximum for the optimal development above which this positive thermal relationship becomes detrimental for the survival and normal development. Further work needs to be conducted to further elucidate the effect of temperature on survival of *P. zelandica*

during the later phases of larval development. In this study, fertilization success increased with increasing temperatures. However, the development of the subsequent embryos showed that a temperature of around 18°C results in a faster developmental rate with a high D-veliger yield.

# CHAPTER 4 - CHANGES IN THERMAL TOLERANCE AND GROWTH DUE TO FOOD AVAILABILITY DURING LARVAL DEVELOPMENT OF THE NEW ZEALAND GEODUCK (*PANOPEA ZELANDICA*)

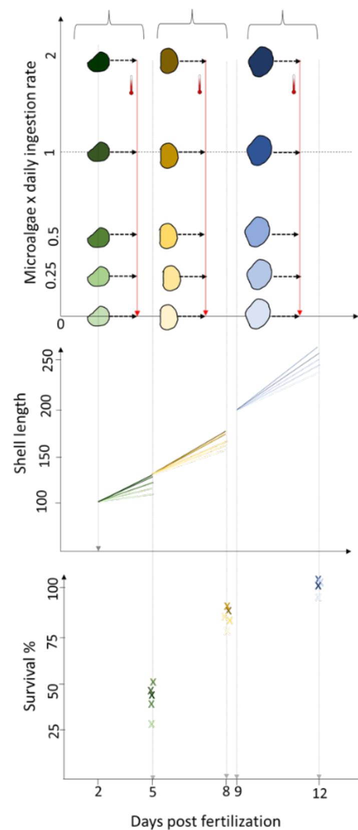


**Figure 4.0** Graphical abstract

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This chapter is under review as:

Sharma, S. S., Alfaro, A. C., Ragg, N. L., & Zamora, L. N. (In Review). Changes in thermal tolerance and growth due to food availability during larval development of the New Zealand geoduck (*Panopea zelandica*).*Aquaculture Research*

# Abstract

It is commonly known that diet has diverse consequences on overall larval growth and survival. The ability for larvae to accumulate exogenous resources is important in decreasing its vulnerability to stressors encountered. In this study, we investigated the vulnerability of different aged pelagic larvae of *P. zelandica* to acute thermal stress after changes in diet concentration. A commercial scale larval production was used as the source of larvae as well as a reference population to evaluate the experimental treatments. On 2, 5, 9 days post fertilization (dpf) *P. zelandica* larvae were removed from the reference population and placed in static systems. The larvae were then fed five different concentrations of microalgae (0.0, 0.25, 0.5, 1.0, 2.0 times the food provided to the reference larval population) for 48h. This was followed by an acute thermal stress at 25°C for 1h and a recovery phase of 24h at ambient temperature of 17°C. After which the growth and survival of the larvae was assessed. Within all age classes larvae growth and survival increased with increasing microalgal concentrations. However, when larvae were provided excess microalgae (2.0x), there was a decrease in both survival and shell growth. This decrease in survival and growth was probably due to bacterial proliferation common in static systems. The vulnerability of *P. zelandica* to thermal stress also decreased with increasing larval age from 88% (2dpf) – 5% (11dpf) regardless of the concentration of microalgae provided. This in part due to the increasing larval size and the ability to accumulate resources. These findings are important in understanding how diet is utilized over the development of *P. zelandica* larvae.

## 4.1 Introduction

The New Zealand geoduck, *Panopea zelandica* (Quoy & Gaimard, 1835), is a large sessile clam found in both the North and the South islands of New Zealand. Panopea genus are amongst the largest living burrowing clams, with a long lifespan and live weight of up to 3kg (Goodwin & Pease, 1989). Geoducks represent one of the most lucrative products in the seafood market, especially in Asia. For example, a top grade live pacific geoduck (*P. abrupta*) can be sold for USD$200 – 300 per kg in Asian restaurants (Shamshak & King, 2015). Therefore, *P. zelandica* has received considerable attention from the New Zealand aquaculture industry (Alfaro et al., 2014) as a new species that can support the country's growth aspirations for the aquaculture sector to generate annual exports exceeding NZ$3 billion by 2035 (Stenton-Dozey et al., 2021).

While this species has excellent potential to achieve a high commercial value, a successful aquaculture industry would require hatchery production of seed (Gribben & Heasman, 2015; Marshall, 2010). One of the most important aspects of hatchery production is a good understanding of the larval biology of the target species (Gosling, 2008). Therefore, a thorough understanding of fundamental aspects, such as functional morphology, development, and survival are required in order to develop reliable larval rearing protocols (Helm, 2004). Most marine invertebrates develop for a certain time in the planktonic stage before metamorphosis into juveniles. The development of *P. zelandica* larvae is similar to other bivalve species. The larvae undergo six developmental stages prior to settlement which includes: Prodissonconch (P) I D-veliger, P II D-veliger, P II early umbo veliger, and Pediveliger; over a period of 16 – 19days (Ferreira et al., 2015; Gribben & Hay, 2003; Le et al., 2017). During this period, planktrophic larvae acquire their food from seawater to obtain energy for growth and

development. *P. zelandica* larvae develop from small eggs with a low energy content and are presumed to be highly dependent on exogenous foods soon after the first feeding stage is reached (King, 1988).

However, growth in the planktonic environment does not go as smoothly. Larvae are often affected by multiple stress factors. In response to stress organisms deploy a suit of mechanisms to repair and protect cells from further damage (Lesser, 2006; Oksala et al., 2014). The upregulation of these stress response mechanisms can be energetically costly (Jeno & Brokordt, 2014). Therefore, food availability may play a crucial role in the ability for an organisms to cope with stressors (Jeno & Brokordt, 2014, Delorme et al., 2020).

Food availability on its own is a common stressor for marine organisms and can have an important effect on the overall larval growth and development. Larvae need a constant supply of exogenous nutrients to undergo metamorphosis. Indeed, reduction in microalgae has been attributed to delayed metamorphosis and an extended larval stage *Teredo navalis* and *Bankia gouldi* (Mann & Gallager, 1985). Reducing the supply of microalgae (food) can also cause mortalities and stunted growth (De Costa et al., 2012; Bassim et al., 2015). Indeed, increasing the number of microalgal cells in the diet has also been shown to increase larval survival and daily growth rate (shell length) in *P. abrupta* (Marshall et al., 2010) and other bivalve larvae, such as mussels (Pettersen, Turchini, Jahangard, Ingram, & Sherman, 2010), oysters (Rico-Villa, Le Coz, Mingant, & Robert, 2006), and scallops (Cerón-Ortiz, Cordero, Arredondo-Vega, & Voltolina, 2009).

Limitation in nutrition can also have an effect on the overall fitness of the organism and consequently impact their response to other environmental stressors such as temperature and pathogens (Wagner et al., 2015). Indeed, when subjected to increasing

temperatures the larvae of *Mercenaria mercenaria*, *Crassostrea virginica*, and *Argopecten irradians* showed increased mortalities, and a decrease in growth, and development (Talmage & Gobler., 2011). In addition, when acute thermal stress was compounded with bacterial stress *Crassostrea gigas* larvae had a decrease in shell growth and an increase in mortalities (Liu et al., 2017). Therefore, it is important to understand how stress factors affect the organism in tandem to fully evaluate the overall performance of an organism.

The present study was designed to assess the ability of different aged *P. zelandica* larvae to cope with acute thermal stress after changes in microalgal concentrations. This study aids in the development of strategies for the aquaculture of *P. zelandica*.

## 4.2 Methods and Materials

### 4.2.1 Acquisition of larvae

Larvae used in this trial was originated from broodstock collected from Golden Bay (South Island, New Zealand) and housed at Cawthron Institute's Aquaculture Park (Nelson, New Zealand) as a part of an ongoing geoduck research program. After spawning induction and completion of embryonic development the newly formed larvae were reared following standard protocols developed at the Cawthron Institute, using CUDLs (Cawthron Ultra Density Larval rearing) (Ragg et al., 2010; Le et al., 2016).

The CUDL systems were stocked with newly hatched D-veligers at a maximum density of 200 larvae/ml supplied with inflowing Filtered Sea Water (FSW) pre-mixed with a microalgal diet. The microalgae initially consisted of 100% *Chaetoceros calcitrans* (CC), with *Isochrysis galbana* (ISO) introduced into the system on day 4 at a ratio of 9:1 (CC:ISO). The concentration of microalgal cells was maintained at 40 cells/µl. This concentration was monitored by measuring the number of microalgal cells in the outflowing water from the CUDL's. A Neubauer haemocytometer (BS748, Hawksly, UK) was used to make daily counts of outflowing cell concentrations from each CUDL tank and these values were used to generate a feed-out rate correction factor required to maintain the desired microalgal concentration. The individual cell counts and total amount of microalgal cells provided to each larvae in the CUDL's and diet manipulation days are shown in Figure 4.1.

## 4.2.2 Experimental procedure

The main study was focused around the pelagic phase of *P. zelandica* larvae during 2 – 12dpf. The experiment consisted of diet ratio treatments (48h) → thermal shock (1h) → recovery (24h) of three different aged larvae of *P. zelandica* (Figure 4.1).

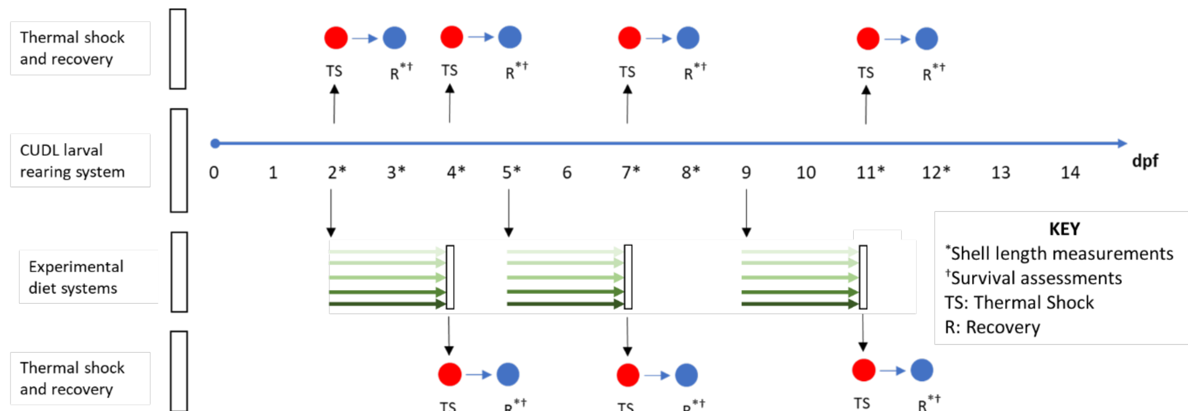



**Figure 4.1.** Experimental setup and procedure during the pelagic phase of *P. zelandica*. Larvae removed from CUDL's (→) on day 2, 5, 9dpf and kept on five different diet concentrations for 48h (→), then subjected to thermal shock for 1h at 25°C (●TS) and a recovery period (●R) for 24h. Shell measurements (*) were taken before diet treatments and after recovery period. Survival (†) was assessed after the recovery period.

Five different microalgal diet ratios were chosen which included: - 0.0 (unfed), 0.25, 0.5, 1, and 2 times the amount of microalgae provided in the CUDL's (Figure 4.2). Each diet ratio treatment consisted of three replicates of 2,500 larvae.

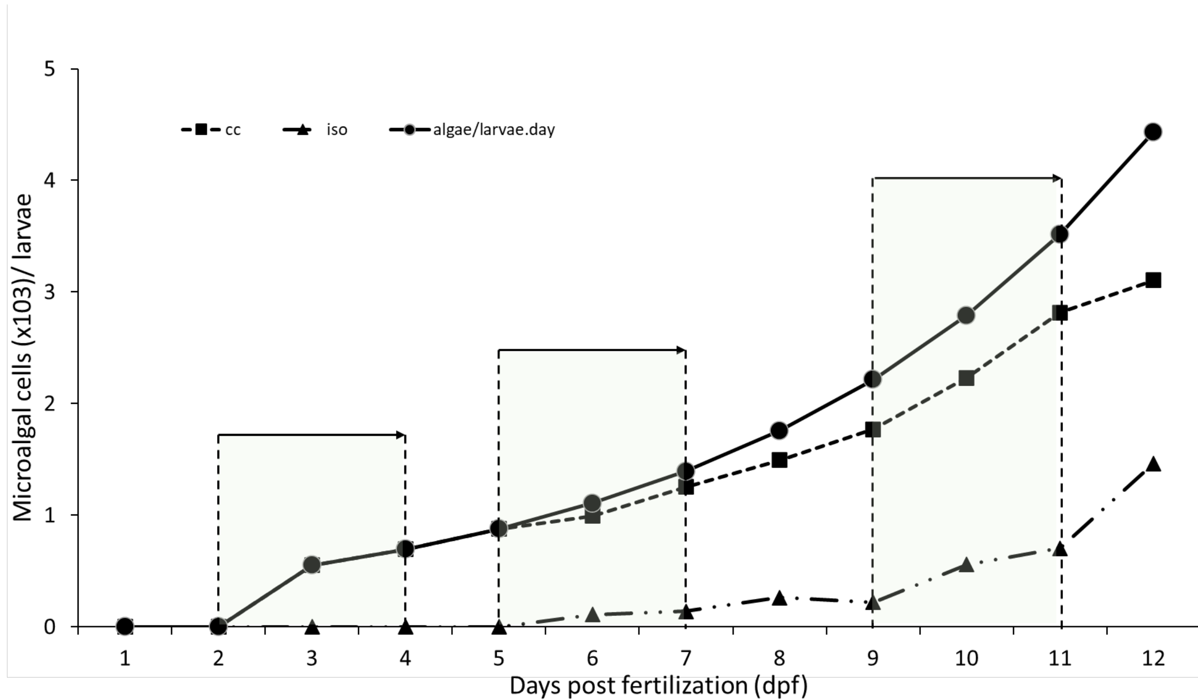



**Figure 4.2**. The concentration of microalgal cells provided in the CUDL's per larva over during the pelagic growth stage. The separate ratios of *Chaetoceros calcitrans* (CC) and *Isochrysis galbana* (ISO) are also shown. Diet manipulation days are highlighted.

To obtain a baseline survival post thermal stress for newly metamorphosised larvae; three replicates of 2,500 larvae were removed from the CUDL's on 2dpf and placed in a 20 ml scintillation vials and subjected to temperature shock and recovery (described in detail below).

On day 2, 5, and 9 post-fertilization larvae were removed from the CUDL's with shell lengths of 50 larvae analysed under an Olympus Omax microscope using the CellSens software®. The larvae were then placed in 1L beakers containing FSW. The volume in each 1L beaker was topped up to 500ml, to give a final concentration of 5 larvae per ml in each beaker. The amount of microalgal cells provided daily in each of the experimental days is shown in Table 4.1.

**Table 4.1.** Concentrations of microalgal cells given to the larvae during the different diet ratio treatments. The percentages of CC and ISO are also provided. * Baseline assessment of unfed larvae

| **Dpf** | **Concentration of microalgal ($10^3$ cells/ml)** | | | | | **Percentage (%)** | |
|---|---|---|---|---|---|---|---|
| | 0.0x (unfed) | 0.25x | 0.50x | 1.0x* | 2.0x | CC | ISO |
| **2 – 2*** | - | - | - | - | - | - | - |
| **2 - 4** | - | 9.66 | 19.32 | 38 | 77.28 | 100 | 0 |
| **5 - 7** | - | 19.35 | 38.69 | 77.38 | 154.77 | 80 | 20 |
| **9 - 11** | - | 48.84 | 97.67 | 195.35 | 390.7 | 70 | 30 |

After 48h on each diet treatment the larvae were removed from the beakers by washing them over a 43µm mesh sieve. The larvae were then transferred into individual 20 ml scintillation vials. These vials were then placed in an aluminum temperature block (Sharma et al., 2020) set to 25°C. The temperature in each vial was raised to 25°C within 10 mins, and the larvae were then exposed to 25°C for 1h. The larvae were then removed and placed in 1L beakers at 17°C where they were left to recover for 24h after which time the survival of larvae was conducted and shell lengths (n = 50) from each replicate was recorded. Additional samples were taken from the CUDL’s (three replicates n=50) to record and assess the shell length of the reference population.

### 4.2.3 Survival analysis

The larvae were placed in 4ml TCD wells and all the shells on the bottom of each plate were counted using an Olympus binocular microscope at 10x. Then, 0.5ml 4% buffered formaldehyde solution was added to stop development of swimming larvae. After 10 mins, the entire plate from each treatment and replicate were counted again. For each treatment, the survival was calculated as follows:

$$survival\ \% = 100 - \frac{\text{number of living larvae}}{total\ number\ of\ larval\ shells}$$

### 4.2.4 Statistical analysis

Larval survival and size were analyzed using Two Way Analysis of Variance (ANOVA), with microalgal concentration (seven levels: initial size, 0.0, 0.25, 0.5, 1, 2x, and CUDL), with survival (%) and shell length (µm) as factors. All data were checked for normality and homoscedasticity using the Shapiro-Wilk and Leven's test, respectively (Quinn & Keough, 2002). Data on larval survival and size were untransformed as they met the assumptions of normaility and homogeneity of variances.

For all analyses, Turkey pairwise comparisons were used to examine the significant differences between the factor levels. All statistical analyses were conducted using the R Studio software R-4.1.0; and the significance was taken at 0.05.

# 4.3 Results

## 4.3.1 2 & 3 days post fertilization (unfed baseline)

Survival of newly metamorphosized larvae was relatively low. This was evident as only 13.84 ± 3.73% were recorded surviving when subjected to thermal stress (Figure 4.3). The overall growth of larvae was also significantly affected (one-way ANOVA, $F_{(2,143)} = 488.7$, $p < 0.001$) by thermal shock. Over a period of 24h the larvae in the CUDL systems grew from an initial size of 104.70 ± 0.65μm to 115.01 ± 0.75. Whereas larvae subjected to thermal stress had significantly (t test, $p < 0.001$) reduced growth rate and were on average 7.95 ± 0.45μm smaller after 24h.

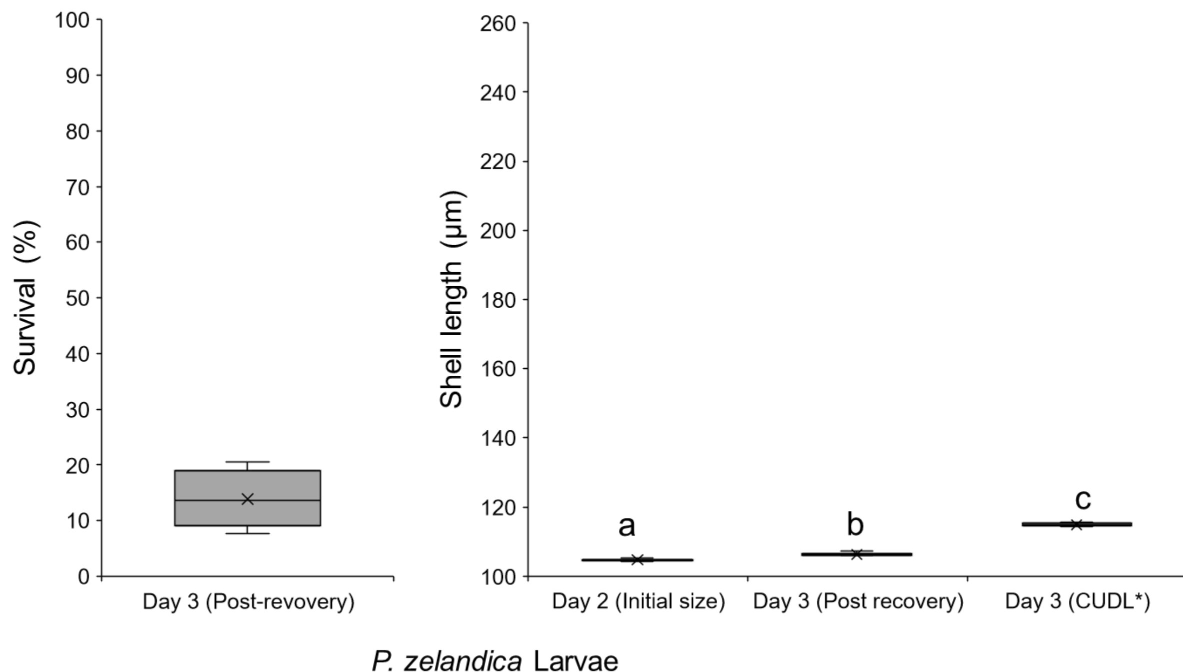



**Figure 4.3**. Survival of larvae post recovery on 3dpf (left). Shell lengths of larvae (right), initial size on 2dpf, and on 3dpf post recovery and reference population (CUDL). Significant differences are denoted by letters above each boxplot. Significance taken at 0.05.

### 4.3.2 2 – 5days post fertilization

The concentration of microalgae provided over 48h (2 – 3 dpf) had significant effect on both survival (two-way ANOVA, $F_{(5,18)} = 6.20$, $p < 0.002$) and shell length (two-way ANOVA, $F_{(5,61)} = 13.38$, $p < 0.001$) of *P.* zelandica larvae after the recovery period (Figure 4.4 top). Unfed larvae on 5dpf had the lowest survival percentage between 27.35 ± 3.19%, with survival of larvae increasing with increasing microalgal concentrations. The highest survival percentage after the recovery period was recorded for the larvae that were obtained from the CUDL's (51.25 ± 1.25%). However, there were no significant (paired t test, p = 1) differences observed in survival of larvae fed microalgal concentrations equal to and above 0.5x (19.3 x $10^3$ microalgal cells).

Over this experimental period (2 – 5dpf), significant growth was recorded within all the dietary treatments including larvae that were unfed (Figure 4.4 bottom). The relative shell size of larvae increased with increasing microalgal concentrations. However, larvae which were fed the highest microalgal concentration of 2x (77 x $10^3$ microalgal cells) appeared to be significantly (paired t test, p < 0.02) smaller than larvae fed at 1x (38 x $10^3$microalgal cells) the concentration of microalgal cells provided in the CUDL's. Largest growth in shell size was observed in the reference population (CUDL's) which had a recorded growth of between 32 ± 2.45μm from an initial size of 104.7 ± 0.65um to 136.78 ± 0.91μm.

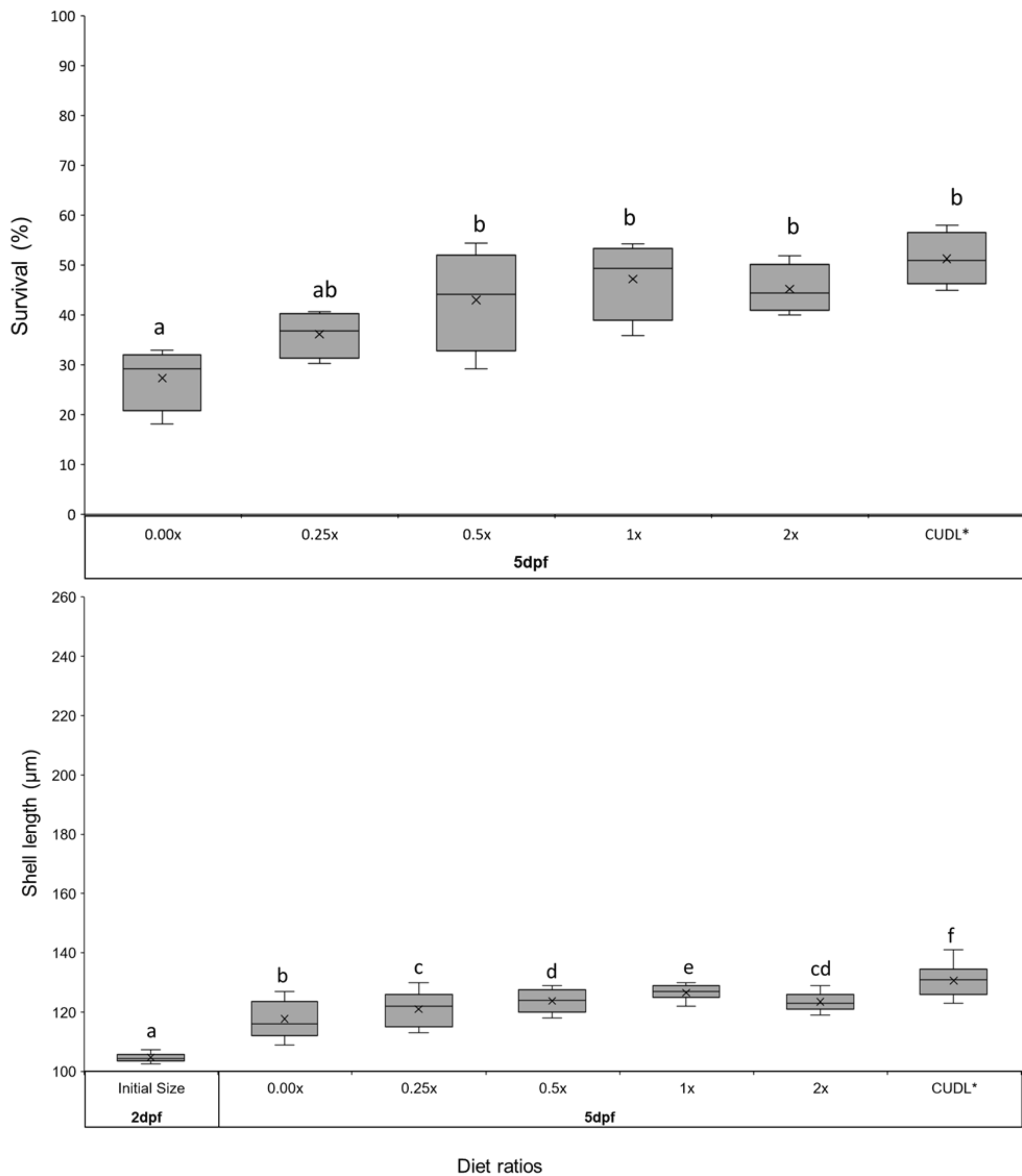



**Figure 4.4**. Survival of larvae post recovery on 5dpf (top). Shell lengths of larvae (bottom), initial size on 2dpf, and on 5dpf post recovery and reference population (CUDL). Significant differences are denoted by letters above each boxplot. Significance taken at 0.05.

## 4.3.3 5 – 8 days post fertilization

Changes in microalgal concentrations over the 48h period on 5 – 6 dpf had a significant effect on both survival (two-way ANOVA, $F_{(5,61)} = 70.84$, $p < 0.001$) and overall shell length (two-way ANOVA, $F_{(5,61)} = 2.96$, $p < 0.001$) of the larvae (Figure 4.5). Unfed larvae

had the lowest survival percentage at 76 ± 1.9% and had an overall smaller shell length at 142.16 ± 3.0μm. An increase in microalgal concentration resulted in an overall increase in larval survival with maximum survival recorded for larvae kept at the same microalgal concentration as the CUDL's (77.38 x $10^3$ microalgal cells) and 0.5x (38.69 x $10^3$ microalgal cells). Larvae provided with twice the highest concentration of microalgae (154.77 x $10^3$ microalgal cells) twice the amount provided for the reference population (CUDL's) showed a decrease in overall survival.

Over the period of 3 days (5 – 8 dpf) the shells of larvae in the CUDL's grew from 145.3 ± 3.71μm to 180.52 ± 0.80μm (Figure 4.5 bottom). There was an increase in shell length recorded for all the late D – veligers from all the treatments. An increase in microalgal concentrations resulted in an increase in overall size. However, larvae that were fed twice (154.77 x $10^3$ microalgal cells) the amount fed to the reference population (CUDL's) had significantly smaller shell lengths than larvae that were fed 1x (77.38 x $10^3$ microalgal cells) concentration of microalgae.

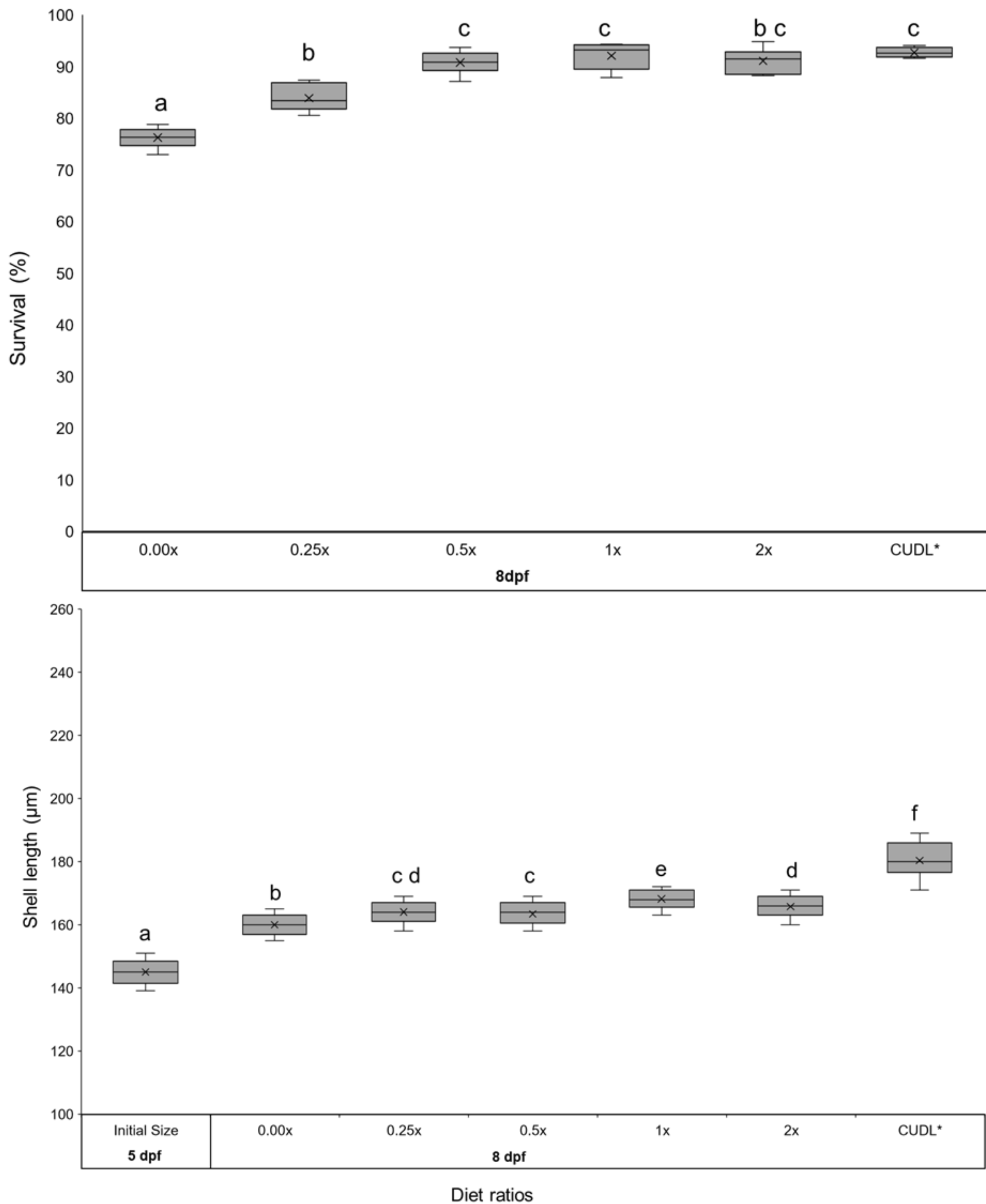



**Figure 4.5**. Survival of larvae post recovery on 8dpf (top). Shell lengths of larvae (bottom), initial size on 5dpf, and on 8dpf post recovery and reference population (CUDL). Significant differences are denoted by letters above each boxplot. Significance taken at 0.05.

### 4.3.4 9 – 12 days post fertilization

Changes in microalgal concentrations over days 9 – 10 dpf had a significant impact on both survival (two-way ANOVA, $F_{(5,43)} = 16.22$, $p < 0.001$) (Figure 4.6 top) and shell length (two-way ANOVA, $F_{(5,177)} = 13.59$, $p < 0.001$) of larvae (Figure 4.6 bottom). On day 12 post recovery unfed larvae had the lowest survival (82.79 ± 0.79%) and had the smallest shell length compared to other treatments (196.53 ± 3.80μm). Both survival percentage and shell length increased with increasing microalgal concentrations. Highest survival and shell length post recovery (12 dpf) were recorded for larvae that were obtained from the reference population (CUDL's) and provided half and same amount of microalgal cells (97.67 x $10^3$ and 195.35 x $10^3$ microalgal cells respectively). However, there was a decrease in both survival and shell growth of larvae fed twice (390.700 x $10^3$ microalgal cells) the concentration of microalgae provided to the reference population (CUDL's).

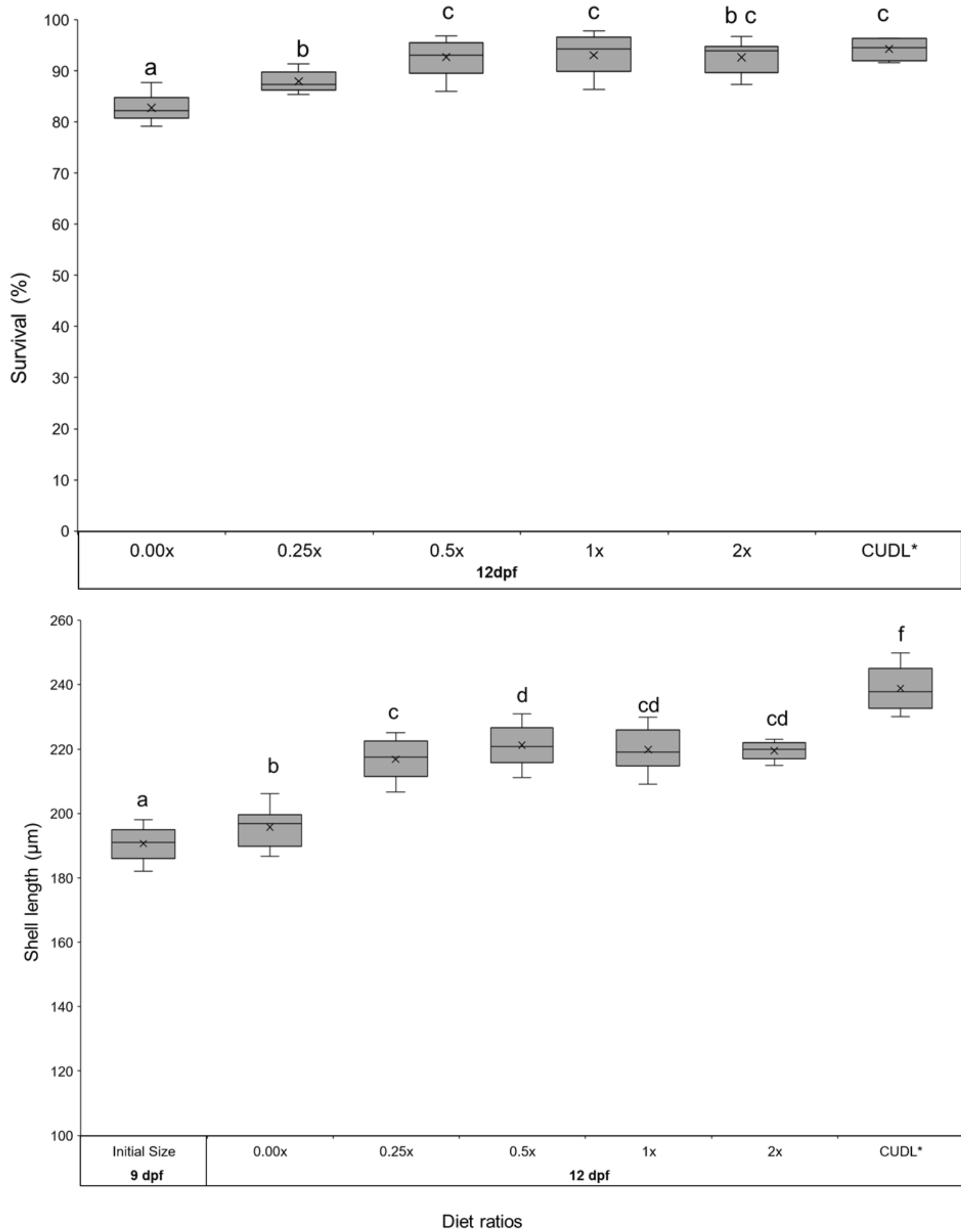



**Figure 4.6**. Survival of larvae post recovery on 12dpf (top). Shell lengths of larvae (bottom), initial size on 9dpf, and on 12dpf post recovery and reference population (CUDL). Significant differences are denoted by letters above each boxplot. Significance taken at 0.05.

## 4.4 Discussion

The results of this study indicate that during the pelagic larval phase of the New Zealand geoduck (2 – 12dpf), not all age classes are equally vulnerable to thermal stress. This was evident as the overall survival of larvae increased with increasing larval age regardless of the concentration of microalgal diet provided. However, microalgal concentration had an important effect within each age class as with increasing microalgal concentration the ability to cope with thermal shock significantly increased. Larval shell length also appeared to be correlated with the concentration of microalgal diet provided, with an increase in microalgal diet resulting in an increase in overall larval shell length. However, when larvae were provided with the highest concentration of microalgae tested (twice the amount provided to the reference population in the CUDL's), there was a reduction in both the survival and overall shell length post recovery.

In marine bivalves, the degree of tolerance to environmental conditions varies during ontogeny (Brown et al., 2004; Ueda & Boettcher, 2009; Verween et al., 2007), with early ontogenic stages tend to exhibit lower tolerance to thermal stressors than the respective later stages. This ability to cope with thermal stress with age was attributed to the ongoing development of the shell and internal tissue in larvae. As larvae later in development are better able to maintain homeostasis and protect their internal organs from the external environment. Indeed, the mussel larvae (*Mytilopsis leucophaeata* & *Dreissena polymorpha*) showed increased ability to cope with thermal changes on 2dpf compared to 4dpf (Verween et al., 2007; Wrightet al., 1996). This is in parallel with the current study as *P. zelandica* larvae regardless of the concentration of microalgae

provided, *P. zelandica* larvae tended to show increased ability cope with thermal stress with increasing developmental age.

However, within each larval age class there was an overall decrease in survival with a decrease in the concentration of microalgae provided. As most of the reserves stored in eggs during gametogenesis are used during embryogenesis (Gallager & Mann 1986; Whyte et al., 1990; Marshall et al., 2010) which coincides with the development of the feeding appendages. This shift from dependence from endogenous reserves to exogenous reserves has been seen in a number of other bivalve larvae such as scallops *Placopectin magellanicus* (Gouda et al., 2004); *P. maximus* (Delaunay et al., 1993), mussels *Mytilus galloprovincialis* (Pettersen et al., 2010), oysters *Crassostrea gigas* (Waldbusser et al., 2013), clams *Ruditapes decussatus* (Albentosa et al., 1999); *Mercenaria mercenaria* (Coutteau & Sorgeloos, 1993) where limitation to external food sources resulted in reduced survival. The capability for synthesis of essential fatty acids in bivalves is very limited and inadequate to meet their nutritional requirements therefore, they must be supplied exogenously (Marshall et al., 2010). Clearly, *P. zelandica* larvae within the same age class appeared to be stressed due to the limitation of food (microalgal diet) and were not able to actively protect from the effects of acute thermal stress.

One important way invertebrates deal with environmental stressors is by the production of heat shock proteins (Hsp's) (Monari, Foschi, Rosmini, Marin, & Serrazanetti, 2011). Heat shock proteins can be induced by heat stress, and the levels of HSP's provide the rapid protection against heat shock (D. Liu & Chen, 2013; Sokołowski & Brulińska, 2018). The heat shock response is an energy consuming process, during the activation of transcription, synthesis of Hsp's, and the ATP dependant chaperoning by HSP's. Hawkins, 1985 (Hawkins, 1985) estimated that the cost of protein synthesis constitutes

to 20 - 25% of the energy budget of the Northern blue mussel, *Mytilus edulis*. Studies on the California native oyster; *Ostreola conchaphila* veliger larvae also showed a significant (fourfold) increase in HSP's expression after 1h heat shock. In this study the *P. zelandica* larvae which were unfed or had a limited microalgal concentration appear to not have had the energy resources to produce HSP's, thus showing lowered resilience to thermal stress.

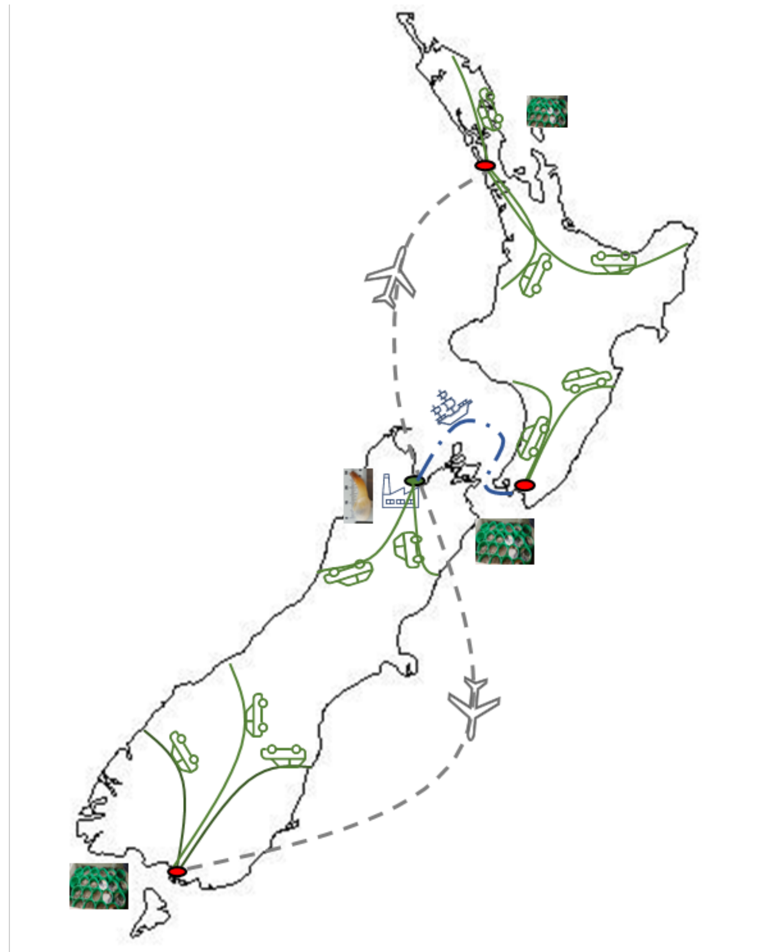
Energy requirements seem to have also played a factor in the different growth rates observed among treatments, as growth appeared to increase with an increase in microalgal concentration. This is due to the increased ingestion rate with increased microalgal concentration. At lower microalgal concentrations, larvae have to spend more energy searching for food while at the same time ingesting less algae (Robert Marshall et al., 2010). This can potentially lead to a depletion of endogenous biochemical reserves and thus ultimately reduced growth (His, Robert, & Dinet, 1989; Ponis, Robert, Parisi, & Tredici, 2003; Tang, Liu, Wang, Zhang, & Xiang, 2006). This is in parallel with the larvae of the scallop *Nodipecten subnodosus* where increased ingestion rates resulted in increased overall growth rates (Angel -Dapa et al., 2021). Increased in ingestion rates with increasing microalgal concentrations was also reported in *P. globosa*, however, the overall growth rate in response to increased diet was not recorded (Ferreira et al., 2015). However, growth rate also corresponds to the maximum ingestion rate as *P. zelandica* larvae provided with extra microalgal cells (twice that of the reference populating in the CUDL's) did not result higher growth rates. This is in agreement with oyster larvae *C. gigas* as there is appears to be maximal growth regardless of the concentration of microalgae provided (Villa et al., 2009).

An interesting trend observed in this study was a reduction of both survival and overall shell growth (length) when larvae were kept on the highest microalgal concentration.

The lower survival can be attributed to the proliferation of opportunistic bacterial and protozoa, which is usually amplified in static systems (Marshall et al., 2014). Also, a major issue with using *C. calcitrans* in the microalgal diet is the extrusion of transparent exopolymers (Corzo, Morillo, & Rodríguez, 2000). At lower concentrations, these exopolymers do not seem to pose any problems, but higher concentration these exopolymers have led to larval clumping and adhesion to surfaces (Ragg, King, Watts, & Morrish, 2010). Larval clumping would have reduced the access to microalgal cells and increased the energy consumption needed to move around in the water column, resulting in higher mortalities and reduced shell growth. This was also observed in *C. gigas* larvae which were reared in static systems, the authors did suggest that the use of a flow-through system is likely to resolve this issue (Villa et al., 2009).

In conclusion, survival, and shell growth of *P. zelandica* larvae post-thermal stress were dependent on the age of the larvae and the concentration microalgae provided. Overall as larvae aged, they tended to perform better in terms of survival and shell growth regardless of the concentration of microalgae provided. This suggested that larvae depend more on exogenous resources acquired daily instead of utilizing stored lipids at earlier larval stages. Within each larval age class microalgal concentrations had a positive impact on both survival and growth, but care must be taken not to over feed the larvae. Indeed, larvae that were provided with the highest concentration of microalgal cells did not have the highest survival and the largest shell growth. At the highest microalgal concentrations, the increased microalgae seemed to impose added stress probably due to exopolymer secretions caused by microalga which resulted in larval clumping which seemed to have impacted larval survival and shell growth.

# SECTION 2: TRANSPORT OF JUVENILES



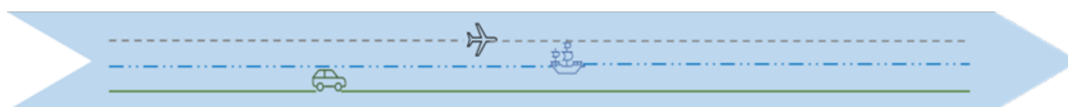


**Figure Sect 2.** Potential transport route and mode of transport from hatchery

# CHAPTER 5 - PHYSIOLOGICAL RESPONSES OF JUVENILE NEW ZEALAND GEODUCK (*PANOPEA ZELANDICA*) FOLLOWING EMERSION AND RECOVERY

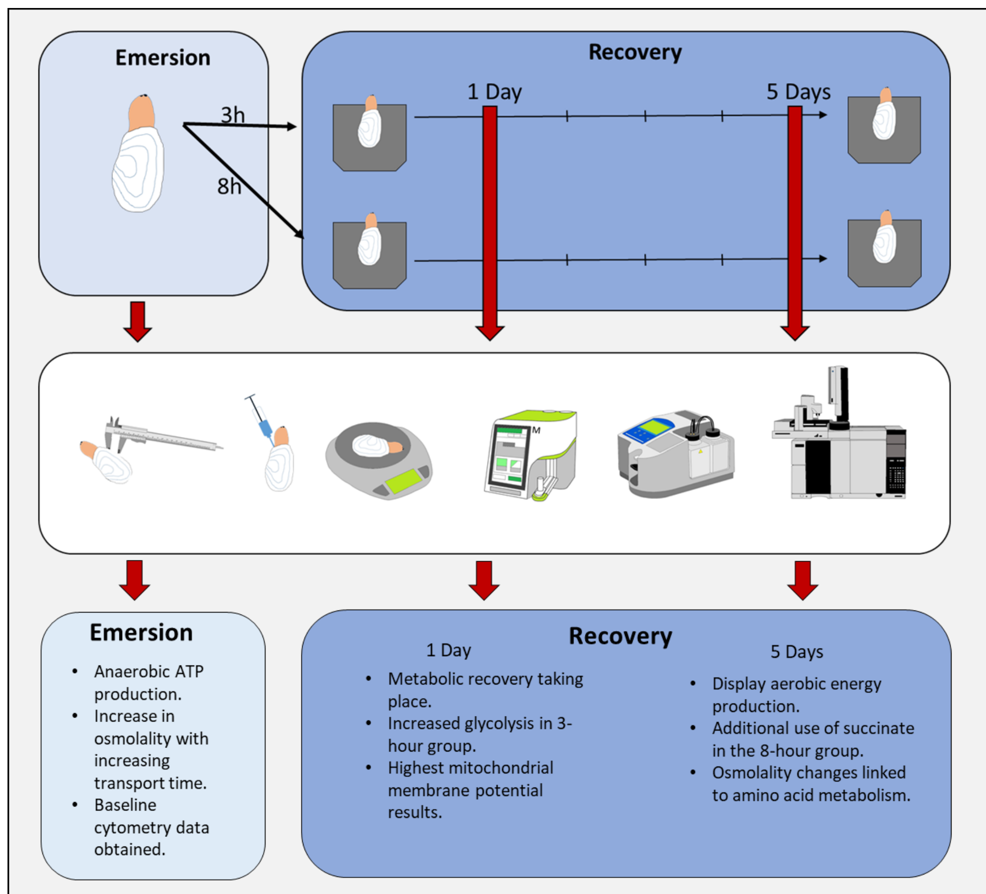



**Figure 5.0** Graphical abstract

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This chapter has been published as:

Sharma, S., Venter, L., Alfaro, A. C., Ragg, N. L., Delorme, N. J., & Zamora, L. N. (2022). Physiological responses of juvenile New Zealand geoduck (Panopea zelandica) following emersion and recovery. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, *41*, 100929.

## Abstract

The New Zealand geoduck clam is a unique seafood delicacy, with animals selling for up to $US 220-330/kg. Stress accumulated during transport of juveniles to grow-out sites represent a bottleneck in the aquaculture process. In this study, the physiological responses of juvenile geoducks following emersion (3- and 8-hours), and recovery (1- and 5-days) were investigated. An integrated approach of flow cytometry, osmolality, and metabolomics, along with behavioural assessments was used. Both cellular and chemical haemolymph parameters and metabolite profiles were recorded for *P. zelandica* juveniles and are reported herein for the first time. An increase in haemolymph osmolality was experienced with an increase in emersion period, with significant differences seen between the 3- and 8-hour emersion groups after 5 days of recovery. Viability measures of haemocytes varied significantly between experimental groups, creating baseline ranges. The proportion of haemocytes undergoing respiratory burst activity did not appear to be affected by emersion and re-immersion. Haemocyte mitochondrial membrane potential was highest following 1-day of recovery, likely linked to metabolic readjustment, and increased glycolysis, taking place following emersion. Metabolomics analyses suggest that protein, lipid, and carbohydrate metabolite classes assist with energy production in geoducks. Activation of anaerobic metabolic pathways, with a high dependence on succinate, were prominent in the 8-hour exposure group, with metabolic recovery still taking place following 5-days of immersion, mainly due to proteins restoring energy reserves. Elucidating the physiological responses of juvenile geoduck subjected to transport stress can aid cultivation methods already underway to develop a novel, high value aquaculture industry.

## 5.1 Introduction

The New Zealand geoduck clam (*Panopea zelandica;* Quoy & Gaimard, 1835) has been receiving considerable attention as an emerging aquaculture species (Alfaro et al., 2014, Gribben and Heasman, 2015), that will contribute to an export target of NZ$3 billion by 2035, set by the New Zealand aquaculture sector (Stenton-Dozey et al., 2020). Geoduck are found in both the North and South islands of New Zealand, with animals selling for $USD220-330 per kg in China (Shamshak and King, 2015). In New Zealand, there is currently no feasible way of obtaining juvenile seed from the wild, highlighting the need for hatchery implementation to supply spat to grow-out farmers (Packer, 2020). As such, considerable advances in hatchery production have been made with *P. zelandica* as a target species. These have been primarily focused on broodstock conditioning (Le et al., 2014, Le et al., 2017a), spawning (Le et al., 2018), and larval rearing (Le et al., 2017b, Sharma et al., 2020). In addition to the successes already achieved to produce spat, the development of culture methods that comply with NZ legislation is ongoing, requiring the production of sufficient spat numbers to allow evaluation of potential grow-out methods (King, 2010).

The grow-out phase of the geoduck farming cycle typically starts when clams are out planted into sediments until the animals reach market size (Vanblaricom et al., 2015). In New Zealand, transport of geoduck spat from a hatchery to grow-out locations in the North Island typically require 8 hours of transport, while the moving of animals to the South Island takes about 3 hours (Cawthron Shellfish Aquaculture Platform, *unpublished data*). For such transport events, the animals need to be removed from their sediment and exposed to air for the duration of the transfer period, until reaching

the designated grow-out destination. Considering that *P. zelandica* is restricted to subtidal habitats and rely on their sediment to keep their valves closed (due to the absence of a well-developed adductor muscle and disproportionately large tissue-to-shell ratio), it is important to minimise the duration of emersion and disinterment. This perspective is reinforced when looking at past findings, where it has been documented that this species does not thrive living out of sediment (Feldman et al., 2004, Gribben and Heasman, 2015). The use of hatchery-reared spat, however, necessitates the removal of animals from their sediment, warranting a deeper look into the stress associated with transport of *P. zelandica* spat to potential grow-out areas.

Stress associated with transport of other bivalves such as mussels (Chandurvelan et al., 2013, Zamora et al., 2019, South et al., 2020), oysters (Dharmaraj et al., 1991) and scallops (Christophersen, 2000) has been well documented, with transport stress ultimately being detrimental to the survival of the animal. For example, simulated transport of great scallop (*Pecten maximus*) spat, showed reduced survival with increasing periods of aerial exposure (Christophersen, 2000). Even though adult geoducks (*P. globosa,* and *P. generosa*) are transported live without seawater to markets (local and overseas) (Shamshak and King, 2015), there is a lack of information regarding the effects of transport on recovery of juveniles. Apart from stressors experienced during transport, the physiological mechanisms implemented by geoduck to survive the subsequent replanting process are also unknown. Typically stress responses can be assessed by considering molecular, cellular, biochemical, physiological and/or behavioural responses (Waller and Cope, 2019). For instance, the presence of siphon apertures in geoducks implies active respiration and feeding; this behavioural observation is currently used as an indicator of general stress

(Davenport and Wong, 1986). In addition, flow cytometry can be used provide useful information regarding haemocyte health (Rolton and Ragg, 2020). Flow cytometry assays such as haemocyte cell count, cell viability and oxidative stress assays, have been used to determine cellular stress responses in other molluscan species (Van Nguyen and Alfaro, 2019, Delorme et al., 2021). To date, flow cytometry has been scarcely applied to geoducks, with the exception of haemolymph sampling previously used in *P. globosa* to characterise haemocyte cell types (Hernández-Méndez et al., 2020). Haemolymph can also be used to track osmolality (electrolyte–water balance), resulting in an indication of the geoducks' capacity to cope with changes in the external environment (Byrne et al., 1989, McFarland et al., 2013). In addition to haemolymph assessments, different geoduck tissues have also been used to investigate physiological changes such as sex identification through specific enzyme-linked immunosorbent assays (Kim et al., 2018), transcriptomic analyses (Juárez et al., 2018), proteomics analyses (Timmins-Schiffman et al., 2020) and now metabolomics analyses. By assessing metabolites, valuable insights into the biochemistry of physiological processes are likely to be gained (Want et al., 2013), as seen in scallops (*P. maximus*) where carbohydrate and glycogen levels were decreased with an increase in emersion time (Fleury et al., 1996, Maguire et al., 1999) and increases in octopine content were documented under oxygen limited conditions (MacDonald et al., 2006).

To better facilitate the link between post-hatchery procedures and pre-grow-out settlement, the aim of this study was to assess the physiological responses of juvenile New Zealand geoduck, *P. zelandica*, following 3- and 8-hour emersion challenge, with a subsequent re-immersion recovery phase of 1- and 5-days, using an integrated

approach consisting of: behavioural observations, haemocyte flow cytometry, haemolymph osmolality measures and full body tissue gas-chromatography/mass spectrometry metabolomics.

## 5.2 Materials and methods

### 5.2.1 Animal husbandry

Six-month-old hatchery-reared *P. zelandica* juveniles produced at Cawthron Institute Aquaculture Park (Nelson, New Zealand) were used for this study. Prior to experimentation, the juveniles were kept in 750 mL holding tanks containing 10 cm depth of substrate (~1 mm grain size sand). These containers were submerged in 50 L tanks in an ambient temperature flow-through seawater system in which they were fed continuously with a standard 1:1 ratio of *Chaetoceros muelleri* (CM) and *Isochrysis galbana* (ISO) diet. The concentration of algal cells in suspension were kept at a constant 40 cells/ µL to ensure that food was always available.

### 5.2.2 Experimental approach

A total of 135 juvenile geoducks (weight 8.3 ± 2.4 g; shell length 28.5 ± 2.9 mm (mean ± SE)) were randomly divided into three experimental groups, allocating 45 individuals to each group: Control (C), Group 1 (G1): 3-hour emersion, and Group 2 (G2): 8-hour emersion (Figure 5.1). The animals from G1 and G2 (90 in total) were removed from their holding tanks by gently separating them from the substrate by irrigating with fresh seawater over a 2 mm sieve while submerged in water to reduce mechanical stress. The remainder of the animals (45 individuals) were kept undisturbed in the tanks and

treated as the control group (C). After recovering the juveniles from the substrate, the G1 and G2 animals were taken out of the water and placed in separated plastic trays with a seawater-saturated cotton-cloth at the bottom and covering the juveniles during aerial exposure. The trays were kept in an incubator set at 13ºC (corresponding to housing temperatures influenced by natural seawater, at the time of the experiment) for emersion simulation of 3 hours (G1) and 8 hours (G2), respectively.

Following each emersion period (3- and 8-hours), 10 animals were taken from the containers and sampled, while the remaining 35 animals were placed back into the seawater tanks for recovery, with two individuals planted in each container and completely buried with sand. Control animals (10) from the undisturbed group were sampled in between the 3- and 8-hour sampling events (about 5-6 hours from the start of the experiment).

During the recovery periods, another 10 animals were sampled from each of the C, G1 and G2 groups at each recovery time (1-day: recovery phase 1 [R1]; 5-days: recovery phase 2 [R 2]). Recovery phases are based on historical data linked to previous grow-out efforts (Cawthron Shellfish Aquaculture Platform person communication).

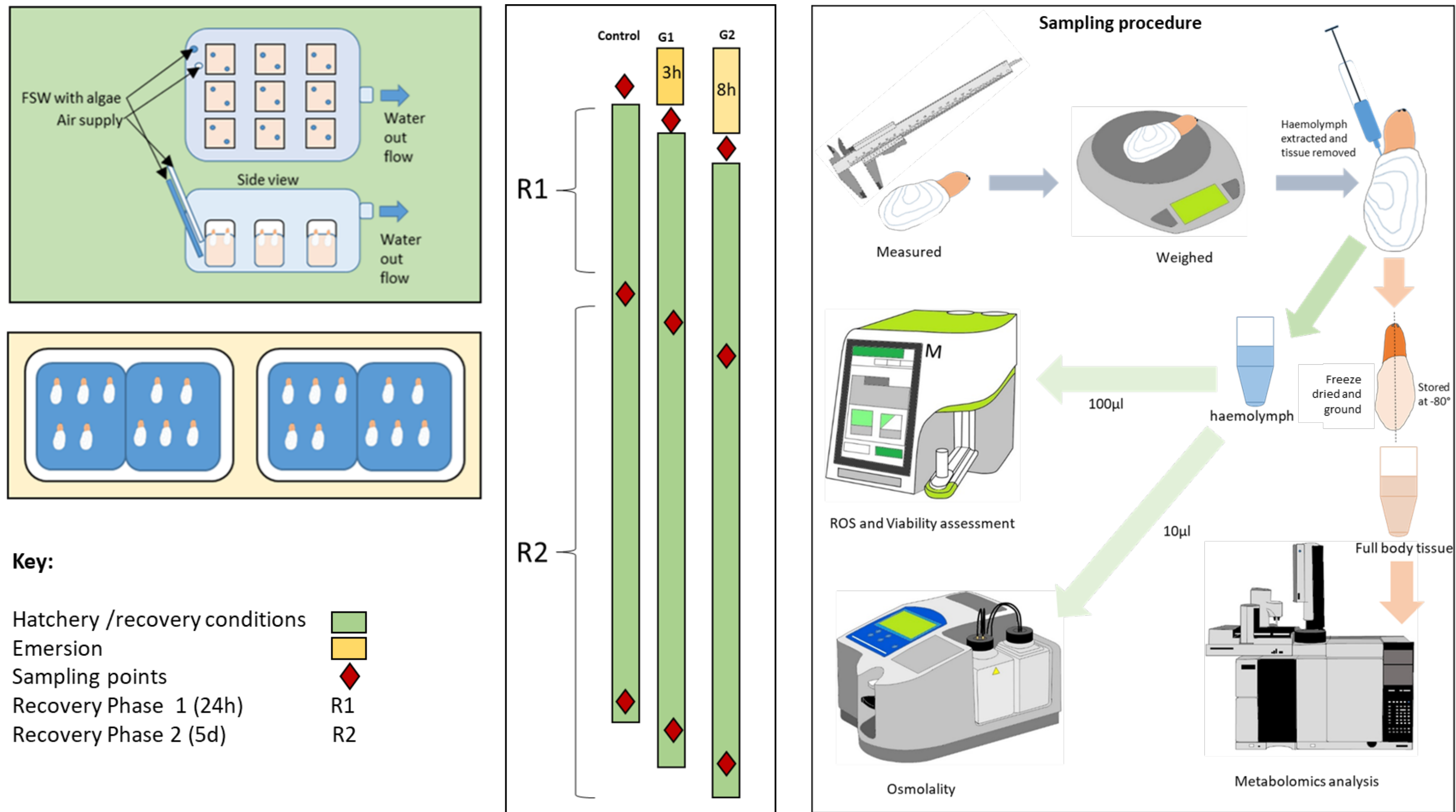


**Figure 5.1:** Experimental approach. Nine groups corresponding to three treatments (C:no emerison, G1:3 hours emersion and G2: 8 hours emersion) and three sampling points for each treatment (at the end of emersion; R1 after 1-day recovery period; R2 after 5-days recovery period) were applied, involving a sampling regime of weight and length measuring, haemolypmh collection, tissue sampling, flow cytometry and osmolality analyses and subesequent GC-MS metabolomics analysis.

### 5.2.3 Behavioural observations after emersion

Animals placed into the recovery tanks were observed every 20 minutes for the first two hours of recovery in seawater and continued twice a day (morning and evening) until day 5 of recovery. The initial observations were used to record the absence or presence of siphons at the surface of the sediment, which was used as an indication that the animals were ready to feed. The daily observations over the period of 5 days considered feeding behaviour of the animals from exposed siphon length and siphon aperture openness (as shown in the supplementary data).

### 5.2.4 Sample collection and analyses

During sampling, all animals in the corresponding group were weighed to the nearest 0.01 g and the shell lengths were measured to the nearest 0.10 mm, along the longest axis, using callipers.

Haemolymph (approximately 500 µL) was extracted from the pericardial cavity at an angle of 45° to the long axis, with a puncture depth of <1 cm. This was performed by placing the animal dorsal side up and using a pre-chilled sterile syringe and needle (Delorme et al., 2020). Immediately after collection, the haemolymph was transferred and stored on ice in pre-chilled microcentrifuge tubes. Pre-chilling the equipment used to collect and store the haemolymph is necessary to maintain haemolymph quality and avoid cell aggregation (Rolton and Ragg, 2020). Haemolymph sub-samples were used for osmolality and flow cytometry measurements (as described below). Animals were opened by inserting a scalpel above the mantle and cutting through the anterior and posterior muscle attachments. The tissue was then separated into two equal samples

by cutting along the dorsal-ventral line. Tissue samples were placed in cryovials, snap-frozen using liquid nitrogen and stored at −80°C until metabolomics analysis was performed.

## 5.2.5 Flow cytometry

A Guava® Muse® Cell Analyzer (Luminex Corporation) was used to determine the total number of haemocyte cells and cell viability, oxidative stress, and mitochondrial transmembrane potential in the haemolymph samples. Commercially available assay kits were used for these analyses (see below). In each case the proportion of cells affected was subsequently expressed at a percentage of the total haemocyte population.

### 5.2.5.1 Cell Viability assay

Cell viability was measured using a Muse® Count and Viability Kit (MCH100102; Abacus Dx, New Zealand), following the manufacturer's specifications. A sub-sample of 20 μL of haemolymph was added to 380 μL Muse® Count and Viability reagent resulting in a working concentration of $5 \times 10^5$ haemocytes $mL^{-1}$, which was vortexed (3 s) and incubated at room temperature (18°C) for 5 min. Analyses were performed using the Muse® Count and Viability assay set to a dilution factor of 20 and acquisition of 1000 events. For threshold settings, a positive control sample was produced via thermal treatment, which was prepared by incubating a haemolymph sub-sample at 100°C for 5 minutes (Lulijwa et al., 2019).

### 5.2.5.2 Oxidative stress assay

Intracellular superoxide (hereafter referred to as ROS) production was measured using a Muse® Oxidative Stress Kit (MCH100111; Abacus Dx, New Zealand). A total of 180 µL of Muse® Oxidative Stress working solution was incubated with 20 µL of haemolymph at room temperature (18°C) for 30 min. Then, the samples were analysed using the Muse® Oxidative Stress assay set to a dilution factor of 10 and acquisition of 3000 events. The setting of the cell size index and gated cell populations on the instrument were verified with the use of a positive control sample. The control sample was prepared by adding 10 µL of a 0.5% hydrogen peroxide solution to 200 µL haemolymph and allowing incubation for 30 min after which 20 µL of this sample was treated as above (Rolton et al., 2020).

### 5.2.5.3 Mitopotential assay

A Muse® MitoPotential Kit (MCH100110; Abacus Dx, New Zealand) was used to measure mitochondrial potential and cellular plasma membrane permeabilization during the early stages of apoptosis. A 100 µL sample of haemolymph was incubated with 95 µL Muse® MitoPotential working solution at room temperature (18°C) for 20 min. Then, 5 µL of 7-aminoactinomycin D (7-AAD) dye were added and incubated for 5 min at room temperature prior to flow cytometric analysis. Cell populations were distinguished prior to analysis by analysing a haemolymph sample treated with carbonyl cyanide m-chlorophenyl hydrazine (CCCP). A 2 µL volume of a 1% CCCP solution was added to 200 µL haemolymph and incubated at room temperature for 60 min to produce a positive control sample. A 20 µL sub-sample was analysed in the

same way as the biological samples described above. Mitopotential results onwards are representative of the total % of depolarised cells.

## 5.2.6 Osmolality

Haemolymph osmolarity was measured using a vapour pressure osmometer (Wescor VAPRO® 5600). For this, a filter paper disc (SS-033, ELITechGroup) was placed in the osmometer's sample holder and 10 µL of haemolymph were dispensed onto the filter, followed by automatic analysis of the sample. Prior to the analyses, a calibration of the osmometer was performed following manufacturer's instructions using a 100, 290 and 1000 mOsmol/Kg osmolality ampule standards (MSPP-OA-010, MSPP-OA-029 and MSPP-OA-100, Opti-Mole™, ELITechGroup). A seawater reference sample was measured every time that samples were collected.

## 5.2.7 GC-MS metabolomics

### 5.2.7.1 Sample processing and metabolite extraction

Geoduck full body tissue samples allocated to metabolomics analysis were freeze-dried overnight and ground using a mortar and pestle following the drying step. Approximately 10 mg of animal tissue were co-extracted with 20 µL L-alanine-2,3,3,3-$d_4$ (10 mM prepared in water) and 500 µL of a cold methanol:water solution (50% MeOH:50% $H_2O$). Samples were vortexed for 1 min and centrifuged for 10 min at 20,800 *g* at -4°C. After transferring of the supernatant to a new microcentrifuge tube, a second extraction step was performed by adding 500 µL cold methanol:water solution (80% MeOH:20% $H_2O$) to the pellet, with vortexing and centrifugation repeated as above. After combining the extracts and overnight freezing of the

supernatants, the samples were freeze dried in a SpeedVac concentrator for 4 h at 0°C under vacuum prior to derivatisation (Young et al., 2019). Quality control (QC) samples were prepared taking a small volume of each biological sample, thoroughly mixed into a homogenous pooled sample (Broadhurst et al., 2018), from which multiple aliquots of 10 mg were prepared as described above.

#### 5.2.7.2 MCF derivatisation

An established methyl chloroformate (MCF) alkylation protocol was used to derivatise the dried extracts (Smart et al., 2010). A volume of 400 µL 1M sodium hydroxide was used to resuspend the extracts and quantitatively transferred to salinized borosilicated glass tubes, followed by the addition of 334 µL methanol and 68 µL pyridine. This was followed by a series of reagent additions and vortexing: 40 µL of MCF reagent - 30s, 40 µL of MCF - 30s, 400 µL of chloroform - 10 s, and 800 µL of 50 mM sodium bicarbonate – 10 s. The mixture was centrifuged at 1,174 *g* for 5 min at 6°C. The upper aqueous layer was discarded, and approximately 30 mg of anhydrous sodium sulphate was added to remove residual water. The chloroform phase containing the MCF derivatives was transferred to 2 mL amber CG glass vials fitted with inserts for GC-MS analyses (Young et al., 2019). Samples were analysed over six days and divided into smaller batches not exceeding a 24-hour analysis window using a randomisation equation (Dunn et al., 2012). Quality control samples were included among the batch which were injected at regular intervals throughout the analytical run of the analysed batch to condition the analytical platform and evaluate daily instrument activity.

#### 5.2.7.3 GC-MS analyses

The MCF derivatives were analysed with an Agilent GC7890B and autosampler coupled to a MSD5977A (Agilent Technologies, USA), with a quadrupole mass selective detector (EI) operated at 70 eV. The system was equipped with a ZB-1701 GC capillary column (30m×250 μm id × 0.15 μm with 5m stationary phase) (Phenomenex, Torrance, CA, USA). Helium was used as the carrier gas (flow of 1 mL $min^{-1}$) and the injector temperature was heated to 260°C. The instrumental setup parameters were conducted according to Smart et al. (2010). Samples (1 μL) were injected under pulsed splitless mode with the injector temperature at 260°C. The helium gas flow through the GC-column was set at a constant flow of 1 mL $min^{-1}$. The GC-oven temperature was initially held at 45°C for 2 min, and then raised with a gradient of 9°C $min^{-1}$ to 180°C. After 5 min, the temperature was increased at 40°C $min^{-1}$ to 220°C. After a further 5 min, the temperature was increased at 40°C $min^{-1}$ to 240°C and held for 11.5 min. Finally, the temperature was increased at 40°C $min^{-1}$ until it reached 280°C, where it was held for a further 2 min. The interface temperature was set to 250°C, the source was set at 230°C and the quadrupole temperature was set at 150°C. The mass spectrometer was operated in scan mode (starting after 6 min; mass range 38–650 amu at 1.47 scans $s^{-1}$). Identification of compounds was carried out using mass spectra acquired in scan mode from 38 to 550 amu, with detection threshold of 100 ion counts. A derivatised sample blank containing the internal standard, a derivatised standard amino acid mix, a non-derivatised standard alkane mix and a sample of pure chloroform solvent were also injected and analysed for QC purposes (Young et al., 2019).

#### 5.2.7.4 Data extraction and processing

Raw spectra were processed using Automated Mass Spectral Deconvolution and Identification System (AMDIS) software integrated with the MassOmics R-based package (University of Auckland) based on protocols described in Smart et al. (2010). Metabolite identifications and peak integrations (relative quantification) were conducted using Chemstation Software (Agilent Technologies) and customised R-XCMS-based scripts (Aggio et al., 2011), based on an in-house mass spectral library of MCF derivatised commercial standards. Compound identifications were based on matches (≥70%) to both the MS spectrum of the derivatised metabolite and its respective chromatographic retention times. Thus, the identified compounds can be assigned a level 1 and 2 identification and the unknown features receiving a level 3 confidence interval (Schymanski et al., 2014). Analyses were carried out in 'R' platform version 3.3.1. A Microsoft® Excel file containing peak height data for each metabolite was generated and manually checked for the presence of contaminants (e.g., MCF derivative artefacts). Data were normalised to sample biomass and, to remove non-biological variation, the data were normalised using the mass spectrometry total useful signal normalisation method (Lindeque et al., 2018). The QC samples analysed among the processed batches were assessed in terms of coefficient of variance percentages and visual interpretations (Wehrens et al., 2016). The data were evaluated for a within batch effect. As no visible effect was seen, no correction methods were applied. The pre-treatment steps were completed by using the webserver MetaboAnalyst (www.metaboanalyst.ca) (Chong et al., 2018). Missing value estimation was performed, whereafter the data were transformed using the generalised logarithm (glog) transformation function before statistical analysis (Lindeque et al., 2015).

### 5.2.8 Statistical analyses

Statistical analyses for animal measures, observations, flow cytometry and osmolality data were done in R Studio (version 1.4.1103) with sampling time (three levels: post emersion, R1, and R2) and treatment groups (three levels: control, G1, and G2) as factors. Tukey pairwise comparisons were used to examine differences between factor levels and significance was taken at 0.05.

#### 5.2.8.1 Animal size and behavioural observations

One-way Analysis of Variance (ANOVA) tests were performed to determine if experimental groups differed statistically based on weight and shell length measurement data. Behavioural data on the presence and absence of visible siphons on the sediment surface were analysed among treatments and at each time point (20 min intervals) using one-way repeated measures ANOVA. Pairwise t tests were used to compare difference in number of siphons visible over time for each treatment group.

#### 5.2.8.2 Flow cytometry data

Results from the flow cytometric analyses (cell counts, viability, reactive oxygen species [ROS], and mitopotential) were each analysed using a Two-Way ANOVA with treatment groups and recovery periods as factors.

#### 5.2.8.3 Osmolality data

Osmolality results were analysed separately for each treatment at each sampling point against the control group using a Two-Way ANOVA with treatment groups and recovery periods as factors.

### 5.2.8.4 Metabolomics data

Univariate statistical analyses were performed with MetaboAnalyst, using one-way ANOVA to identify significant differences between the experimental groups (Control, Group 1, and Group 2). This was followed by post-hoc analyses (Fisher's LSD). Features with a *p*-value of <0.05 and *q*-value (false discovery rate [FDR] adjusted p-value) of <0.10 were considered statistically different among the groups (Figueroa et al., 2013). Findings were cross referenced with the online server, Kyoto Encyclopaedia of Genes and Genomes (KEGG), to assign metabolite classes to each significant feature. This classification was used to manually construct a broad metabolic map of the metabolite findings.

# 5.3 Results

## 5.3.1 Animal size

The wet weight and shell length data (Table 5.1) of the control group were not statistically different (ANOVA, $F_{(2,42)} = 1.35$, $p = 0.26$; $F_{(2,42)} = 2.70$, $p = 0.07$, respectively) when comparing G1 and G2, irrespective of treatment (Supplementary Fig.1S). Therefore, differences described below can be attributed to the physiological response and not biological size variation.

**Table 5.1**. Wet weight and shell length data of juvenile geoducks from different treatment groups.

| Experimental Group | | Mean Wet Weight ± SE (g) | Mean Shell Length ± SE (mm) |
|---|---|---|---|
| **Control** | 0h emersion (C E) | 7.8 ± 2.2 | 28.2 ± 2.3 |
| | 1d recovery (C R1) | 8.0 ± 2.2 | 28.3 ± 2.1 |
| | 5d recovery (C R2) | 7.6 ± 2.3 | 27.0 ± 3.1 |
| **3-Hour emersion group (G1)** | 3h emersion (G1 E) | 8.3 ± 2.5 | 29.3 ± 2.3 |
| | 1d recovery (G1 R1) | 8.1 ± 1.9 | 29.7 ± 3.1 |
| | 5d recovery (G1 R2) | 9.9 ± 3.2 | 29.3 ± 4.7 |
| **8-Hour emersion group (G2)** | 8h emersion (G2 E) | 9.1 ± 2.2 | 29.8 ± 2.7 |
| | 1d recovery (G2 R1) | 7.5 ± 2.8 | 27.0 ± 1.9 |
| | 5d recovery (G2 R2) | 8.5 ± 1.1 | 27.6 ± 2.1 |

## 5.3.2 Behavioural observations

Clear statistical differences were observed in the number of siphons visible over time among controls and the emersion after re-immersion (ANOVA, $F_{(7,57)} = 24.91$, $p < 0.05$). After 18h of immersion there were no difference in the number of siphons visible between the experimental groups (t-test, p = 1). After 2 days of behavioural

observations during recovery, 100% of siphons from all the treatment groups were visible with no significant differences among them (Figure 5.2).

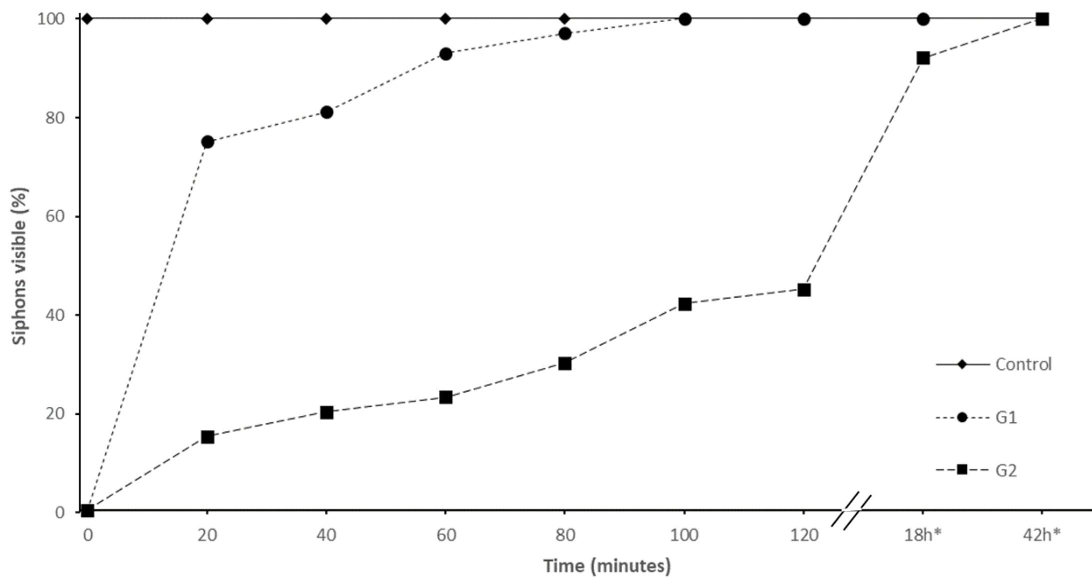


**Figure 5.2.** Percentage of juvenile geoduck siphons visible in holding tanks during recovery in seawater following emersion (*hours instead of minutes, i.e., 18 and 42 hours) with the undisturbed control group (◆), 3-h emersion (G1) group (•) and 8-h emersion (G2) group (▪) represented.

### 5.3.3 Physiological response to emersion

Similar responses in the proportion of viable haemocyte were observed for the control (C: 59%), G1 (51%), and G2 (63%) groups following emersion (ANOVA, $F_{(2,27)} = 0.87$, $p = 0.43$) (Figure 5.3A). No significant differences were observed in the proportion of haemocytes determined as 'ROS-positive' among the different experimental groups (ANOVA, $F_{(2,27)} = 0.74$, $p = 0.49$), with the control, G1, and G2 producing 39, 47 and 39%, respectively. The proportion of haemocytes demonstrating depolarised mitochondrial membranes in the control, G1, and G2 groups were 32, 28 and 31%, respectively (ANOVA, $F_{(2,27)} = 0.34$, $p = 0.72$).

The haemolymph osmolality was significantly different among emersion groups (ANOVA, $F_{(2,27)} = 23.07$, $p < 0.001$), with control, G1 and G2 groups having an osmolality (mean ± SE) of 1024 ± 1.8, 1026 ± 1.6, and 1033 ± 1.8, mOsmol $kg^{-1}$, respectively (Figure 5.3C), while seawater (reference sample) had an osmolality of 1018 mOsmol $kg^{-1}$. Pairwise comparisons revealed that haemolymph osmolality increased significantly between 3 (G1) and 8 h (G2) emersion.

Following emersion (3- and 8-hours), a total of 55 significantly different metabolites were detected by GC-MS (Supplementary Table 5.1S). When comparing the 3-hour emersion (G1) group to the control group the following metabolites showed an increase in relative abundance (Figure 5.3C): 10-heptadecenoate (C17:1); 2-aminobutyric acid; 3-hydroxyisobutyrate; 4-aminobutyrate; 4-methyl-2-oxopentanoate; aminomalonate; dihomo-gamma-linolenate; eicosapentaenoic acid; glutamate; hexadecanoate; homocysteine; linoleate; LL-2,6-diaminopimelate; oleate; palmitoleic acid; succinate and undecanoic acid, while aspartate and 2-oxobutanoate showed a decrease. In contrast, compared to the control group, G2 (8-h emersion) displayed significantly lower levels of: 2-aminobutyric acid; 2-aminoisobutyric acid; 2-oxobutanoate; aspartate; azelaic acid; benzoate; carbamate; cis-4-hydroxy-L-proline; cystathionine; docosahexaenoate; docosanoate; eicosapentaenoic acid; fumarate; gamma-linolenic acid; glutarate; glycine; heptadecanoic acid; hexadecanoate; linolenate; LL-2,6-diaminopimelate; octanoate; oxalate and tetradecanoate. All the metabolites detected after 8 hours of emersion showed a decrease in relative abundance in the G2 group when compared to G1.

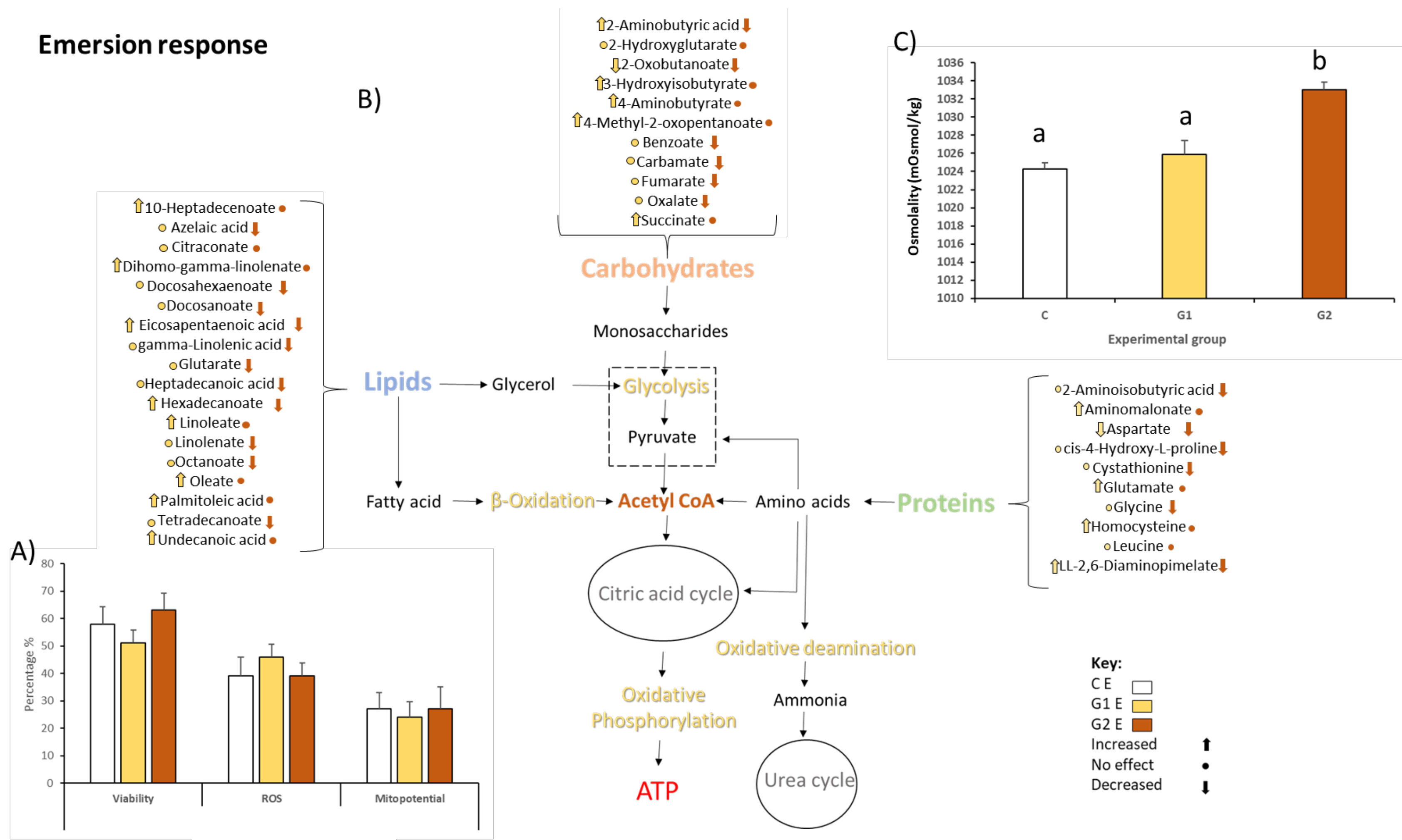


**Figure 5.3.** The haemocyte (A), metabolic (B) and osmolality (C) response of juvenile *Panopea zelandica* following emersion. Statistical differences among treatments ($p<0.05$) are denoted by the lowercase letters above each bar, while indicators (↑•↓) to the left of the metabolites represent the response from the 3-h emersion (G1 E) group those found on the right-hand side are representative of the 8-h emersion (G2) group.

### 5.3.4 Physiological response of recovery phase 1 (R1: 1-day)

Haemocyte viability after 1-day recovery (R1) showed no significant differences between the control (50%), G1 (46%) and G2 (55%) groups (ANOVA, $F_{(2,27)} = 0.61$, $p = 0.55$) (Figure 5.4A). The proportion of ROS-positive haemocytes was 44% for the control group, 39% for G1 and 48% for G2, with no significant differences among groups (ANOVA, $F_{(2,27)} = 1.54$, $p = 0.23$) (Fig. 4A). The percentage of cells with depolarised mitochondrial membranes significantly increased from 24% in both the control and G1 groups to 35% in G2 (ANOVA, $F_{(2,27)} = 8.5$, $p < 0.05$) (Figure 5.4A).

Haemolymph osmolality showed no significant differences between the experimental groups (ANOVA, $F_{(2,27)} = 0.70$, $p > 0.5$) following the initial recovery phase (Figure 5.4C). Mean osmolality (±SE) of haemolymph was 1020 ± 1.4 mOsmol $kg^{-1}$ for the control group, 1021 ± 2.2 mOsmol $kg^{-1}$ for G1, 1022 ± 1.7 mOsmol $kg^{-1}$ for G2 and 1014 mOsmol $kg^{-1}$ for the reference seawater sample (Figure 5.4C).

The metabolic response following one day of re-immersion resulted in 18 significantly different metabolites. When comparing G1 to the control group, metabolite increases were seen in lysine, phosphoenolpyruvate, threonine and tryptophan, while decreases were seen in the metabolite abundance of: 3-hydroxyisobutyrate, aspartate, citraconate, glyoxylate, heptadecanoic acid and hydroxybenzoic acid. Compared to the control group, G2 resulted in increased 2-oxobutanoate and threonine and decreased levels of: 3-hydroxyisobutyrate; aspartate; citraconate; fumarate; glyoxylate; heptadecanoic acid; LL-2,6-diaminopimelate; lysine; malate; stearate and succinate.

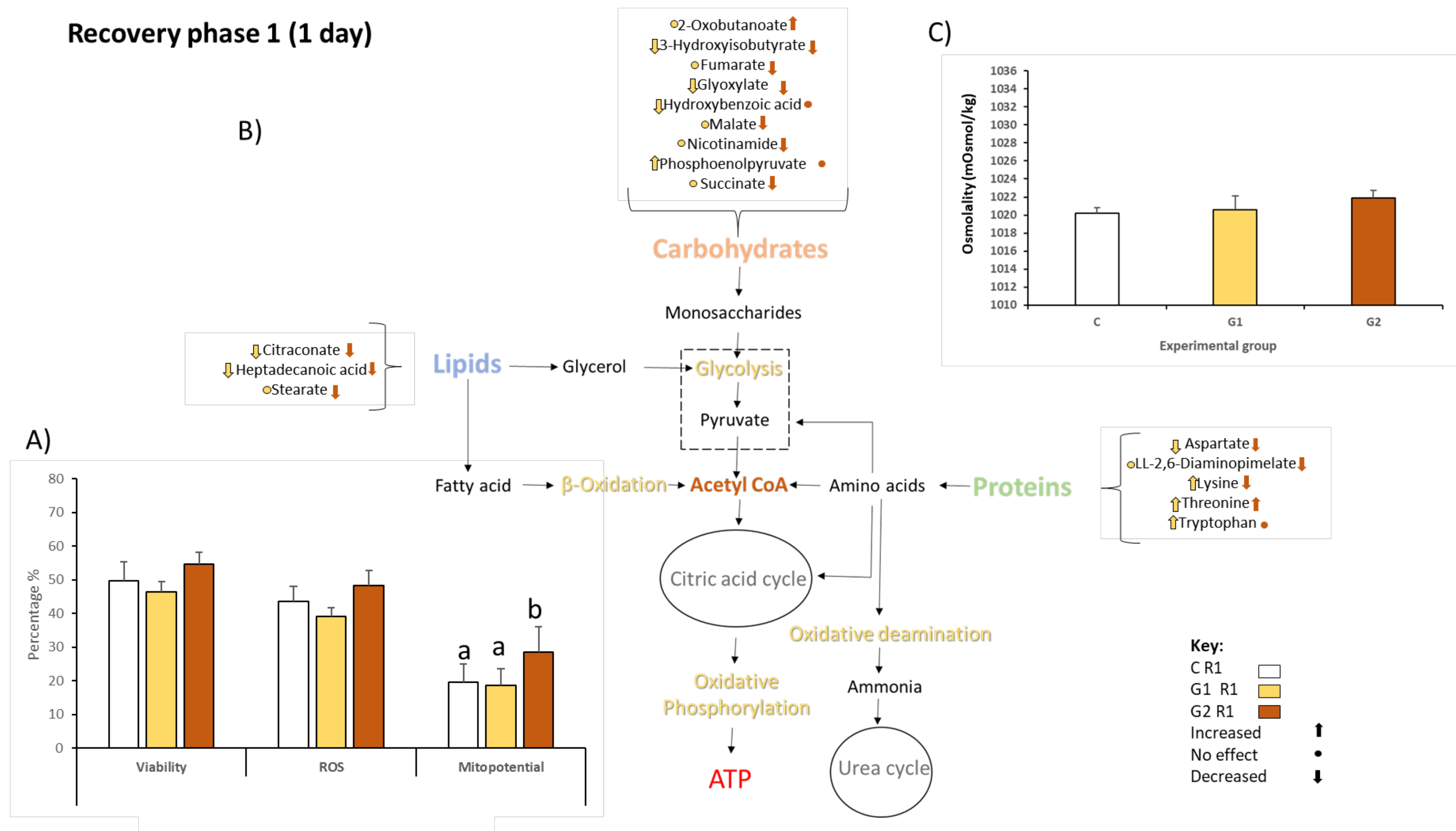


**Figure 5.4.** The haemocyte (A), metabolic (B) and osmolality (C) response of juvenile *Panopea zelandica* following the first recovery phase (1-day). Statistical differences among treatments (*p<0.05*) are denoted by the lowercase letters above each bar, while indicators (↑•↓) to the left of the metabolites represent the response from the 3-h emersion (G1 E) group those found on the right-hand side are representative of the 8-h emersion (G2) group.

### 5.3.5 Physiological response of recovery phase 1 (R2: 5-day)

Following 5-days of recovering in standard housing conditions, results from flow cytometry assays showed no statistical differences for any of the three assays performed (ANOVA, $F = 3.4$, $p = 0.05$). The number of viable haemocytes was 35, 48 and 38% for the control group, G1 and G2, respectively (Figure 5.5A). The proportion of ROS-positive haemocytes was very similar among groups (C = 49%, G1 = 47% and G2 = 44%) (ANOVA, $F_{(2,27)} = 1.54$, $p = 0.23$) (Figure 5.5A). The percentage of cells exhibiting depolarised mitochondrial membranes was 27%, 30% and 20% for control, G1 and G2, respectively (ANOVA, $F_{(2,27)} = 0.39$, $p = 0.68$) (Figure 5.5A).

Haemolymph osmolality was significantly different among groups (ANOVA, $F_{(2,27)} = 3.9$, $p = 0.04$), with values for the control group of 1018 ± 1.6 mOsmol $kg^{-1}$, for G1 of 1017 ± 1.7 mOsmol $kg^{-1}$, and for G2 of 1014 ± 1.6 mOsmol $kg^{-1}$, with osmolality being lower in the G2 group compared to G1 (t test, $p < 0.05$) (Fig. 5C). The reference seawater sample had an osmolality value of 1017 mOsmol $kg^{-1}$.

Metabolomics analyses after five days recovery resulted in 13 significantly different metabolites, somewhat fewer than for previously sampled treatment groups. Comparing G1 to the control group resulted in increased: 2-aminobutyric acid, glutamine, homocysteine, leucine, ornithine, tryptophan and tyrosine. Comparisons between G2 and the control group resulted in increased: 2-aminobutyric acid, glutamine, leucine, lysine, ornithine, tryptophan and tyrosine, while fumarate, malate

and succinate were decreased. Comparisons between treatment groups can be found as supplementary material (Table 5.3S; Figure 5.5B).

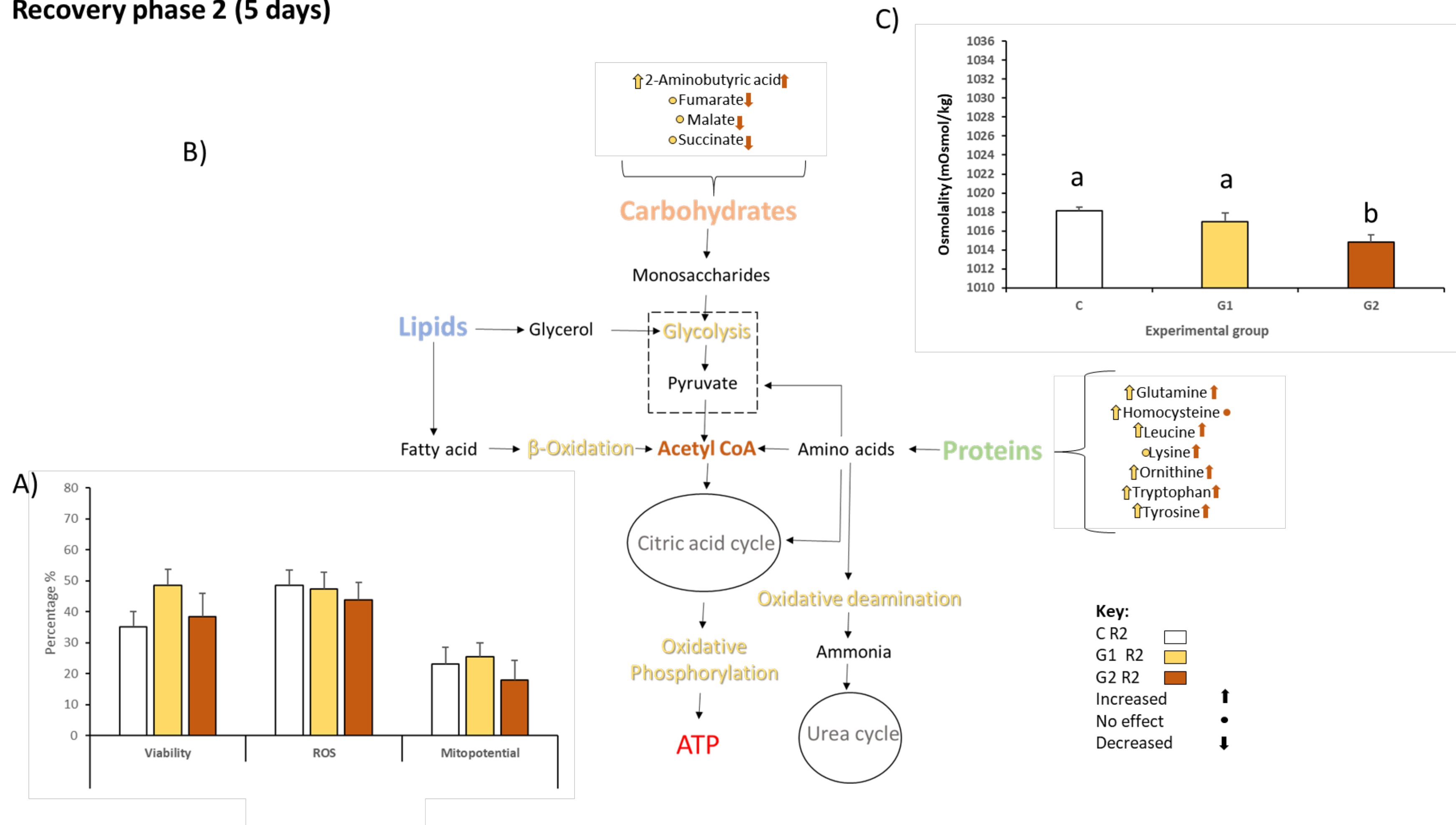


**Figure 5.5.** The haemocyte (A), metabolic (B) and osmolality (C) response of juvenile *Panopea zelandica* following the second recovery phase (5-days). Statistical differences among treatments (*p<0.05*) are denoted by the lowercase letters above each bar, while indicators (↑•↓) to the left of the metabolites represent the response from the 3-h emersion (G1 E) group those found on the right-hand side are representative of the 8-h emersion (G2) group.

## 5.4 Discussion

The monitoring of cellular and chemical properties of haemolymph, the metabolite profiles of tissues and behavioural observations, in response to transportation and subsequent recovery in juvenile *P. zelandica* are reported here for the first time. Following 3- and 8-hours of simulated transport, no significant differences were observed for the three sets of flow cytometry results, while haemolymph osmolality showed an increase in the 8-hour group and metabolomics revealed an altered metabolic response for both treatments. The recovery of experimental animals (8-hour group) after 1-day resulted in a significant increase in mitochondrial membrane potential response. After 5 days of recovery there were still differences observed within haemolymph osmolality and metabolic response. The physiological responses of geoduck after each of the experimental phases: 1) emersion; 2) 1-day recovery (R1) and 3) 5-day recovery (R2) (will be characterised and discussed separately in the following sections, in terms of osmolality, amino acid (protein), carbohydrate, and lipid metabolites, and haemocyte responses, focusing on the response of both the 3-hour (G1) and the 8-hour transported groups (G2).

### 5.4.1 Emersion response

Following emersion an increase in haemolymph osmolality was observed with an increase in transport time. The lack of seawater used in the transport phase potentially resulted in a significant increase in haemolymph osmolality, in the 8-hour transported group, which can be attributed to mild dehydration or a lack of gas exchange, causing low molecular organic substrates to be retained (Natochin and Parnova, 1987, Dong et al., 2019). Typically, osmoconformers like geoduck will increase their respiration

rates when an increase in the osmotic environment is experienced, with organic osmolytes (such as amino acids) utilised to increase intracellular osmolality (Rivera-Ingraham and Lignot, 2017).

As a result of changes in osmolality metabolism will be geared towards amino acid production, as seen in the 3-hour emersion group where most of the amino acids detected are increased relative to the immersed controls, as previously been reported in *Mytilus edulis* (Zurburg and De Zwaan, 1981). Apart from their osmolyte function, invertebrates can utilise amino acids to generate nicotinamide adenine dinucleotide ($NAD^+$) (Venter et al., 2018a) and to balance $NADH/NAD^+$ ratios (Carroll and Wells, 1995) to support aerobic and anaerobic glycolysis pathways, supported by the metabolite response in both the 3-and 8-hour groups. The involvement of amino acids in reactive oxygen species (ROS) regulation is also seen in the current study, where metabolites like homocysteine, cystathionine, glutamate, glycine and 3-hydrioxyisobutyrate (affected in the both 3 and 8-hour groups), are used to produce glutathione through a transsulfuration pathway. The glutathione pathway is associated with ROS regulation, and prevention of cell damage (Timmins-Schiffman et al., 2020), as also seen in *Crassula aequilatera* (Alfaro et al., 2019). An initial increase in ROS production was seen in geoduck following 3-hours of aerial exposure with a decrease found after 8-hours, as indicated by modest changes in the proportion of ROS-positive circulating haemocytes. Oxygen variability is known to cause higher ROS levels (Spencer et al., 2019) and together with the fact that molluscan haemocytes undergo respiratory burst producing ROS in response to stressors (Timmins-Schiffman et al., 2020) an increase in ROS production can be expected following 3-hours of aerial exposure. A similar respiratory burst response has been reported in the larvae of *P.*

*generosa,* in response to ciliates (Timmins-Schiffman et al., 2020). The subsequently decreased ROS in the 8-hour group can be attributed to a state of metabolic depression associated with exposure to acute stressors where the metabolic rate is restrained (Wang et al., 2018).

In addition to amino acids, carbohydrates support glycolysis as seen in the 3-hour group where an increase in carbohydrates (sugars) were found. The metabolite findings in the 3-hour emersion group corresponds to that seen in *Mytilus galloprovincialis*, where air exposure showed that endogenous fuel (like adenine triphosphate (ATP) and phospho-arginine) are being utilised and anaerobic metabolites (like succinate found in *P. zelandica* and propionate) are accumulating to overcome the oxygen-poor scenario (Babarro and De Zwaan, 2008). Furthermore, changes in aminomalonate, glycine and oxalate (which are precursors of pyruvate) from the current study (3-hour group), supports glycolic functions, and the synthesis of pyruvate (and subsequent $NAD^+$ production) (Venter et al., 2018c) Decreased metabolic reactions (linked to carbohydrate and protein metabolites) were observed in the 8-hour group. A reduction in net metabolic rate is a common characteristic of anaerobic metabolism, resulting in increased carbohydrate consumption (De Zwaan and Wijsman, 1976), as supported by the findings of the 8-hour group. The decrease in citric acid cycle metabolites and precursors (fumarate, glutamate and aspartate) demonstrates the usage of carbohydrates clearly. Amino acids like glutamate and aspartate are well known metabolic fuels during anaerobic conditions (Venter et al., 2018b), and their reduced levels in *P. zelandica* links to a replenishing role, to ensure citric acid cycling for ATP production, even in smaller quantities with prolonged transport time.

Unlike proteins and carbohydrates, lipid metabolites were mostly affected in the juvenile geoducks subjected to transport in the current study. This is in contradiction to research done on *P. generosa,* where carbohydrates were more abundant than lipids in both the siphon and the mantle of the adult animals studied (Hernández-Méndez et al., 2020). Yet, accumulation of lipids have been shown to be critical for post-metamorphic survival and early juvenile performance in bivalves (Przeslawski et al., 2008), supporting the notion of abundant lipids in the 3-hour transported juvenile geoducks. Also, an increase in the unsaturated fatty acids, dihomo-gamma(□)-linolenate (20:3n6), oleate (18:1n9) and palmitoleic acid (16:1) in this study links to stabilising effects of membrane lipids, to facilitate functioning of membrane-bound proteins during periods of short-term anoxia, as seen in *Mytilus edulis* (Fokina et al., 2017). Increases in the fatty acids: hexadecanoate (C16); linoleate (C18:2), and undecanoic acid (C11) suggest sufficient lipid synthesis prior to aerial exposure, to be used via beta (ß)-oxidation to produce acetyl-coenzyme A (CoA) and eventually NADH and flavin adenine dinucleotide ($FADH_2$) for electron transport chain functioning to support ATP production (Lopaschuk et al., 2010). The lipid metabolites in the 8-hour group showed reduction in free and bound fatty acids (azelaic acid; docosanoate; hexadecanoate; linoleate) suggesting a high fatty acid release rate during the prolonged time out of water, to meet increased energy demand during stress (Sonawane and Lomte, 2015). Essential fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoate (DHA) have been found to be important contributors to gametogenic development in geoduck (Le et al., 2017a). However, in the current study, reduced levels of EPA and DHA following 8-hours of aerial exposure verifies their highly regulated metabolic roles (for survival and adaptive responses to

environmental effects), as they cannot be *de novo* synthesised in marine bivalves (Fokina et al., 2017).

As well as input from carbohydrate, protein and lipids sources, varying cellular energy needs can be regulated by the electrochemical potential across the inner mitochondrial membrane, which is seen as a reflection of the bioenergetic state of the cell (Huang, 2002). Ectotherms are said to generally exhibit a low mitochondrial membrane potential (Abele et al., 2002) and here the number of total haemocytes with depolarised mitochondrial membranes detected following both 3 and 8-hours of emersion was relatively low and insignificant to the control. Furthermore, the haemocyte viability data displayed no statistical differences between the control, and exposure groups, but can be used to accumulate baseline data for *P. zelandica.* Interestingly, in *P. globosa* two main haemocyte types have been identified, namely hyalinocytes and granulocytes, with a reported cell viability of 90% (Hernández-Méndez et al., 2020); in contrast, mean viability in the present study was relatively low and variable (35 – 59%), suggesting that baseline condition may have been compromised and/or the assay may need refinement for application to this species.

### 5.4.2 Recovery phase 1 response (R1, 1-day)

As in the case of the emersion groups, the 1-day recovery groups showed an increase in haemolymph osmolality with an increase in aerial exposure time (however, this increase is negligible and not significant). A similar response was observed in the freshwater bivalve *Corbicula fluminea*, with osmolality returning to normal within 6-hours after emersion (Byrne et al., 1989).

As for the metabolic response displayed by the recovery groups, the 8-hour emersion group showed the most changes, with decreases seen in carbohydrates, proteins, and lipids. This links to the findings of de Zwaan et al. (1983), where recovery taking place following anaerobic metabolism resulted in re-synthesis of energy reserves and anaerobiosis substrates (De Zwaan, 1983). This is apparent when focusing on the citric acid cycle metabolites, succinate, fumarate, malate and aspartate (precursors) which were all decreased in this study, linking to the glucose/aspartate-succinate pathway, responsible for the accumulate reduced equivalents for synthesis of $NAD^+$ and $FAD^+$ (Venter et al., 2018a). In effect, the low citric acid cycle metabolites observed in the both the 3- and 8-hours groups, are likely to be replenishing metabolite stores to ensure oxidation of acetyl-CoA for oxidative phosphorylation. In the clam *Scapharca inaequivalvis*, a recovery period of 1-day in seawater was not long enough for restoration of energy reserves following anaerobiosis (Isani et al., 1989), as seen in the geoduck subjected to 8-hours of aerial exposure.

The 3-hour exposure group responded differently to 1-day of recovery with an increase in phosphoenolpyruvate, activating phosphoenolpyruvate carboxykinase to produce succinate via oxaloacetate (Isani et al., 1989), seen as one of the most obvious differences in this group, Resultantly, increased ATP yields from glucose are experienced as extra substrate-level phosphorylation reactions take place (Venter et al., 2018a). Despite the 3-hour exposed group using different routes for ATP production, an increase in the amino acids, lysine, threonine and tryptophan was also experienced. In marine bivalves, cell volume changes are quickly restored by amino acid levels in the cytoplasm and some are also considered important substrates for oxidative metabolism (Ballantyne and Storey, 1985). Lysine is classified as a

ketogenic amino acid forming acetoacetyl-CoA as end-product which feeds into the citric acid cycle. The most prominent role of threonine is the ability to be catabolised to pyruvate. Catabolism of tryptophan results in important precursors for the synthesis of $NAD^+$ and $NADP^+$ (Salway, 2016). In all instances elevation of these amino acids supports respiratory fuel for aerobic ATP production and highlights the increased need of geoduck to fulfil metabolic losses following anaerobic conditions.

Lipids are generally used as an energy source, once protein and carbohydrates sources are depleted, whereafter lipids are metabolised to glycerol and fatty acids, and used as metabolic intermediates to assist with ATP production (Venter et al., 2018a). The decreased fatty acids, citraconate, stearate and heptadecanoic acid seen in the recovery phase of both the 3- and 8-hour exposure groups attests to the likelihood that fatty acids were utilised by the animals during aerial exposure, leaving these sources depleted in the 1-day recovery phase.

The flow cytometry results reflected no significant differences between the control, 3- and 8-hour groups following 1-day of recovery, when looking at cell viability and ROS production. The number of live cells with depolarised mitochondrial membranes, however, differed between the 3- and 8- hour groups, suggesting that a 1-day recovery period is generally sufficient to obtain the similar haemocyte activity between controls and aerial exposed groups (but a slight increase in subsequent cell death may be apparent beyond one day of re-immersion). Results from the literature suggest that re-oxygenation in *Crassostrea gigas* caused oxidative burst, leading to an increase in ROS production (Sussarellu et al., 2013). In *Mytilus galloprovincialis* a subsequent decrease in ROS, ascribed to a decrease in biochemical activity, was seen after 24-

hours of recovery following cold and heat stress (Wang et al., 2018). It has been determined that the lifetime of free radicals in the cells is very short due to the instability of their chemical structure (Phaniendra et al., 2015). Therefore, the non-significant effect of emersion or recovery in haemocyte ROS production observed in this study, may be the result of the animal's fast oxidative metabolic response, dealing with the reoxygenation-associated oxidative burst shortly after the animals are immersed in seawater and returning to baseline levels within hours of re-immersion. The proportion of haemocytes determined as 'ROS-positive' may also provide an incomplete picture of ROS levels affecting other tissues. This creates an opportunity to target both tissue and haemocyte ROS in future studies to truly understand the mechanisms involved during reoxygenation during recovery in seawater.

### 5.4.3 Recovery phase 2 response (R2, 5-days)

A significant decline in haemolymph osmolality in the 8-hour emersed animals, were experienced, likely to be linked to changes observed in the concentrations of osmotic effectors like amino acids or organic ions (Rivera-Ingraham and Lignot, 2017), indicating that lingering effects of emersion remain apparent after five days of recovery.

After emersion and 5-days of recovery the metabolomics results showed carbohydrate levels remained affected in the 8-hour group, with most metabolites decreased below control levels. Both the 3- and 8-hour emersion groups showed an increase in amino acids, but among lipid classes no metabolites were found to be statistically different from the control, plausibly due to the fact that end-products from anaerobic metabolism, phosphagen and ATP pools can be oxidised as substrates for aerobic

metabolism (Ellington, 1983). As in other invertebrates, the recovery phase in juvenile geoduck seems largely aerobic, considering the absence of significant glycolytic metabolites and the lack of increased anaerobic end-products (i.e., lactate, succinate, alanine).

The increases in amino acids can be linked to enhanced amino acid oxidation and protein breakdown, together with suppressed protein synthesis (Ishikura et al., 2013). In this instance, amino acids (from protein sources) can be used to restore energy reserves to ensure homeostasis (Isani et al., 1989). Abundant amino acids found in the recovery groups included: glutamine, which plays and important role in intracellular protein synthesis; leucine, which has the potential to activate cell-signalling pathways that stimulate protein synthesis; homocysteine, a precursor of 2-aminobuytric acid (elevated after 5 days), that has been found as a oxidative stress marker in bivalves; lysine, known to produce acetyl-CoA for gluconeogenesis; ornithine, which plays a part in ammonia detoxification; tryptophan, which can be oxidised as a respiratory fuel and act as a precursor for $NAD^+$ synthesis; and tyrosine; which is linked to protein phosphorylation (Wu, 2009, Garrett and Grisham, 2010, Wu et al., 2014, Salway, 2016, Haider et al., 2020). Amino acids also display remarkable metabolic and regulatory versatility, making it difficult to pinpoint the exact use thereof in the current study. Oliveira et al. (2011) investigated geoduck siphon and mantle, and found a total of seven amino acids (glutamate, aspartate, lysine, arginine, alanine, glycine and leucine) as most abundant and accounted for approximately 60% of the total amino acids detected (Oliveira et al., 2011). These geoducks were wild-caught animals of commercial size, providing some insight into their chemical composition, something

which is unknown for *P. zelandica,* especially when focusing on hatchery-produced juveniles.

The citric acid cycle acts as the central hub connecting carbohydrate, lipid and protein metabolism, and is the main pathway to ensure energy homeostasis, but also provides carbon species for biosynthetic processes (Garrett and Grisham, 2010, Chen et al., 2018). The decrease of succinate, fumarate, and malate in the 8-hour group following 5-days of recovery, suggests that the citric acid cycle is suppressed in this recovery phase. Pyruvate, which is carboxylated to malate, fumarate and then succinate through a reversal of the citric acid cycle is again apparent, as previously seen in *Crassostrea virginica* (Hammen, 1969). As succinate is the end-product of this pathway it can be hypothesised that reduction in metabolite levels reflect an increased use of succinate (to fuel the respiratory chain), together with a decreased resupply thereof due to a suppressed citric acid cycle.

Interestingly, the portion of cellular energy utilised for lipid metabolism is relatively low when compared to the overall costs of carbohydrates and proteins, as only a fraction of the fatty acids and sterols absorbed from the diet undergo *de novo* lipid synthesis (Lawrence, 2013). In this sense large changes in lipid metabolism are not expected in the 5-day recovery groups. However, the control group showed slightly (non-significantly) higher ROS (mean 49% haemocytes ROS-positive) than the previously emersion treatments; this observation is surprising given increasing oxidative stress is an expected outcome in the recovery phase following hypoxia (Sokolova et al., 2012). As discussed above, the presence of ROS in haemocytes may not fully represent oxidative stress in the whole organism; a broader examination of oxidative damage

markers may therefore be valuable. For example, it has been observed in other shellfish species that increases in lipid peroxidation occur due to higher ROS production following exposure to a stressful condition (de Almeida et al., 2007, Carregosa et al., 2014, Delorme et al., 2021). Peroxidation of lipids exposed to oxygen is responsible for deterioration of foods and tissue damage *in vivo*, due to free radicals, typically reactive oxygen species (Murray et al., 2014). As lipid (and so presumably lipid peroxide) levels are low in this geoduck, oxidative damage products from proteins and deoxyribonucleic acid would be particularly informative (Gasparovic et al., 2013).

The results obtained from mitochondrial membrane potential showed no differences among the 5-day recovery groups. Mitochondrial membrane potential can never be fully dissipated as it is required for protein transport and mitochondrial maintenance during the recovery phase following stress (Sokolova et al., 2012). The 3-hour emersion group following 5-days of recovery showed the highest cell viability (48%), but was not found to be significantly different to the control or 8-hour emersed animals, suggesting that immunocompetency was largely unaffected by the emersion and recovery treatments (Gopalakrishnan et al., 2009).

### 5.4.4 Conclusions

The physiological responses displayed by juvenile New Zealand Geoduck, *Panopea zelandica,* following emersion and subsequent recovery in seawater are consistent with the implementation of cellular changes to reduce oxygen demand, while metabolic mechanisms provided energy via additional pathways and defence mechanisms allowed protection against damaging effects.

The group of geoduck juveniles subjected to emersion showed an increasing haemolymph osmolality, with an increase in aerial exposure time. The 3-hour exposure group produced ATP aerobically, utilising additional $NAD^+$ production. In contrast the 8-hour emersion group utilised anaerobic metabolism for ATP production. Flow cytometry results remained within narrow ranges, suggesting minimal impact upon immune status and creating baseline data for these conditions.

A 1-day recovery phase following emersion displayed depleted lipid sources with additional energy reserves and anaerobic substrates apparently experiencing re-synthesis to replenish metabolic losses following anaerobic conditions in both the 3- and 8-hour exposure groups. Small differences in haemocyte mitochondrial membrane potential are likely to be linked to metabolic readjustment taking place following aerial exposure.

Following 5-days of recovery in standard conditions juvenile geoduck displayed aerobic metabolism as the main route of energy production with additional use of succinate to fuel the respiratory chain in the 8-hour emersion group. The enhanced use of amino acid metabolism suggests restoration of energy reserves still taking place following emersion. Changes in the osmolality were consistent with changes in levels of these amino acid osmotic effectors.

Overall, this study demonstrated that juvenile geoduck have the potential to be transported to different seeding grounds providing that sufficient recovery time is allowed to refuel metabolic energy stores. This a reassuring prospect and introduces an opportunity to assess alternative transport scenarios in future.

## Supplementary data

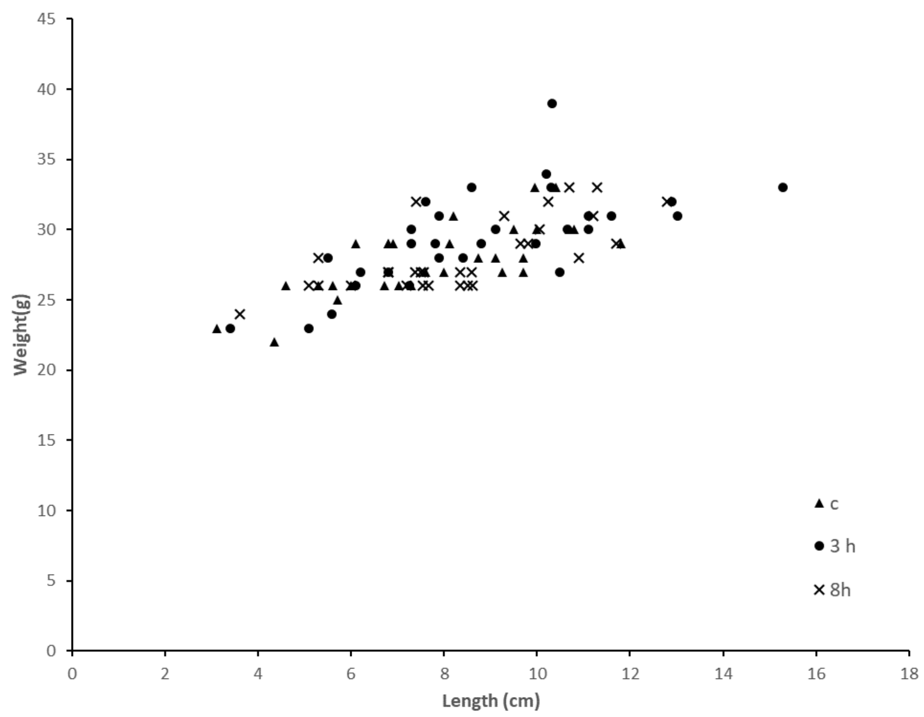


**Figure 5.1S:** Plot showing the relationship between wet weight and shell length of juvenile geoducks sampled from the different experimental groups.

**Siphon extension**

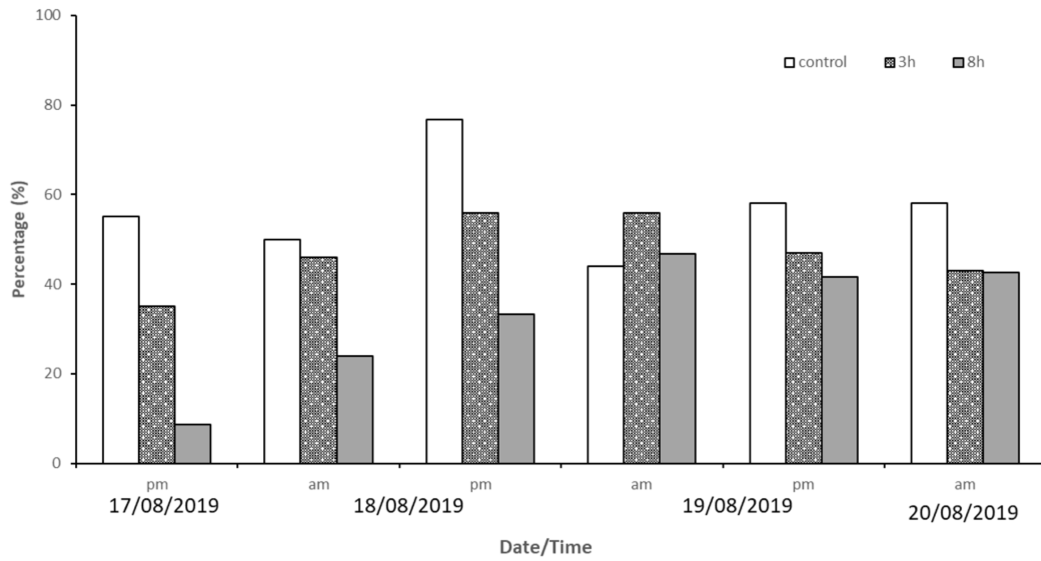


**Figure 5.2S**. Percentage of juvenile geoducks with fully extended siphons during recovery after emersion for each experimental group.

**Siphon openness**

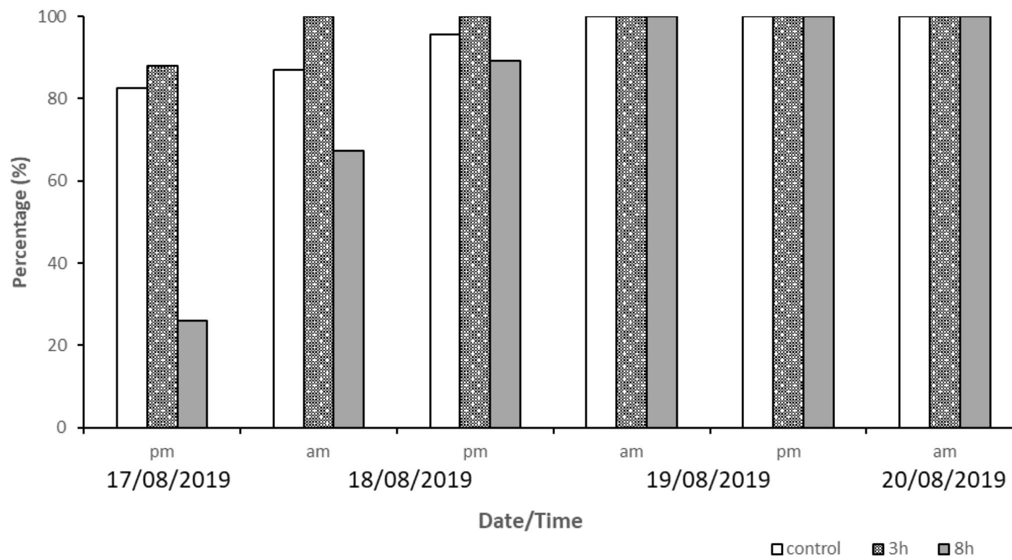


**Figure 5.3S**. Percentage geoduck juveniles with siphons visibly opened during recovery after emersion for each experimental group.

The siphons visible/not visible at the surface of the sediment of each animal was given a numeric value.
0 – Still buried
1 – Short extension (<2.5cm)
2 – Medium extension (>2.5 <5.0cm)
3 – Long extension (>5cm)
The values were added and converted to percentages to show the proportion of animals in each group with fully extended siphons.

**Table 5.1S:** Metabolites found in *Panopea zelandica* juveniles following emersion. Metabolites are listed in alphabetical order and arrows indicate an increase (↑) or decrease (↓) in metabolite abundance. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to classify the metabolites according to metabolite class.

| Feature | *p*.value | FDR | Fisher's LSD | G1 - 3H reaction from control | G2 - 8H reaction from control | G2 from G1 | Classification | KEGG nr |
|---|---|---|---|---|---|---|---|---|
| 10-Heptadecenoate (C17:1) | 5.70E-03 | 3.71E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Fatty acid | NA |
| 2-Aminobutyric acid | 2.91E-03 | 3.29E-02 | G1 - C; C - G2; G1 - G2 | ↑ | ↓ | ↓ | Animo fatty acid | C02261 |
| 2-Aminoisobutyric acid | 9.27E-03 | 4.26E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Amino acid | C03665 |
| 2-Hydroxyglutarate | 5.98E-03 | 3.71E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Short-chain hydroxy acid | C02630 |
| 2-Oxobutanoate | 6.73E-03 | 3.82E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Short-chain keto acid | C00109 |
| 3-Hydroxyisobutyrate | 2.04E-02 | 6.83E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Alpha hydroxy acids | C01188 |
| 4-Aminobutyrate | 3.21E-02 | 8.58E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Amino fatty acid | C00334 |
| 4-Methyl-2-oxopentanoate | 2.40E-03 | 3.20E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Short-chain keto acid | C00233 |
| Aminomalonate | 4.56E-03 | 3.37E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Amino acid | C00872 |
| Aspartate | 2.79E-02 | 7.60E-02 | C - G1; C - G2 | ↓ | ↓ | - | Amino acid | C00049 |
| Azelaic acid | 2.43E-02 | 7.24E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Medium-chain fatty acid | C08261 |
| Benzoate | 1.76E-02 | 6.41E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Benzoic acid | C00539 |
| Carbamate | 4.09E-03 | 3.34E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Carbonic acid derivative | C01563 |
| cis-4-Hydroxy-L-proline | 2.17E-02 | 6.86E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Amino acid | C01015 |
| Citraconate | 1.79E-02 | 6.41E-02 | G1 - G2 | - | - | ↓ | Methyl-branched fatty acid | C02226 |
| Cystathionine | 6.76E-03 | 3.82E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Amino acid | C02291 |
| Dihomo-gamma-linolenate | 1.70E-02 | 6.41E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Long-chain fatty acid | C03242 |
| Docosahexaenoate | 3.89E-02 | 9.85E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Very long-chain fatty acid | C06429 |
| Docosanoate | 4.58E-03 | 3.37E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Very long-chain fatty acid | C08281 |
| Eicosapentaenoic acid | 3.78E-03 | 3.34E-02 | G1 - C; C - G2; G1 - G2 | ↑ | ↓ | ↓ | Long-chain fatty acid | C06428 |
| Fumarate | 3.55E-03 | 3.34E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Fatty acyl | C00122 |
| gamma-Linolenic acid | 2.59E-02 | 7.33E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Long-chain fatty acid | C06426 |
| Glutamate | 2.19E-02 | 6.86E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Amino acid | C00025 |
| Glutarate | 2.89E-03 | 3.29E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Fatty acid | C00489 |
| Glycine | 5.74E-03 | 3.71E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Amino acid | C00037 |
| Heptadecanoic acid | 1.84E-02 | 6.41E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Long-chain fatty acid | NA |
| Hexadecanoate | 6.49E-04 | 1.45E-02 | G1 - C; C - G2; G1 - G2 | ↑ | ↓ | ↓ | Long-chain fatty acid | C00249 |
| Homocysteine | 1.50E-05 | 2.20E-03 | G1 - C; G1 - G2 | ↑ | - | ↓ | Amino acid | C00155 |
| Leucine | 2.31E-02 | 7.06E-02 | G1 - G2 | - | - | ↓ | Amino acid | C00123 |

| Linoleate | 8.10E-03 | 3.97E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Long-chain fatty acid | C01595 |
|---|---|---|---|---|---|---|---|---|
| Linolenate | 2.19E-02 | 6.86E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Long-chain fatty acid | C06427 |
| LL-2,6-Diaminopimelate | 1.05E-04 | 5.13E-03 | G1 - C; C - G2; G1 - G2 | ↑ | ↓ | ↓ | Amino acid | C00666 |
| Octanoate | 1.35E-02 | 5.38E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Fatty acids | C06423 |
| Oleate | 7.45E-03 | 3.90E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Fatty acids | C00712 |
| Oxalate | 1.87E-02 | 6.41E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Carboxylic acid | C00209 |
| Palmitoleic acid | 7.31E-03 | 3.90E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Long-chain fatty acid | C08362 |
| Succinate | 2.07E-04 | 7.62E-03 | G1 - C; G1 - G2 | ↑ | - | ↓ | Organic acids | C00042 |
| Tetradecanoate | 6.06E-03 | 3.71E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Long-chain fatty acid | C06424 |
| Undecanoic acid | 1.11E-02 | 4.81E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Medium-chain fatty acid | C17715 |
| Unknown1 | 1.03E-02 | 4.60E-02 | G1 - G2 | - | - | ↓ | - | - |
| Unknown2 | 1.86E-03 | 2.73E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | - | - |
| Unknown3 | 6.86E-04 | 1.45E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | - | - |
| Unknown4 | 1.35E-02 | 5.38E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | - | - |
| Unknown5 | 7.69E-03 | 3.90E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | - | - |
| Unknown6 | 8.95E-03 | 4.24E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | - | - |
| Unknown7 | 1.05E-04 | 5.13E-03 | C - G2; G1 - G2 | - | ↓ | ↓ | - | - |
| Unknown9 | 3.55E-02 | 9.15E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | - | - |
| Unknown11 | 1.77E-02 | 6.41E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | - | - |
| Unknown12 | 2.77E-02 | 7.60E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | - | - |
| Unknown13 | 2.50E-02 | 7.24E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | - | - |
| Unknown14 | 6.89E-04 | 1.45E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | - | - |
| Unknown15 | 3.47E-03 | 3.34E-02 | G1 - C; G2 - C | ↑ | ↑ | - | - | - |
| Unknown16 | 3.97E-03 | 3.34E-02 | G1 - C; G2 - C | ↑ | ↑ | - | - | - |
| Unknown17 | 1.29E-02 | 5.38E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | - | - |
| Unknown18 | 8.26E-04 | 1.52E-02 | G1 - C; G2 - C | ↑ | ↑ | - | - | - |
| | | | | | | | | |

**Table 5.2S:** Metabolites found in *Panopea zelandica* juveniles following 1-day of recovery after emersion. Features are listed in alphabetical order and arrows indicate an increase (↑) or decrease (↓) in metabolite abundance. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to classify the metabolites according to metabolite class.

| **Feature** | ***p*.value** | **FDR** | **Fisher's LSD** | **G1 - 3H reaction from control** | **G2 - 8H reaction from control** | **G2 from G1** | **Classification** | **KEGG nr** |
|---|---|---|---|---|---|---|---|---|
| LL-2,6-Diaminopimelate | 7.26E-03 | 8.11E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Amino acid | C00666 |

| 2-Oxobutanoate | 3.01E-03 | 4.63E-02 | G2 - C; G2 - G1 | - | ↑ | ↑ | Short-chain keto acid | C00109 |
|---|---|---|---|---|---|---|---|---|
| 3-Hydroxyisobutyrate | 1.33E-02 | 9.17E-02 | C - G1; C - G2 | ↓ | ↓ | - | Alpha hydroxy acids | C01188 |
| Aspartate | 1.06E-02 | 8.75E-02 | C - G1; C - G2 | ↓ | ↓ | - | Amino acid | C00049 |
| Citraconate | 1.34E-02 | 9.17E-02 | C - G1; C - G2 | ↓ | ↓ | - | Methyl-branched fatty acid | C02226 |
| Fumarate | 1.80E-05 | 1.11E-03 | C - G2; G1 - G2 | - | ↓ | ↓ | Fatty acyl | C00122 |
| Glyoxylate | 1.02E-02 | 8.75E-02 | C - G1; C - G2 | ↓ | ↓ | - | Monocarboxylic acid | C00048 |
| Heptadecanoic acid | 5.17E-03 | 6.36E-02 | C - G1; C - G2 | ↓ | ↓ | - | Long-chain fatty acid | NA |
| Hydroxybenzoic acid | 6.55E-04 | 1.26E-02 | C - G1; G2 - G1 | ↓ | - | ↑ | Benzoic acid | C00156 |
| Lysine | 4.29E-04 | 1.26E-02 | G1 - C; C - G2; G1 - G2 | ↑ | ↓ | ↓ | Amino acid | C00739 |
| Malate | 1.37E-04 | 5.62E-03 | C - G2; G1 - G2 | - | ↓ | ↓ | Short-chain hydroxy acid | C00149 |
| Nicotinamide | 1.07E-02 | 8.75E-02 | C - G1; G2 - G1 | ↓ | - | ↑ | Vitamins and cofactors | C00153 |
| Phosphoenolpyruvate | 5.47E-04 | 1.26E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Phosphoric acid ester | C00074 |
| Stearate | 7.19E-04 | 1.26E-02 | C - G1; C - G2 | - | ↓ | ↓ | Long-chain fatty acid | C01530 |
| Succinate | 4.79E-07 | 5.89E-05 | C - G2; G1 - G2 | - | ↓ | ↓ | Organic acids | C00042 |
| Threonine | 4.81E-03 | 6.36E-02 | G1 - C; G2 - C | ↑ | ↑ | - | Amino acid | C00188 |
| Tryptophan | 1.16E-02 | 8.88E-02 | G1 - C; G1 - G2 | ↑ | - | ↓ | Amino acid | C00806 |
| Unknown8 | 1.01E-02 | 8.75E-02 | C - G1; G2 - G1 | ↓ | - | ↑ | ¬ | ¬ |
| | | | | | | | | |

**Table 5.3S:** Metabolites found in *Panopea zelandica* juveniles following 5-days of recovery after emersion. Features are listed in alphabetical order and arrows indicate an increase (↑) or decrease (↓) in metabolite abundance. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to classify the metabolites according to metabolite class.

| **Feature** | ***p*.value** | **FDR** | **Fisher's LSD** | **G1 - 3H reaction from control** | **G2 - 8H reaction from control** | **G2 from G1** | **Classification** | **KEGG nr** |
|---|---|---|---|---|---|---|---|---|
| 2-Aminobutyric acid | 3.49E-03 | 5.45E-02 | G1 - C; G2 - C | ↑ | ↑ | - | Amino fatty acid | C02262 |
| Fumarate | 1.02E-02 | 9.79E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Fatty acyl | C00122 |
| Glutamine | 6.60E-03 | 9.16E-02 | G1 - C; G2 - C | ↑ | ↑ | - | Amino acid | C00819 |
| Homocysteine | 3.82E-07 | 4.77E-05 | G1 - C; G1 - G2 | ↑ | - | ↓ | Amino acid | C00155 |
| Leucine | 8.15E-03 | 9.26E-02 | G1 - C; G2 - C | ↑ | ↑ | - | Amino acid | C00123 |
| Lysine | 1.89E-03 | 3.94E-02 | G2 - C; G2 - G1 | - | ↑ | ↑ | Amino acid | C00739 |
| Malate | 5.47E-04 | 2.28E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Short-chain hydroxy acid | C00149 |
| Ornithine | 1.00E-03 | 2.50E-02 | G1 - C; G2 - C; G2 - G1 | ↑ | ↑ | ↑ | Amino acid | C00077 |
| Succinate | 9.98E-04 | 2.50E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | Organic acid | C00042 |
| Tryptophan | 3.44E-05 | 2.15E-03 | G1 - C; G2 - C | ↑ | ↑ | - | Amino acid | C00806 |
| Tyrosine | 7.72E-03 | 9.26E-02 | G1 - C; G2 - C | ↑ | ↑ | - | Amino acid | C00082 |

| Unknown4 | 2.28E-03 | 4.07E-02 | G1 - C; G2 - C | ↑ | ↑ | - | - | - |
|---|---|---|---|---|---|---|---|---|
| Unknown10 | 1.00E-02 | 9.79E-02 | C - G2; G1 - G2 | - | ↓ | ↓ | - | - |
| | | | | | | | | |

**Table 5.4:** Data (mean, STDEV, SE) obtained via flow cytometry for emersion, and recovery groups, corresponding to cell viability, oxidative stress, and mitochondrial transmembrane potential results.

| **Group** | **Code** | **%Viability** | | | **% ROS** | | | **% MMP** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Emersion** | | Mean | SD | SE | Mean | SD | SE | Mean | SD | SE |
| CONTROL 1 | C E | 58.8 | 20.3 | 6.4 | 39.4 | 15.2 | 4.8 | 31.6 | 22.8 | 7.2 |
| 3H EXPOSURE | G1 E | 51.2 | 22.0 | 7.0 | 46.7 | 15.0 | 4.7 | 27.9 | 17.1 | 5.4 |
| 8H EXPOSURE | G2 E | 63.0 | 18.6 | 5.9 | 38.8 | 18.1 | 5.7 | 31.0 | 28.5 | 9.0 |
| **Recovery 1 day** | | | | | | | | | | |
| 24H RECOVERY CONTROL | C R1 | 49.8 | 17.5 | 5.5 | 43.6 | 9.3 | 2.9 | 23.8 | 12.3 | 3.9 |
| 24H RECOVERY 3H | G1 R1 | 46.5 | 14.1 | 4.5 | 39.0 | 8.1 | 2.6 | 23.6 | 19.1 | 6.0 |
| 24H RECOVERY 8H | G2 R1 | 54.5 | 17.4 | 5.5 | 48.2 | 16.1 | 5.1 | 35.4 | 26.7 | 8.4 |
| **Recovery 5 days** | | | | | | | | | | |
| 5D SETTLED CONTROL | C R2 | 35.0 | 16.1 | 5.1 | 48.5 | 16.3 | 5.2 | 26.8 | 27.8 | 8.8 |
| 5D SETTLED 3H | G1 R2 | 48.4 | 15.9 | 5.0 | 47.3 | 17.3 | 5.5 | 30.1 | 22.7 | 7.2 |
| 5D SETTLED 8H | G2 R2 | 38.3 | 16.9 | 5.3 | 43.8 | 14.6 | 4.6 | 20.3 | 25.5 | 8.1 |

# SECTION 3: GEODUCK FIELD GROWOUT

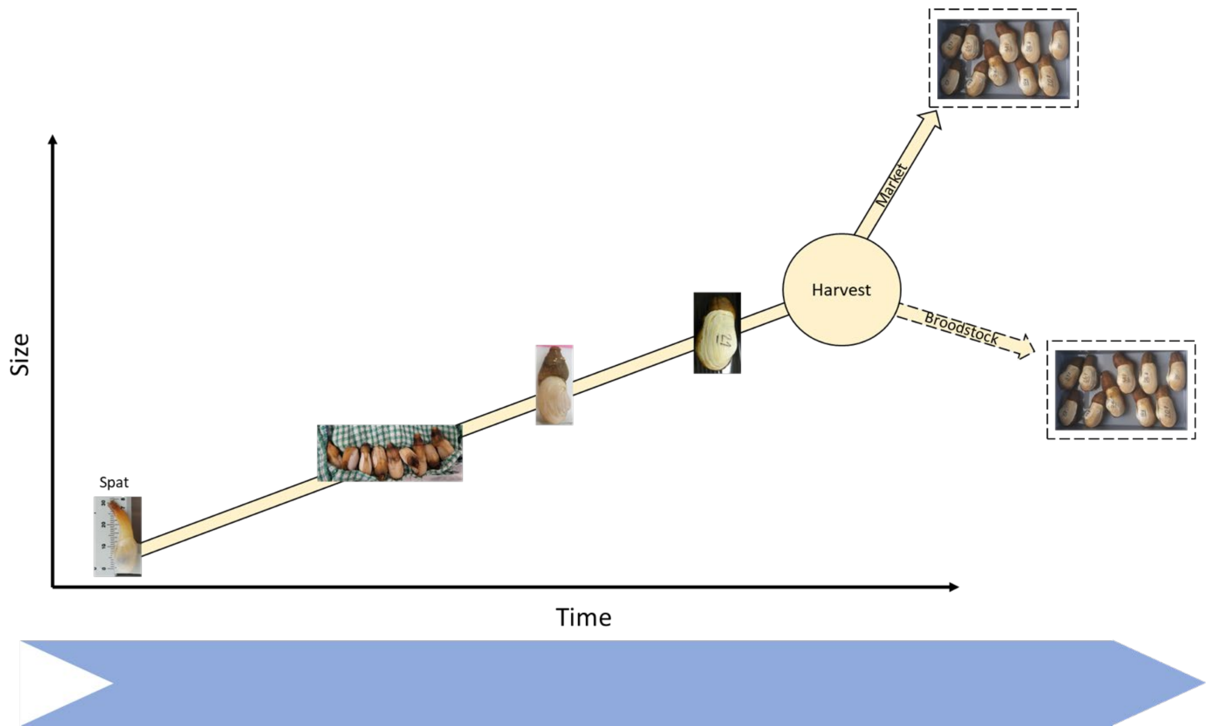


**Figure sect 3.** Outline of growout phase of *Panopea zelandica*

# Chapter 6 - Characterizing selected response mechanisms of juvenile geoduck (*Panopea zelandica*) following acute thermal shock

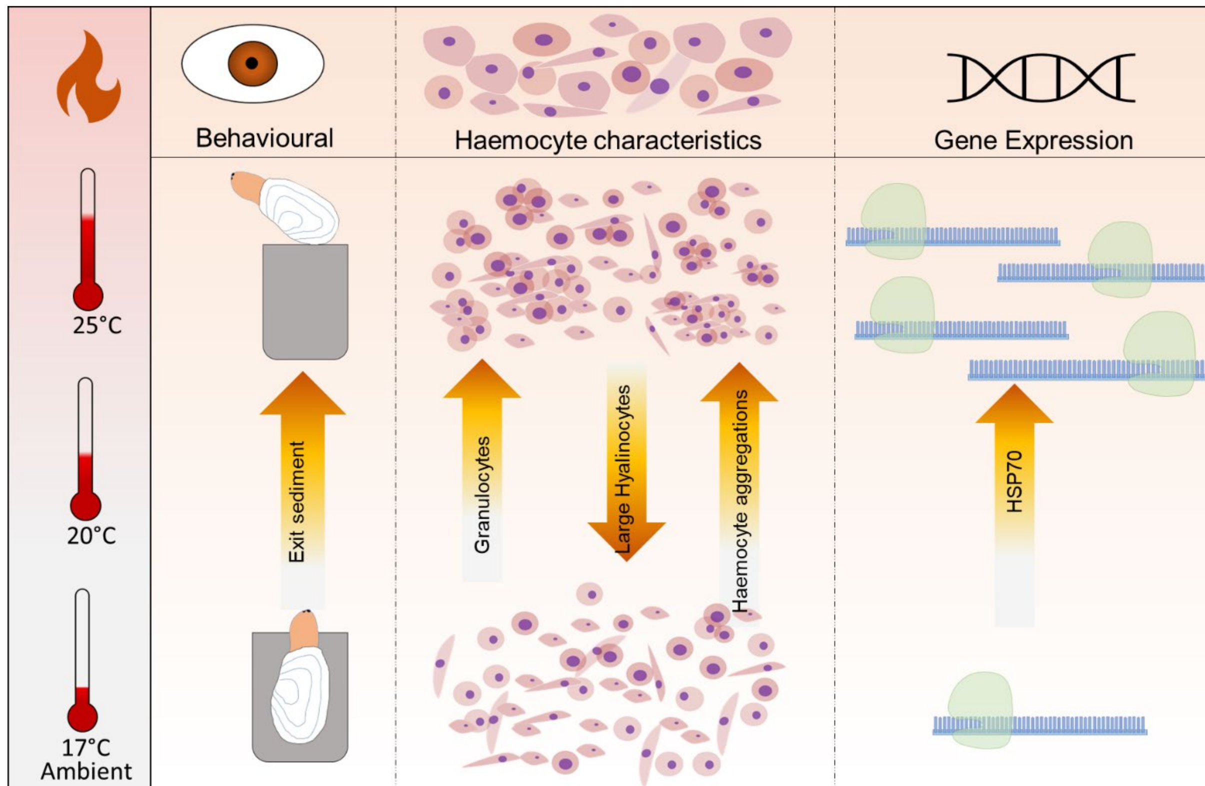


**Figure 6.0** Graphical abstract

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This chapter has been published as:


Sharma, S., Venter, L., Alfaro, A. C., Ragg, N. L., Frost, E., & Zamora, L. N. (2024). Physiological responses of juvenile New Zealand geoduck (Panopea zelandica) following emersion and recovery. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*,269,110892.

# Abstract

Climate extremes, such as heatwaves, are expected to become more intense and of longer duration in the near future. These climatic conditions may have a significant impact on the prospects of establishing a new aquaculture industry for the endemic New Zealand geoduck, *Panopea zelandica*. This study focused on characterising animal behaviour, haemocyte characteristics, and heat shock protein (HSP70 & HSP90) mRNA expression following exposure to elevated temperatures, such as those encountered during marine heatwaves around 20°C and an extreme scenario of 25°C, contrasted to an ambient temperature of 17°C. After 24h of heat challenge, *P. zelandica* were found to be significantly influenced by the thermal changes, as there were differences recorded in all the responses examined. With increasing temperatures, juvenile geoducks were observed to fully emerge from the sediment a behaviour that has not previously been quantified nor associated with stress in this species. The ability of *P. zelandica* juveniles to re-bury still warrants further investigation, as adults are unable to do so. Haemocyte analyses revealed an increase in the abundance of granulocytes, cellular aggregations, and size of these aggregations at the highest temperature exposure. Increased expression of the *hsp70* gene in the haemolymph after exposure at 25°C for 24h was detected and attributed to attempts to mitigate protein denaturation caused by thermal stress. The inter-individual variability in the response of heat shock proteins recorded could aid in future selective breeding programs if it is reflected in net thermotolerance. *P. zelandica* shows great potential for growing in subtidal habitats around New Zealand, and this study highlights the importance of temperature considerations when selecting potential farm and reseeding locations.

## 6.1 Introduction

New Zealand spans a large latitudinal distance across its two main Islands. The oceans around the main islands are influenced by subtropical water in the upper north and by polar Antarctic currents down south. This temperature range provides a vast array of the habitats for a variety of aquaculture species. Indeed, aquaculture is the fastest growing primary sector in New Zealand with interests in expanding to new species in the near future (McGinnia & Collins., 2013, Stenton-Dozey et al., 2021). However, the aquaculture industry could face a major challenge due to the current global climate change crisis. Indeed, reports have confirmed that oceans around some regions in New Zealand are warming, and that climate extremes such as marine heatwaves are expected to become more intense and longer compared to the present day (Behrens et al., 2022 & Stevens et al., 2022). Events, such as marine heatwaves have significant implications for aquaculture and fisheries in New Zealand (Burgh-Day et al., 2022). Increased temperatures and temperature anomalies affect the metabolic and physiological responses of commercially important fish and shellfish species (Rolton et al., 2022). However, the effects of sudden temperature fluctuations on the endemic geoduck clam *Panopea zelandica*, are largely unknown.

The New Zealand geoduck, *P. zelandica*, is found in subtidal areas around the North and South Islands. Even though *P. zelandica* has a huge geographically spread (Gribben et al, 2004), there is limited information about its population dynamics and distribution (Gribben & Heasman, 2015). Currently, there are only a few known populations of *P. zelandica* which support wild harvests (Gribben and Heasman 2015). Establishment of a geoduck aquaculture industry is still pending, with previous

research efforts placed on investigating different aspects of successful hatchery production of seed (Le et al., 2017; Sharma et al., 2020). Due to the current increase in global sea temperatures and the increased chances of marine heatwaves continuing in the future, temperature responses remain an important factor for consideration. Indeed, bivalves exposed to temperatures above their optimal ranges tend to exhibit lower borrowing capacity, decreased feeding and growth rates, and increased rates of mortality (Macho et al., 2016). A common behavioural response to thermal stress in bivalves includes valve closure and/or increased borrowing (Sakurai et al., 1996, Domínguez et al., 2021). Even though valve closure provides an escape from temperature extremes as seen in mussels, *Limnoperna fortune* (Andrade et al., 2018), valve closure is not an option for geoducks as these bivalves are unable to fully close their shells. Considering that sediment at depth is cooler than the surface layers, moving deeper within the sediment may provide animals an escape from surficial temperature stress (Macho et al., 2016). Consequently, Macho et al. (2016) documented the change in subsurface burrowing frequency and siphon activity as a useful measure of stress in clams.

As pointed out by Hernández-Méndez et al. (2020), haemocyte assessment allows one to detect the impact of environmental changes on animal health, and can be used a valuable measure of physiological responses due to stressors. Elevated temperatures have also been shown to affect clam haemocyte parameters, such as cell viability, adhesive capacity, and cell types (Monari et al., 2007; Pérez–Velasco et al., 2022). Haemocytes constitute the cellular component of haemolymph, which moves through the circulatory system, migrating to locations, such as connective tissue and epithelia (Hine 1999). Apart from their main function in host defence mechanisms, bivalve

haemocytes partake in physiological functions, such as nutrient digestion, transportation and distribution, wound healing, detoxification, shell mineralisation and excretion (de la Ballina et al., 2022). Bivalve haemocytes vary in size and abundance but are generally made up of three main cell types, which include small hyalinocytes, large hyalinocytes, and granulocytes, as demonstrated in *P. globosa* (Hernández-Méndez et al., 2020).

At the molecular level, cells react to heat stress by up-regulating genes, for example, chaperone proteins, involved in cell stress defence mechanisms. Typically, heat shock proteins (HSPs) play on important role in protein folding and biosynthesis, with HSP70 controlling cell homeostasis, proliferation, differentiation, and cell death, while HSP90 is involved in immune regulation, signal transduction, and cell cycle regulation (Xu et al., 2022). In a previous study on *P. generosa*, heat shock proteins were increased in larvae following exposure to ciliates as a typical stress response (Timminus-Schiffman et al., 2020). Targeted proteomics were used to investigate pH variations in *P. generosa*, providing a peptide database for quantifying multiple proteins simultaneously (Spencer et al., 2019). Ultimately, HSPs play a key role in controlling protein homeostasis and are among the main indicators of stress-induced protein damage (Tomanek, 2008).

Assessing the effects of elevated temperatures on biological parameters, such as health, growth, and nutrition is important for understanding and predicting resilience (Ewere et al., 2021). Temperature has been the focus of several geoduck studies. For example, *P. lobose*, had increased metabolic rates to temperatures of 29°C (Juarez et al., 2018). Whereas in *P. generosa*, 19°C was deemed as a temperature resulting in significant growth rates (Arney et al., 2015). On the other hand, at 19°C, both *P. zelandica* (Le et al., 2017) and *P. japonica* (Nam et al., 2015) had reduced aerobic

scope and increased mortalities, respectively. However, acute temperature stress is poorly described. The use of acute stress (hours to days) experiments enables one to compare the range over which a physiological stress response is observed with the actual body temperature animals experience under field conditions (Tomanek, 2008). This study provides the first report on the behavioural and physiological responses of juvenile *P. zelandica* to acute thermal stress, aiding the development of strategies for aquaculture planning and fisheries management, of geoduck clams.

## 6.2 Methods and materials

To evaluate the thermal stress response mechanisms of *P. zelandica*, juveniles of this species were exposed to acute changes in temperature for a period of 24h. The temperatures selected went from current summer ambient temperatures in their natural distribution (i.e., 17°C) to two potential heatwave scenarios in which the temperature quickly rises by either 3 or 8 °C i.e., respectively representing mean temperature anomalies seen in recent marine heatwaves and extreme localised warming (Salinger et al.,2019; NIWA). Overall responses were examined at 3 organisational levels: behavioural observations were conducted by taking hourly photographs of the experimental tanks to evaluate the borrowing status of the juvenile geoducks; changes in haemocyte subpopulations across different temperatures were investigated and expression of heat shock protein genes (*hsp70* & *hsp90*) were assessed in both haemolymph and gill samples

### 6.2.1 Animal acquisition and acclimation

Nine-month-old hatchery reared *P. zelandica* produced at the Cawthron Aquaculture Park (Nelson, New Zealand) were used for this study (n=27, wet weight of 16.98 ± 0.41g (mean ±S.E.), shell length of 32.96 ± 0.37 mm, shell width of 31.91 ± 0.23 mm). The juveniles were transported to the Auckland University of Technology (AUT) and were acclimated for two weeks prior to experimentation (Sharma et al.,2022). At AUT, the juveniles were kept in individual 760 mL containers (10 cm x 8.5 cm) with 9 cm depth of substrate (~500 µm grain sand). These holding containers were submerged in 2 x 180 L tanks in an 800 L recirculation system. Water temperature and salinity were maintained stable at 17 ± 0.5°C and 35 ppt, respectively. Individuals were exposed to a

light:dark cycle of 12h:12h throughout the two weeks using a 90 cm Blue Planet® LED track light set on a timer. No mortalities were recorded during the acclimation period.

### 6.2.2 Water parameters

Six HOBO® Onset UA-002-08 pendant temperature data loggers were placed in each of the treatment tanks: 3x in the water column and 3x in holding pots. The holding pots containing the temperature loggers did not contain any animals and were only filled with sediment. This was done to not disturb the animals. Temperature was recorded every 15 min throughout the 24h period. The salinity of the individual experimental tanks was measured before and after using a handheld ATC salinity refractometer- IC-RHS. Parameters such as pH, ammonia ($NH_3/NH_4^+$), nitrite ($NO_2^-$), and nitrate ($NO_3^-$) were also measured before and after using an API saltwater master test kit.

### 6.2.3 Thermal stress exposure

At the end of the acclimation period, the twenty-seven containers with animals were removed from the 180 L recirculation system and divided among three 40 L static tanks (nine replicates per temperature treatment) with aerated seawater (at 35 ppt) connected to an Aqua One® 200-watt heater. An Aqua One® Maxi Power head 101 submersible pump was placed in each 40L static tank to keep the water circulating (Fig. 1A). The static tanks were set up 12h prior to the experiment to provide ample time for the tanks to reach the proposed temperatures of 17, 20, and 25°C. Once the animals were placed in their temperature treatment the experimental time started (time 0). After 24h of

temperature exposure, all the  animals were removed from the experimental tanks for sample collection giving 9 replicates (n = 9) for all the analysis conducted.

### 6.2.4 Behavioural observations

To record the behaviour of each individual geoduck during the experiment, each tank had a GoPro® hero 5 placed in the water column for continuous recording at 1 frame per second for the entire duration of the experiment (24h). The wide-angle setting was used to ensure all geoducks were in the recording frame. Video recording and time stamps were used to obtain the behaviour of individual geoduck over the 24h period (Fig. 1B). Position of the geoducks (infaunal or epifaunal) was assessed and counted at hourly intervals over 24h. This was assessed as there was anectodical accounts of geoducks actively exiting the sediment when stressed.

### 6.2.5 Sample collections

For each temperature treatment  a haemolymph sample (approximately 400 µL) was collected from all nine animals by inserting a pre-chilled 1 mL syringe and needle placed at a 45° angle to the long axis to the ventricle (Sharma et al.,2022). Immediately after collection, a subsample of 300 µL of haemolymph was transferred to a cryovial and snap frozen in liquid nitrogen and stored at -80°C for further HSP analysis. The remaining 100 µL of haemolymph was prepared for microscopic observations of haemocytes (see below). Next, geoducks were opened by inserting a scalpel above the mantle and cutting through the anterior and posterior muscle attachments. A subsection of gill tissue was

collected, placed in cryovials, snap-frozen using liquid nitrogen and stored at -80° C until HSP expression analysis was performed.

### 6.2.6 Hemocyte preparations and assessment

A volume of 100 µL of freshly collected haemolymph (9 animals per treatment) was mixed 1:1 with 0.2 µm filtered artificial seawater (ASW) containing 0.38% sodium citrate as an anticoagulant, and placed on ice (Preziosi & Bowden, 2016). Haemolymph slides were prepared according to protocol described by Carballal et al. (1997). In brief, a monolayer of haemocytes was obtained by transferring 50 µL of 1:1 diluted haemolymph onto clean microscope slides. Cells were left to adhere to the slides for 30 min in a moist chamber at room temperature. Excess haemocytes were washed with filtered ASW, and the slides were then air dried. Once dried, the slides were fixed with analytical grade methanol for 5 min and stained with 10% Giemsa stain (in phosphate buffer solution) for 10 min. After 5 min, any excess stain was washed away with distilled water, and the slide were air dried again (Matozzo & Bailo, 2015). The haemocytes were observed under an Olympus Omax light microscope at 1000x magnification.

Haemocyte subpopulations were differentiated based on descriptions by Hernández-Méndez et al.(2020). In brief, haemocytes were identified and classified as granulocytes, and small and large hyalinocytes. A differential haemocyte count was conducted in triplicate by tallying up the first 200 cells observed while moving from left to right on the microscope. Haemocyte cellular aggregations were separated into 3 categories based on number of haemocytes and the size of aggregations. Aggregations were classified as 'small' if 4 – 10 cells with a net diameter of 10 – 80 µm were present, as 'medium' if

11 – 30 cells of 80 – 150 µm were seen and as ‘large’ if >30 cells of >150 µm were detected (Fig. 1C). Individual haemocyte and aggregation diameters were determined using CellSens® software (Version 1.16). Targeted images were taken with a camera (OMAX 14.0mp attached to the microscope).

### 6.2.7 Heat Shock Protein (HSP) analyses

Total ribonucleic acid (RNA) was isolated from replicate gill and haemolymph with TRIzol Reagent (Invitrogen, New Zealand), in accordance with the manufacturer’s instructions (Invitrogen user guide Pub. No. MAN0001271). Purified RNA was reconstituted in RNase-free water and any contaminating deoxyribonucleic acid (DNA) was removed by treating with Turbo DNase (Ambion, TX, USA) following the manufacturer’s recommendations (Ambion user guide Pub No. 1907M). The extracted total RNA pellet was re-suspended in nuclease-free water (Sigma, New Zealand) and cleaned using a TURBO DNase Free TM Kit (Invitrogen, New Zealand). The quality and quantity of the supernatant was then determined using a Qubit 2.0 fluorometer (Life technologies, Oregon, USA) and Qubit RNA BR Assay Kit (Life technologies, Oregon, USA). A total of 500 ng of RNA was synthesised into cDNA using Superscript VILO VI (Invitrogen, New Zealand) and stored at -20˚C for later analysis.

Specific gene primers for *hsp70* and *hsp90*, and the housekeeper β-Actin, were designed using the Geneious Prime (version 2022.0). A single housekeeper β-Actin was used in the analysis as it had an adequate efficiency of 91% and demonstrated stable amplification and efficiency across temperatures and treatments.Target sequences were designed based on the alignment of conserved domains aligned against the

assembled genome for the closely related *Panopea generosa*. Resulting target and housekeeper amplicons were Sanger sequenced, with sequences confirmed through the National Center for Biotechnology Information (NCBI) database, nucleotide BLAST (https://blast.ncbi.nlm.nih.gov, accessed on 30 Nov 2022). Quantitative Polymerase Chain Reaction (qPCR) analyses were performed in duplicate using 2 μL of 10 ng $\mu L^{-1}$ template cDNA in a total reaction volume of 20 μL, using SYBR® Green PCR Master Mix (Applied Biosystems™) according to manufacturer's instructions (publication Part Number 4310251 Rev. G). Three non-template controls (Delorme et al., 2020). *hsp70*, *hsp90* and *β-Actin* (Table 6.1) were tested in triplicate along with controls in the Mastercycler Realplex 2, programmed with the Sybr Green I detection system as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 50°C or 60°C for 1 min. The relative mRNA expression fold change for all target genes was determined by the delta-delta Ct (2- ddCT) method (Livak & Schmittgen, 2001)(Fig. 1D).

**Table 6.1.** qPCR Primers for HSP 70, HSP 90 and B-Actin

| **Primer target** | **Primer type** | **Sequence (5′–3′)** |
|---|---|---|
| *B-Actin* | Forward | GCTATCCAGGCTGTCCTCTC |
| *B-Actin* | Reverse | ATCTCTCTCTCAGCGGTGGT |
| HSP70 | Forward | CTAAGAACGGCATGCGAACG |
| HSP70 | Reverse | TCGGTATGCGAGTTGATCCG |
| HSP90 | Forward | TGAAACTACTGGGCCAAATT |
| HSP90 | Reverse | GCTCTAAATGCTTCGGTTTC |

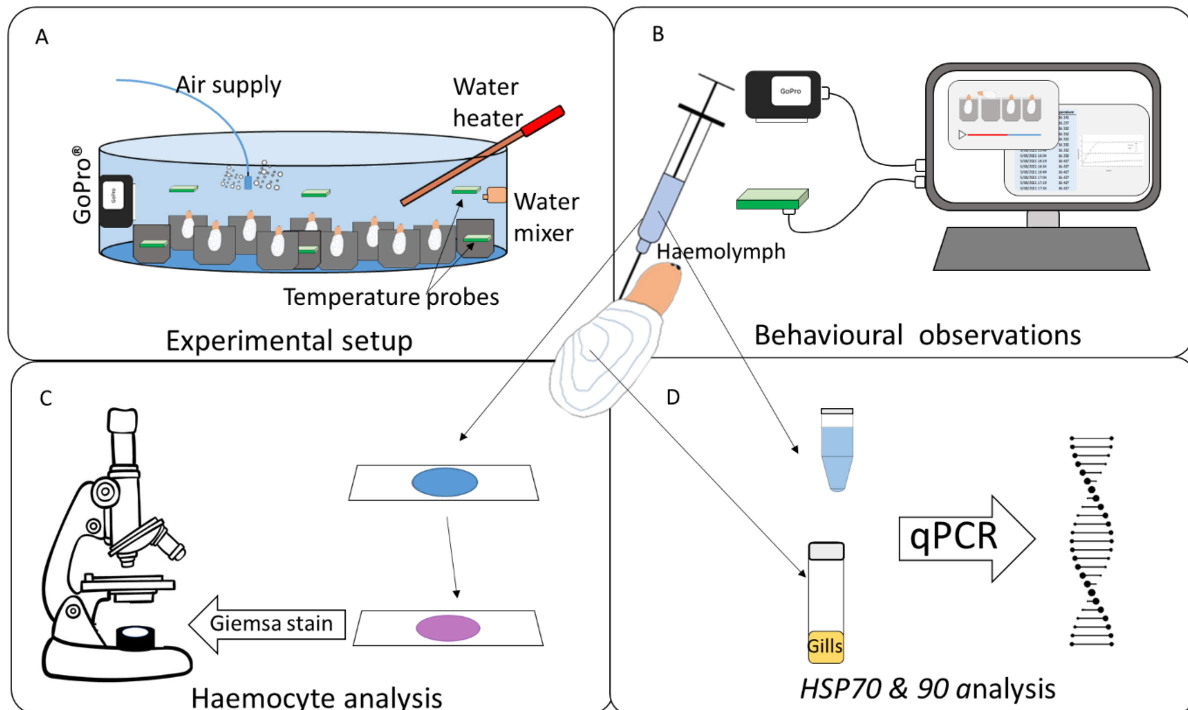


**Figure 6.1.** Experimental outline starting with A) housing of geoduck (n=9) at three different temperatures (17, 20, 25°C) for 24-hours, B) Video recording and temperature data were analysed, C) haemolymph collection and microscope slide preparation D) haemolymph and gill samples were processed for HSP analyses.

### 6.2.8 Statistical analyses

Different haemocyte subpopulations (small hyalinocytes, large hyalinocytes, granulocytes) percentages, frequency of haemocyte aggregates, and the size of haemocyte aggregates were analysed using One-Way Analysis of Variance (ANOVA), with different temperature treatments as factors. Fold changes in the protein and gene expression of *hsp70* and *hsp90* relative to the 17°C experimental group were conducted by first log-transforming the data and then analyzed using One-Way ANOVA.

All data were checked for normality and homoscedasticity using the Shapiro-Wilk and Levene's test, respectively (Quinn & Keough, 2002). Significant differences among treatments for all the analysis were determined using Tukey pairwise comparisons with significance taken at 0.05. All statistical analysis were conducted in R Studio® (version 1.4.1103).

# 6.3 Results

## 6.3.1 Experimental parameters

The water temperature in the experimental tanks remained constant at an average ± S.E of 16.75 ± 0.5°C in the 17°C experimental tank, 19.85 ± 0.5 °C in the 20°C experimental tank, and 24.95 ± 0.5°C in the 25°C experimental tank throughout the 24-h experimental period. The sediment temperature for all experimental groups started at 17°C at the onset of the experiment. Sediment housing animals within the medium experimental group reached 20°C after 2-hours of experimental time, while sediment with animals within the high experimental group reached 25°C after 6-hours of experimental time. Once the target temperature was reached, the sediment temperatures remained constant for the remainder of the experiment (Fig. 6.2). Water parameters [salinity (35.2ppt), pH (8), ammonia (<0.1 ppm), nitrite (< 0.25ppm) and nitrate (<5.0ppm)] remained constant before and after the experiment.

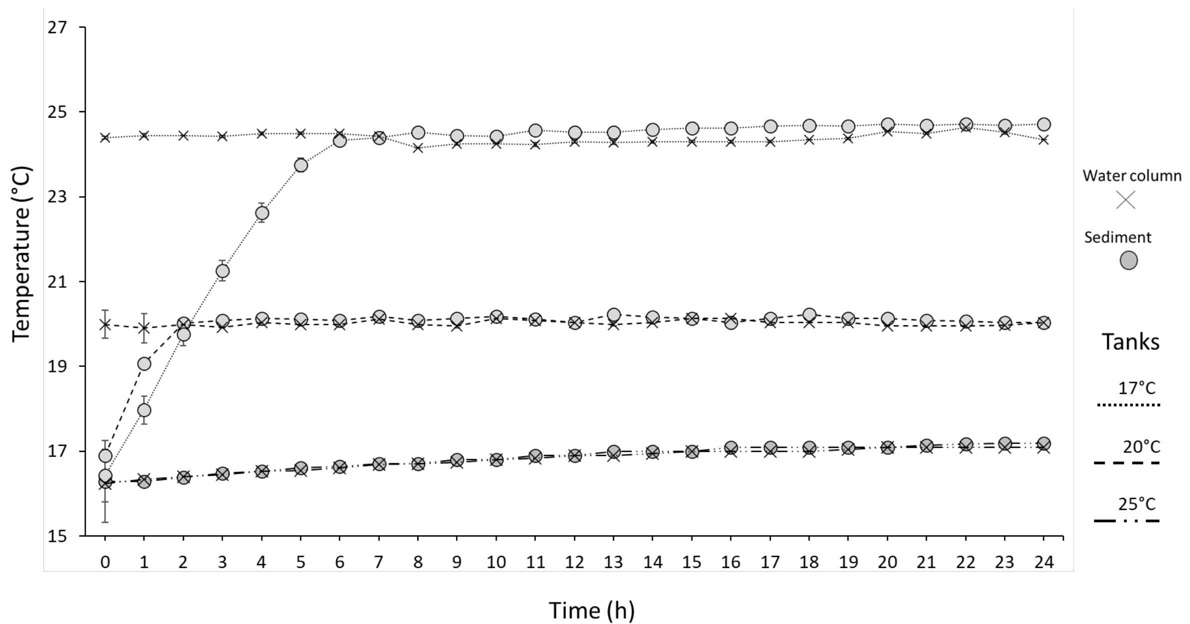


**Figure 6.2.** Temperature monitoring during acute thermal stress experiment. In all instances, water temperature (water column X) was set at the target temperature of 17, 20 and 25°C prior to the start of the experiment. The temperature of the sediment (sediment ●) housing pots is also shown.

## 6.3.2 Behavioural observations

Behavioural observations of geoducks across treatments indicated that within the 17°C experimental tank, all nine individuals remained within the sediment for the duration of the experiment. In the 20°C experimental tank, the first geoduck moved out from the sediment completely and ended up in a horizontal resting position on top of the sediment after 13-hours. By 24-hours in the 20°C experimental tank, 4 animals (44.4%) had unburied themselves and laid on the sediment surface. Animals in the 25°C experimental tank started surfacing from the sediment at 8h, with all 9 animals (100%) being out of their sediment by 24-hours (Fig. 6.3). There were no mortalities recorded in any of the experimental tanks.

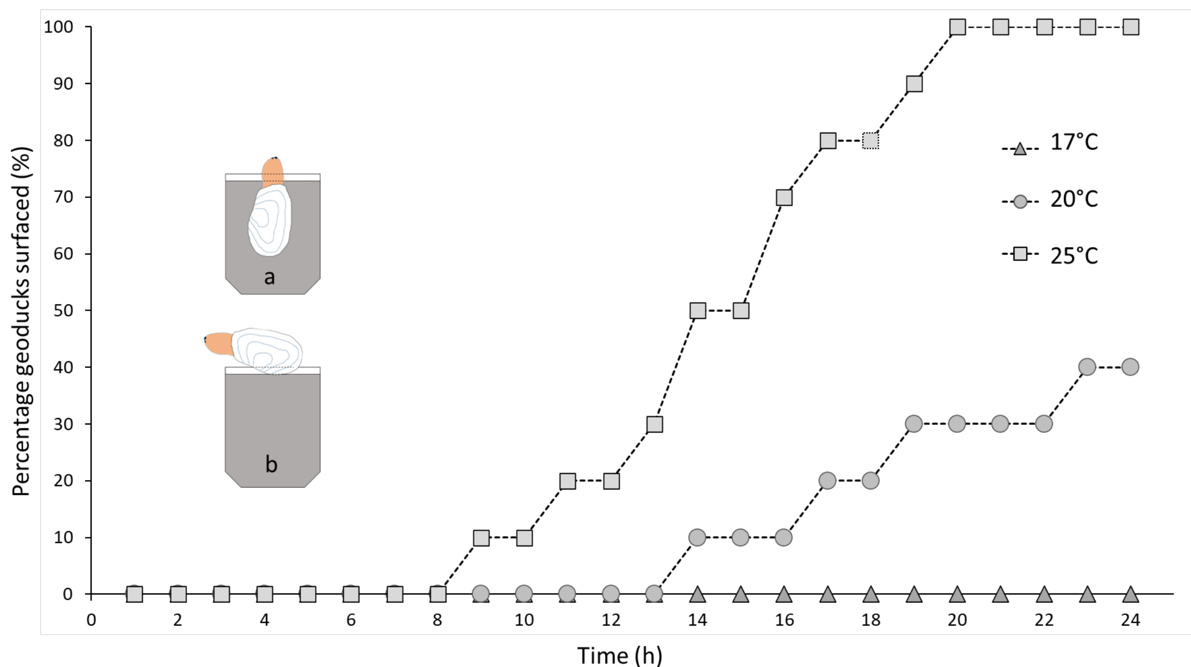


**Figure 6.3.** Behaviour of *Panopea zelandica* juveniles with regards to their position within the sediment (a –geoduck buried in the sediment; b – emerged from the sediment) was monitored for 24h amongst all experimental temperatures (17°C ▲, 20°C ●, 25°C ■).

## 6.3.3 Hemocyte characterization

The three subpopulations of haemocytes were characterised as granulocytes, and large & small hyalinocytes. Granulocyte ranged in size between 10 – 30 µm, and exhibited a round, eccentric basophilic nuclei with abundant granules (Fig. 4A). The hyalinocytes were primarily acidophilic and were differentiated into different subpopulations based on size and morphological differences. Large hyalinocytes (15 – 55 µm) had a crescent shape with a central or eccentric nucleus with cytoplasm spread out in a crescent shape (Fig. 4Bi). The small hyalinocytes (5 – 18 µm) were characterised by small round cells with a central or eccentric nucleus (Fig. 4Bii). Haemocyte aggregations were mainly comprised of a central core of hyalinocytes surrounded by granulocytes (Figure 4 Ci - iii).

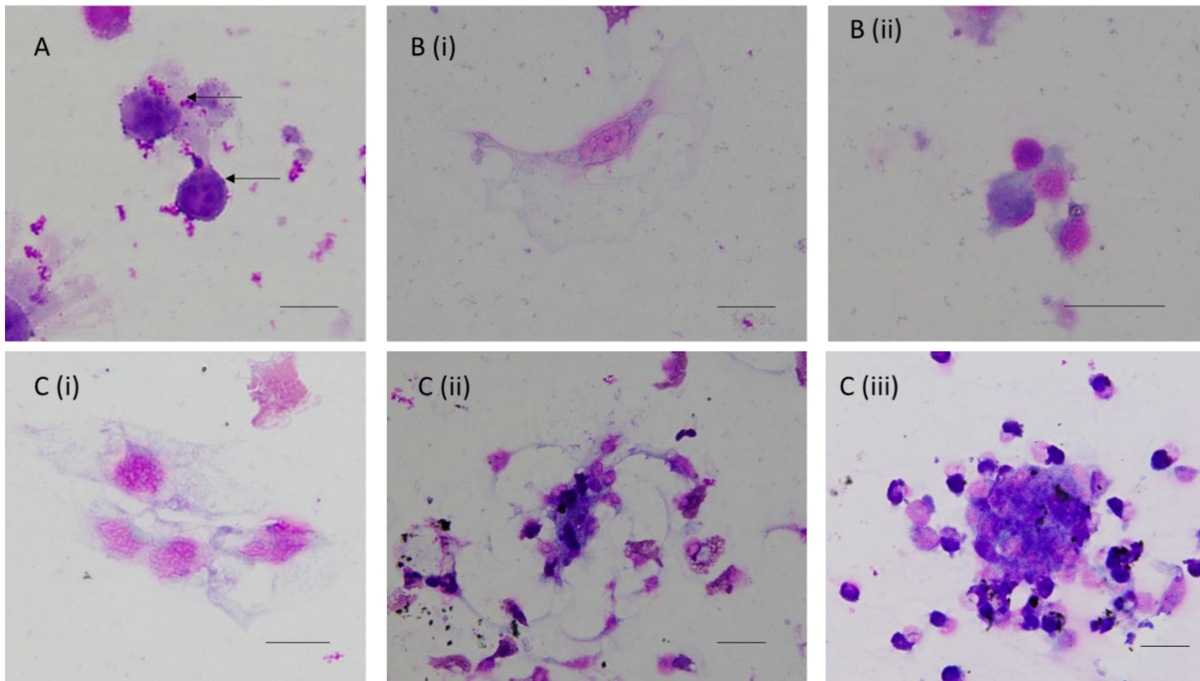


**Figure 6.4.** Hemocyte cell types and aggregations observed in the hemolymph of *Panopea zelandica* stained with Giemsa stain. A: granulocyte- arrows indicate granules, B(i): large hyalinocyte, B(ii): small hyalinocyte, C(i-iii): small, medium, and large hemocyte aggregations (left to right) (Scale bar: 20μm).

Hemocyte subpopulations differed among the different experimental temperatures, with the percentage of different populations presented in figure. 5 (based on 9 geoduck per temperature). There was a significant (ANOVA, $F_{(2,11)} = 5.17$, $p < 0.03$) increase in abundance of granulocytes with increasing temperature, whereas there was a significant (ANOVA, $F_{(2,11)} = 5.59$, $p < 0.03$) decrease in abundance of small hyalinocytes with increasing temperatures. Pairwise comparisons of granulocytes and small hyalinocytes subpopulations showed that the significant differences lay between 17 and 25°C. Even though there was a decrease in abundance of large hyalinocytes with an increase in temperature, the differences were not significant.

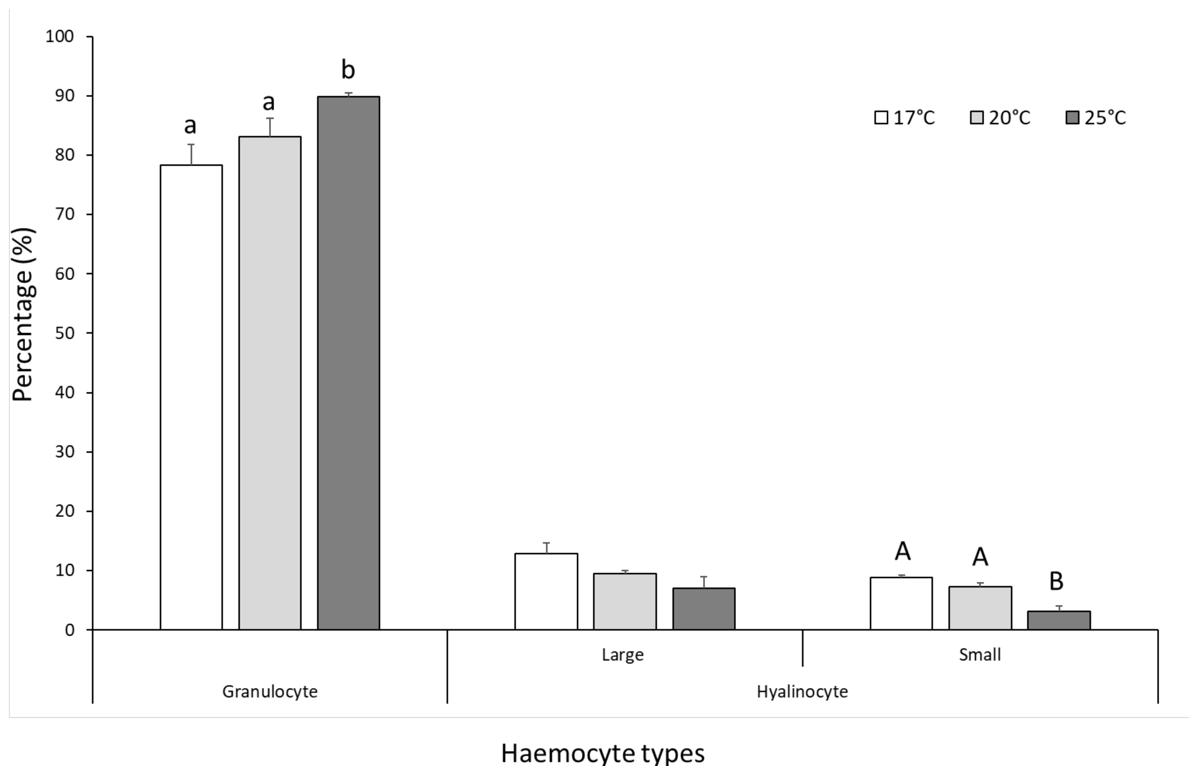


**Figure 6.5.** Percentage of individual haemocyte subpopulation types: granulocytes (left) large hyalinocytes (middle) small hyalinocytes (right), recorded in *Panopea zelandica* juveniles (n = 9) at different temperatures. Significant differences in the proportion of haemocyte cell type are denoted by upper- and lower-case letters above each bar within each subpopulation (significance taken at $p < 0.05$).

A significant increase in the number of haemocyte aggregations (ANOVA, $F_{(2,21)} = 57.9$, $p < 0.001$) was recorded with increasing temperatures. At 17°C, 3 ± 10.5 average ± S.E haemocyte aggregations were detected, while at 20°C, 15 ± 3 and at 25°C, 35 ± 2 cell aggregations were recorded (Fig. 6A). Small and medium haemocyte clusters were observed mainly comprising of large hyalinocytes, while large clusters were observed as a mass of granulocytes. There was a significant increase (ANOVA, $F_{(2,78)} = 10.68$, $p < 0.001$) in the respective size of these aggregations with 58.31 ± 3.85 µm (average ± S.E) seen at 17°C; 106.61 ± 8.10µm at 20°C and 172.82 ± 20.55µm at 25°C (Fig. 6B). Pairwise comparisons showed differences in number of aggregations at all tested temperatures, whereas the size of the aggregations were only significantly different between the 17 and 25°C treatments.

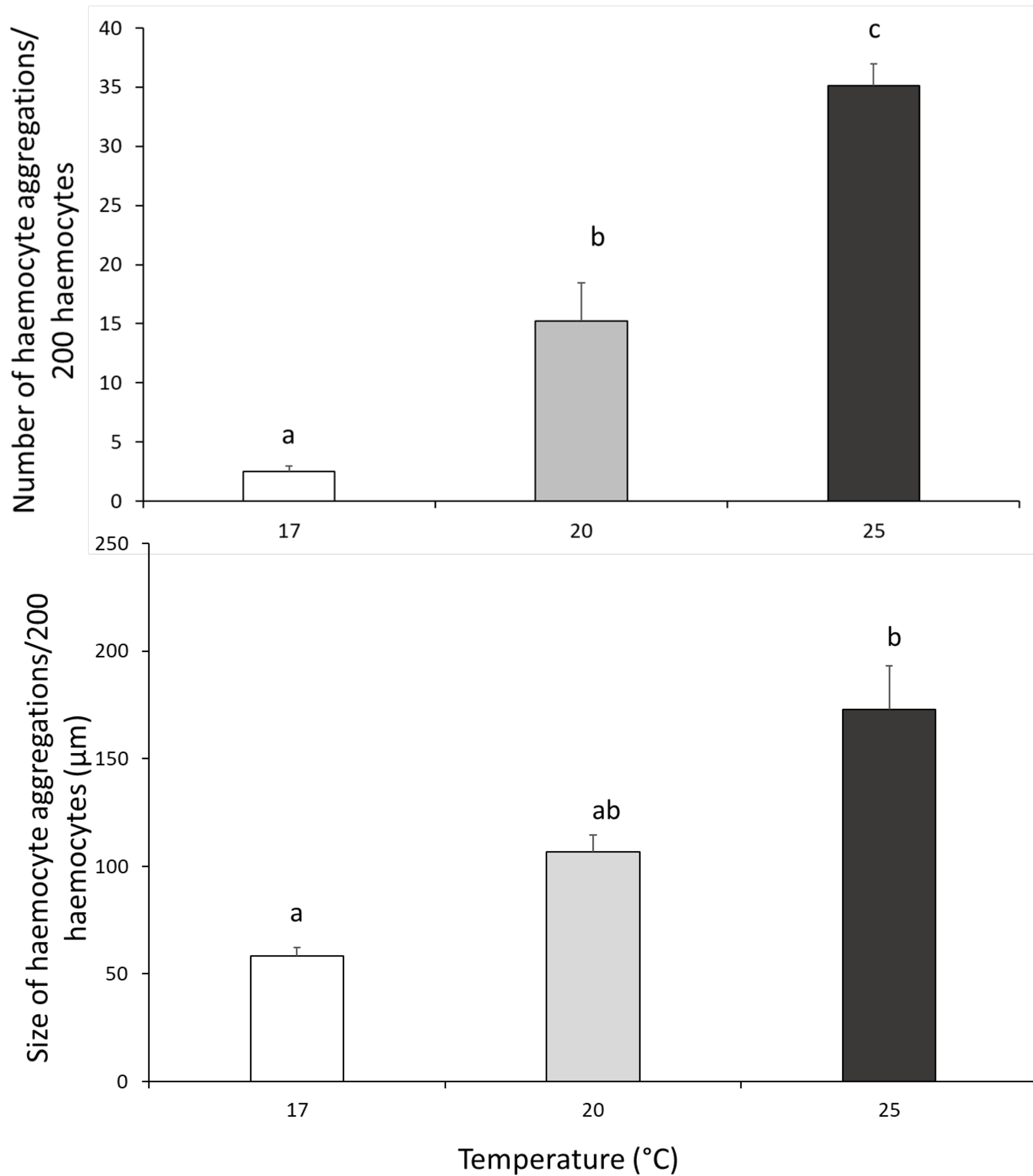


**Figure 6.6**. Number (top) and size of haemocyte aggregations (bottom) recorded per 200 haemocytes in *Panopea zelandica* juveniles (n = 9) at different experimental temperatures. Significant differences ($p < 0.05$) are denoted by lowercase letters above each bar.

### 6.3.4 HSP 70 and 90 expressions

There were no significant differences in the expression of *HSP70* in the gill tissue at the different experimental temperatures (Fig. 7A), however the expression of *HSP70* was highest at 25°C, with a 16-fold increase from 17°C to 25°C. In haemolymph samples, the expression of HSP70 was recorded to significantly increase with increasing temperatures (ANOVA, $F_{(2,20)} = 15.17$, $p < 0.001$). Relative comparisons between 17°C and 25°C showed a 543-fold increase in HSP70 expression within the haemolymph (Fig. 7B). Results from *HSP90* expression showed no significant differences in either gill (Fig. 7C) or haemolymph (Fig. 7D) samples. During the experimental treatments, the levels of *HSP90* remained similar at different experimental temperatures. There was an increase in *HSP90* expression in haemolymph samples at 25°C, but this increase was not statistically significant.

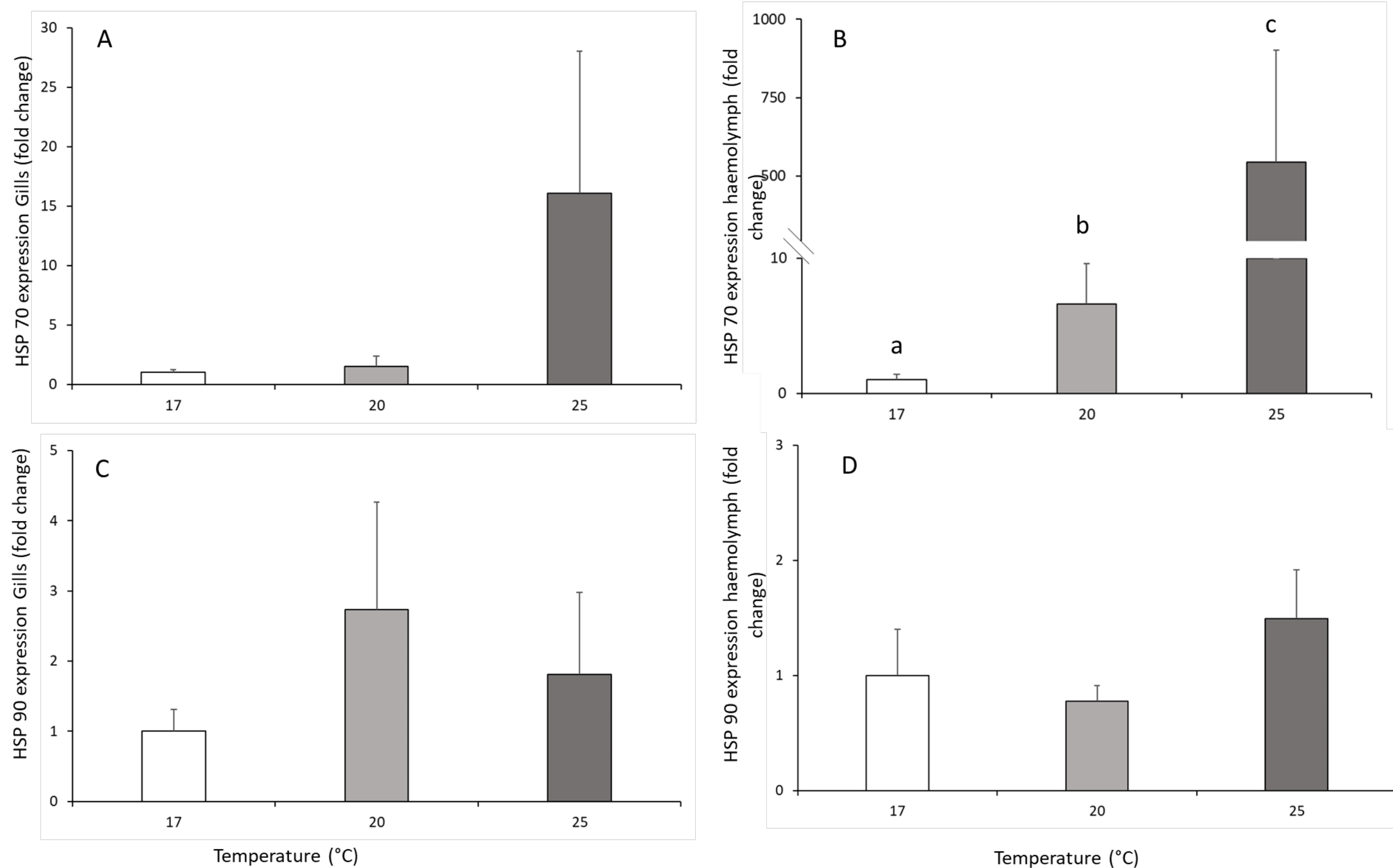


**Figure 6.7.** The gene expression of heat shock proteins in *P. zelandica* juveniles (n = 9) exposed to 17, 20 and 25°C. Fold change values of *hsp70* gene expression detected in A) gill and B) haemolymph samples, along with gene expression of *hsp90* in C) gill and D) haemolymph samples. Significant differences ($p < 0.05$) are denoted by lower case letters on top of each bar.

## 6.4 Discussion

The adaptive capacity of marine organisms to heat stress usually involves a combination of responses that interact in tandem (Leung et al., 2019). With this in mind, the present study focused on a myriad of responses to thermal shock in juvenile *Panopea zelandica*. These responses included, behavioural assessments, haemocyte changes, and gene expression of heat shock proteins (HSP) 70 and 90 within the gills and haemolymph, characterized following a 24h of thermal exposure at 17°C (ambient), 20°C and, 25°C. There were changes within all parameters measured, which suggests that *P. zelandica* is affected by heat stress from the molecular level to the whole animal level.

Behavioural responses are often difficult to quantify, especially for animals with limited visibility, such as infaunal clams (Woodin et al., 2020). In the majority of thermal studies (Rubio-Portillo et al., 2016; Whiteley & Mackenzie, 2016; Pansch et al., 2018; Miranda et al., 2019; Shanks et al., 2020), bivalves are subjected to continuous thermal exposure as opposed to considering the buffering effects of the sediment, thus limiting relevant information behavioural adaptations (such as burial speed and depth). The previously mentioned approach appears to be useful in providing the fundamental mechanisms, but it does not provide information in response to realistic conditions. With limited studies, the common consensus is that bivalves tend to dig into the sediment as the sediment tends to partially buffers the changes in temperature (Archambault et al, 2013, Zang et al., 2020, Domínguez et al., 2021. As in a natural setting, the ocean water and sediment pore water are usually separated, and temperature changes are gradual due to pore water circulation

(Rato et al., 2022). In the present study, geoducks were left in situ, and with increasing temperatures the proportion of geoducks exiting the sediment significantly increased. This behaviour has not been documented for *P. zelandica* prior to this study. However, in a study on the Manila clam, *Ruditapes philippinarum*, it was found that at higher sediment temperatures the clam actively reduced its burial depth closer to the sediment-water interface (Liu et al., 2022). The response of *R. philippinarum* was due to the rapid consumption of dissolved oxygen due to increased temperatures resulting in an increase in hydrogen sulfide concentrations around the clam. In other studies, temperatures above the sublethal limit have also been documented to affect the browning ability of several bivalves such as, *Cerastoderma edule*, *Venerupis corrugat*, *Lampsillis siliquoidea*, *L. cariosa*, *L. fasciola,* and *L. abrupta* (Archambault et al, 2013, Domínguez et al., 2021). Therefore, the geoducks in this study could have been subjected to toxic sediment environments or lethal temperatures causing the animals to exit the sediment in search of a more suitable environment. However, the ability of geoducks to re-bury themselves decreases as the animal grows, partly due to their poorly developed foot muscles (Reidy & Cox, 2013). Therefore, it is not known at what size/ age class would reburial not be possible for *P. zelandica,* as this behaviour is not ubiquitous to all size/age classes. The behaviour of exiting the sediment has not been observed in the natural environment, thus making this an interesting topic for future research.

High water temperature has been reported to influence haemocyte parameters, such as hemocyte motility and viability in bivalves (Fısıer, 1988; Monari et al., 2007). The higher percentage of granulocytes detected in geoducks with increasing temperatures, has also

been documented in other bivalve species, such as the oyster *Crassostrea virginica* (Chu & La Peyre, 1993), the clam *Ruditapes philippinarum* (Liu & Zhao, 2018), and the mussel *M. virgata* (Chen et al., 2007). Monari et al. (2007) hypothesised that the increase in granulocytes due to increased temperature exposure is an immune response as bacteria tend to proliferate at higher temperatures. Indeed, Monari et al. (2007) observed a significantly higher number of bacteria surrounding granulocytes of the clam *C. gallina* exposed to a water temperature of 30°C. Therefore, the increase in percentage of granulocytes recorded in *P. zelandica,* in the present study, could be an evolutionary immune response to prepare for an oncoming possible bacterial infection. On the other hand, in the present study, there was a decrease in the percentage of hyalinocytes observed with increasing temperatures. One likely cause for the decrease of hyalinocytes is the increase in the size of haemocyte aggregations with increasing temperatures, which made it harder to get an accurate number of hyalinocytes. Bivalve hyalinocytes have been reported to play an important role in haemocyte aggregations processes due wounding (Ruddell, 1971; Suzuki et al., 1991 ; Aladaileh et al., 2007 ; Gosling, 2015; Lauet et al., 2017). Nakayama et al. (1997) found that when the haemolymph of the giant clams (*Tridacna crocea*) is exposed to seawater, the haemocytes tended to coagulate, with hylanocytes forming the core of these clots. Therefore, the cause of haemocyte aggrations observed in the present study appears to be due to wounding. Unlike other infaunal clams, *Panopea* genus has poorly developed abductor muscles and thus actively requires sediment to keep its valves fully closed. Once out of the sediment (in the case of animals in the high experimental group), the abductor muscles are extended beyond the normal amount, potentially causing muscle tears. Interestingly, an increase in the size of hemocyte

aggregations with increasing temperatures was also observed in Manila clam (*R. philippinarum*) where the authors suggest that since bivalves are poikilotherms, hemocyte activity may scale up with temperature, thus explaining the correlation between aggregate size and temperature (Flye-Sainte-Marie et al., 2009).

In the present study, the expression of heat shock proteins (HSP70 and HSP90) in the gills and haemolymph were analyzed. Heat shock protein expression has been reported to change in response to external stressors, such temperature, potentially as a response to stress-induced tissue and protein damage (Fabbri et al., 2008, Liu et al., 2014, Velez et al., 2017, Jahromi et al., 2020, Timminus-Schiffman et al 2020). From the current investigation, higher temperatures influenced the upregulation of HSP70 in the haemolymph. This is particularly important, considering the role the haemolymph plays eliciting mounting cryoprotection against severe cellular stress during times of heat shock (Silver & Noble, 2012). Although there was a trend of increasing *HSP70* in the gill tissue with increasing temperatures *of P. zelandica*, this was not significant. This result could be attributed to the difference in peak HSP gene expression at different times in different tissues. Jun et al., (2016) found that in the pearl oyster *Pinctada martensii* the expression of HSP was faster in in the hemolymph compared to the gills. Moreover, it has been found that different tissues upregulate *HSP70* at different rates, with peak *HSP70* expression achieved in the gills of the scallop *C. noblis* by 6 hours, declining thereafter (Cheng et al., 2020).

There was no significant difference in the expression of *HSP90* in either the gills or the haemolymph of *P. zelandica* in the current study. A number of investigations have shown

that the expression of *HSP90* behaves differently compared to *HSP70*, with expression either not changing, or only being upregulated immediately following the initiation of heat-shock, declining thereafter (Lyons et al., 2003; Musanja et al., 2022). In *Pinctada maxima*, *HSP90* expression in the gills and hemolymph were upregulated within the first 6 hours after exposure to heat-shock, peaking at 6 hours and declining to the end of the experiment at 72 hours (Musanja et al., 2022). Conversely, in a study on the Venus clam *Paphia undulata*, *HSP90* was highly detected in the gonad and gills, and poorly expressed in the haemocytes following two hours of heat stress. It has been suggested that this could be as a result of modifying biochemical reactions and changes in the resource energy allocation, with energy being reallocated towards more beneficial proteins, such as HSP70 (Musanja et al., 2022). It is possible that in the current investigation, HSP90 may be involved in the acute stages of heat stress with HSP70 being upregulated when *P. zelandica* experiences chronic stages of heat-shock However, as sampling did not incorporate the acute periods of heat shock, this cannot be confirmed. Considering that samples for HSP90 analyses were collected after 24-hours of thermal exposure, expression levels might have been bypassed, necessitating smaller sampling intervals in future.

In both HSP70 and HSP90, changes in gene expression related to heat-stress showed high individual variability among the geoducks. This is particularly so when *P. zelandica* was exposed to 25ºC. This level of variability suggests that *P. zelandica* may have the ability to acclimate to increased temperature as a result of the upregulation in the gene expression of heat shock proteins. Such variability has been demonstrated in other marine invertebrates, such the urchin *Evechinus chloroticus*, and can indicate that some

individuals are naturally pre-conditioned to tolerate chronic heat-shock compared to others (Delorme et al., 2020). This variability is an important mechanism for the population in the face of ocean warming as it will allow resilient individuals or genotypes to be selected for future conditions. This can also be valuable for future aquaculture ventures involving *P. zelandica* as it can act as a screening tool for future selective breeding to generate resilient *P. zelandica* stock.

The novel findings from this study demonstrate that *P. zelandica* has a heightened response to thermal changes. As the temperature increased from 17 – 25°C, there were changes in behaviour, haemocytes, and expression of HSP70 & HSP90. Behaviourally, there was an increase in the number of animals exiting the sediment with increasing temperatures. However, it is not known if the juveniles within the current experiment would have been able to rebury when the conditions became favourable again. Internally within the haemolymph, there was an increase in the proportions of granulocytes and cellular aggregations and a decrease in the proportions of small hyalinocytes in animals at 25°C. At the molecular level, there was an increase in the expression of HSP70 within the haemolymph, indicating that *P. zelandica* was actively trying to prevent damages to proteins and tissues. The current study contributes to our understanding of how juvenile *P. zelandica* respond to thermal shock, providing scientific knowledge for fishery management decisions and aquaculture planning phases, since *P. zelandica* shows great potential for growing in subtidal habitats covering a reasonable temperature range around New Zealand.

# CHAPTER 7 - DISCUSSION AND CONCLUSIONS

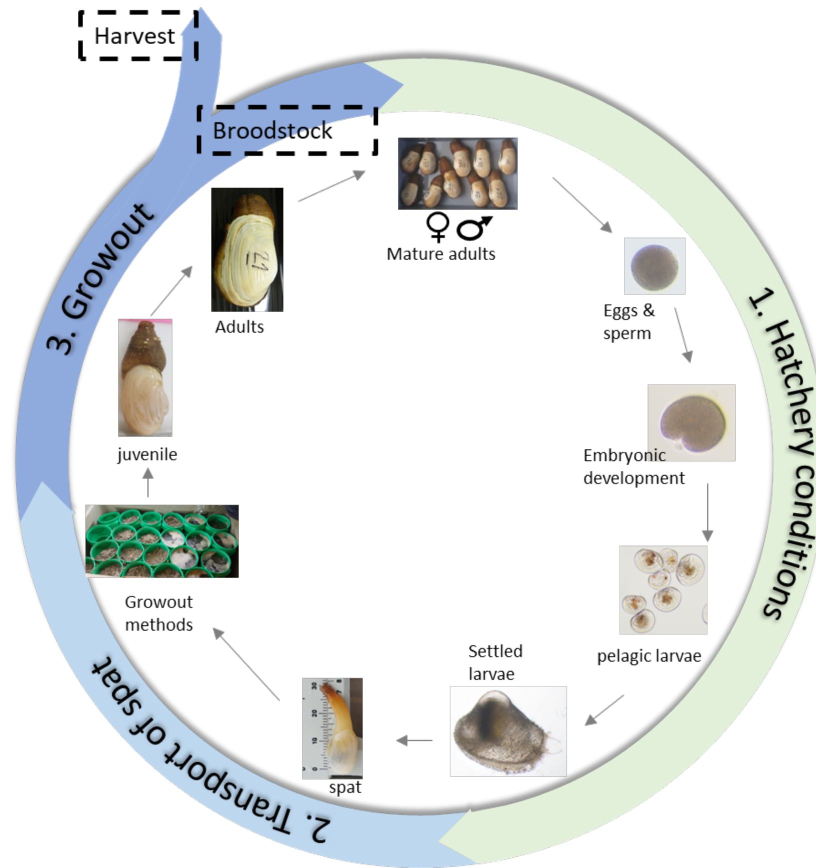


Figure 7.0 . Linking the components of the thesis together

## 7.1 Thesis background

Aquaculture has been established in New Zealand as early as the 1980s (FAO). Since then, the aquaculture industry has primarily been dominated by Greenshell™ mussel (*Perna canicalus*), pacific rock oyster (*Crassostrea gigas*), and the king salmon (*Oncorhynchus tshawytscha*). Even though the potential for geoduck aquaculture was recognized in the 2000's by Gribben et al. (Gribben & Hay, 2003; Gribben et al., 2004; Gribben et al., 2014), there were no studies conducted on hatchery techniques and farming conditions until mid-2010s (Le et al., 2016; Le et al., 2017). This interest in geoducks was primarily due to a strategy by the New Zealand aquaculture industry and government to achieve export earnings of NZ$3 billion by 2030 (MPI 2022). Thus, this thesis work was conducted under a collaboration between the Aquaculture Biotechnology Research Group at the Auckland University of Technology, Auckland and the Cawthron Institute, Nelson to optimising conditions that can support aquaculture initiatives of *P. zelandica.*

The studies presented investigate different aspects of geoduck aquaculture (from hatchery to growout) and potential bottlenecks during all areas of production. The findings in this thesis provide critical *P. zelandica* knowledge on larval culture, and the effect of stressors associated with transport and field grow out methods. Next I discuss the results from my PhD work, address the strengths and limitations, provide recommendation for P. zelandica aquaculture and suggest potential areas for next experiments. It is envisaged that the information gained from this thesis will form the basis of protocols to develop a successful aquaculture from hatchery to growout of *P. zelandica* in New Zealand. In addition, this

thesis also provides information that will enable better fisheries management of *P. zelandica*.

## 7.2 Broodstock conditioning and larval rearing

Hatchery grown spat would form the basis of geoduck aquaculture in New Zealand. Unlike the geoduck aquaculture industry in the USA and Canada where ripe broodstock are collected every season from the wild, in New Zealand due to low densities and small population sizes (Gribben & Heasman, 2015) a population of *P. zelandica* must be kept in the hatchery and conditioned annually to produce spat. The present studies (chapter 2 - 4) provide much needed information on successful conditioning of *P. zelandica* broodstock and subsequent larval development. This study (chapter 2) is the first to report the transgenerational effects of broodstock conditioning. The broodstock were successfully kept on a diet heavily consisting of *Tisochrysis lutea* (ISO) and *Chaetoceros muelleri* (CM), however the results indicate that conditioning broodstock with equal ratios of ISO and CM produces high quality embryos. As the embryos produced from mixed diet conditioning have an overall higher survival, transition into D – veligers, size of D - veligers, with low incidence of abnormalities within the D – veligers and a greater salinity tolerance. Findings of this chapter have immediate applications on reducing hatchery costs as this study show that broodstock can be kept on a single algal culture of ISO or CM during non-conditioning period and switched to a mixed diet of ISO and CM during the conditioning period. Constantly keeping broodstock on mixed cultures of ISO and CM is not optimal for the hatchery as culturing algae on its own is highly expensive. Future studies could benefit from developing artificial feeds which may reduce the overall cost of keeping broodstock all year round and feeding during broodstock conditioning.

After obtaining embryos, the next step is to optimise the development of larvae by altering larval rearing conditions. Temperature appears to be one such factor, since in hatcheries temperature is often manipulated to reduce the incubation time for larvae to develop from embryos (Helm, 2004). However, the degree to which temperature can be manipulated is species-specific and there was no information available on *P. zelandica* prior to our study (chapter 3). The study clearly demonstrated an increase in fertilization and development of embryos increased with increasing water temperatures. However, there appears to be a thermal maximum for the optimal development of *P. zelandica* embryos as an increase in temperature also resulted in an increased incidence of abnormalities. The major strength of this study was the utilization of a wide temperature scale with small temperature increments giving a fine scale view into the development of the embryos. The results indicate that *P. zelandica* embryos have a narrow temperature range during development and in hatcheries this should not exceed 18°C. This study (chapter 3) has wider implications for the wild populations of *P. zelandica* as adults spawn when the ambient sea temperatures reach 15°C in spring and autumn (Gribben et al., 2004), these months are also susceptible to marine heat waves, and with the projected increase in sea temperatures larval development of natural stocks of *P. zelandica* could be greatly affected. Therefore, hatchery produced spat could form a viable alternative to re-establishing wild stocks.

Once *P. zelandica* embryos have transitioned into D – veligers, they remain within the water column for up to 19 days prior to settlement. The newly transitioned D – veligers are no longer dependant on the provisions provided for in the egg and thus larvae have to actively acquire food for growth and development. Therefore, it is no surprise that food availability on its own is a common stressor for the developing larvae. The next study (chapter 4)

addressed food availability and associated vulnerability of the developing *P. zelandica* larvae. This study showed that susceptibility of larvae to thermal stress reduces with larval age, suggesting that older larvae are better able to acquire and store exogenic resources. This information is important as it shows that a greater level of care is needed during the early stages of larval development to assure a high survival rate. This study also showed that there is a daily maximum growth rate for *P. zelandica* larvae and providing excess microalgae does not increase daily growth rates but can be detrimental to both growth and survival. Microalgae tend to extrude transparent exopolymers (Corzo et al., 2000) at lower concentrations. These exopolymers do not seem to pose any problems, but higher concentration of these exopolymers have led to larval clumping and adhesion to surfaces (Ragg et al., 2010). Findings of this chapter have immediate applications to hatchery operations as by matching the concentration of microalgae with the daily ingestion of larvae a high turnover of settled larvae could be achieved with minimal algal waste.

## 7.3 Transport of juveniles

In New Zealand, the current consensus is to construct a single geoduck hatchery and transport spat to growout and re-seeding sites across the two main islands. This experiment provides an understanding of the underlying mechanisms during transportation, reporting for the first time on the behavioural, haemocyte, and metabolic effects of transportation events and recovery in growout sites. The first clear finding from this research is that the duration of emersion had a significant effect on overall *P. zelandica* response and recovery. Following 3-hours of transport, smaller changes within haemolymph osmolality, haemocyte parameters and metabolism were experienced (in comparison with the responses after 8-hours of transport). Also, animal behaviour returns to normal faster within the 3-hour transport group. These results suggest that *P. zelandica* respond and recover well with the current transport protocols established for other bivalves which include out of substrate and in air transport. This is important for growout sites closer to the hatchery as *P. zelandica* has shown the potential to adapt to transport for 3h, by activating defence and repair mechanisms to ensure survival post transport.

As the transportation/ emersion time extends to 8 hrs, there is a greater change in the metabolic profile and behaviour of the animals. The animals tended to stay hidden in sediment and did not display any signs of feeding for a longer period. This was seen in the metabolic profiles as the animals actively switched from aerobic to anaerobic respiration to reduce oxygen demand thus depleting internal reserves to produce energy. After 5 days of recovery in standard conditions, juvenile geoducks started to display a return to aerobic metabolism as the main source of energy production. However, the effects of emersion

were still evident with the use of succinate to fuel the respiratory chain within the 8h exposure group. To prevent added stress to the animals, future research could be focused on modifying the existing methods or implementing other novel ways to transport the animals. To reduce the metabolic stress on the animals, one suggestion is to reduce the temperature at which the animals are transported at, since with lower temperatures the energy demand for bivalves is greatly reduced. The size of the animals should also be considered as larger animals tend to fare better when dealing with stressful conditions of transportation. Since *P. zelandica* is an obligate infaunal species other practical methods which include in-sediment or in-water transport should also be explored. However, it does appear that given the right conditions and enough food resources the animals will be able to successfully restore internal energy reserves. It must be recognized that there is a practical problem in looking at recovery over an abbreviated period in a laboratory. In a laboratory setting the environment is controlled and the integration of natural factors, such as temperature and food availability is not apparent which is dynamic in the wild. Novel findings of this study do indicate that juvenile *P. zelandica* can be successfully transported from hatcheries to growout sites across New Zealand. To further reduce the metabolic stress and aid in acclimation, one suggestion is that these transportation events occur in winter months. This study is a reassuring prospect and introduces an opportunity to assess alternative transport scenarios in future. Ultimately this information is important for predicting the long-term success of juvenile planting and growing.

## 7.4 Field Growout

Once geoducks arrive to potential growout sites, there lies an additional 3 - 6 years before reaching market size. Even though geoducks remain fully immersed in substrate they are not fully protected from biotic and abiotic factors. Mass mortalities are a common occurrence in bivalve aquaculture and not only reduce production but can also have a negative effect on natural populations (Son & Ransangan, 2019). With reports of increase in global sea temperatures and the increased chances of marine heatwaves, temperature could pose a substantial challenge to the geoduck industry in New Zealand. Chapter 6 reports the first study on the behavioural and physiological response of *P. zelandica* to a simulated heat wave. Behaviour can potentially be used as a non-destructive, non-invasive biomarker of physiology in this clam species. By recording behaviour overtime, it becomes possible to identify when individuals are experiencing increased stress and hence serves as a sign when intervention would be needed. An increase of seawater temperature to 25°C resulted in all geoducks emerging from the sediment, arguably as a method to escape the warming conditions. This is the first recorded observation of *P. zelandica* actively exiting the sediment. It is well known that that geoducks have poorly developed foot muscles and their ability to rebury is highly limited. Future work needs to be conducted on the capacity of *P. zelandica* to rebury itself when disturbed. There are two major issues that could result with the animals exiting the sediment, firstly the entire population could be lost as once out of the sediment they could potentially “move” out of the growout sites or result in mortalities if they are unable to rebury and when geoducks exit the sediment they are left vulnerable to the elements as they can be easily preyed upon when exposed. Understanding the

capacity of geoducks to rebury could help formulate predator protective measures during such events or finding deeper and less heatwave susceptible growout locations to prevent geoducks from exiting the sediment.

A simulated marine heatwave also resulted in haemocyte changes in *P. zelandica* showing a decrease of hyalinocytes and an increase in granulocytes and haemocyte aggregations when temperatures increased from 17 to 25°C. The overall health of *P. zelandica* appeared to decline with increasing temperatures as increase in both granulocytes and haemocyte aggregations is associated with a loss of health in bivalves (Ballina et al., 2022). Thus, extended periods of higher than ambient temperatures could result in high mortalities within individual affected sites. Even small heatwaves could have a longer lasting effect on the animal as a reduction in health can leave the animal vulnerable to pathogenic infections. To date there have been no studies conducted on the pathogens that affect *P. zelandica*. Therefore, future research should focus on the resilience and pathogens that infect *P. zelandica*.

The measurement of genes (*hsp70* and *90*) expressing heat shock proteins are herein reported for the first time in *P. zelandica* following subjection to thermal stress. At the molecular level, there was an only increase in the expression of HSP70 within the haemolymph, indicating that *P. zelandica* was actively trying to prevent damage to proteins and tissues. However, the high changes in the gene expression of both HSP70 and HSP90 with increasing temperatures show the individual variability among the geoducks. This level of variability suggests that *P. zelandica* may have the ability to acclimate to increased temperatures as a result of the upregulation of heat shock proteins. The varibility of heat shock protein expression is an important mechanism for the population in the face of ocean

warming as it will allow resilient individuals or genotypes to be selected for future conditions. This can also be valuable for future aquaculture of *P. zelandica* as it can act as a screening tool for future selective breeding to generate resilient *P. zelandica* stock.

Ultimately the findings from this research are an important first step to support management decisions for limiting the effects of environmental changes. The behavioural and physiological response of P*. zelandica* to thermal stress in a natural environment without the constraints of the laboratory is an important next step to select suitable grow out locations and optimize geoduck production.

## 7.5 Future Recommendations

"The more you know, the more you realize you don't know"
Aristotle

While answering the research questions proposed in this thesis, new questions and possible avenues of research became apparent in different areas of geoduck production. In hatcheries, the main source of expenditure is based around the production of microalgae for broodstock (as broodstock remain in the hatchery all year round), larvae and juvenile feed. Switching to artificial diets could curb the associated costs within the hatchery. To date there have been no successful artificial diets formulated for geoducks research within this area. Research into artificial diets is highly needed as it can aid not only geoducks within hatcheries but could pave the way to possibly grow geoducks in land based growout ponds. There are still crucial areas of research required around broodstock conditioning mainly on developing non-invasive methods, such as, magnetic resonance imaging, endoscopy, and vitellogenin in haemolymph to determine sex and gonad developmental stages. There appears to be a significant loss of larval population prior to settlement, which currently can cause more than 90% larval mortalities which remains a mystery. An in-depth analysis of the processes involved during this period is needed to ensure a high turnover of spat from spawning events. During the growout phase, mass mortalities due to biotic and abiotic are a major issue for other bivalve species. Even though the effects of short-term temperature changes were reported in this thesis. Future work may be needed to investigate the ability for geoducks to acclimate to increasing global sea temperatures. As of now, there is no information on the pathology of bacterial and viral infections in geoducks.

Research in this area would be highly beneficial in order to determine optimal farm densities and locations.

## 7.6 Conclusions

The studies in this thesis successfully investigated selected factors crucial for the successful establishment of aquaculture of *P. zelandica* in New Zealand. Within hatcheries, this thesis characterised ideal microalgal diet ratios during conditioning, optimal thermal range for embryonic development, and the effects of dietary constraints on developing larvae. These studies could help hatcheries better manage costs associated with algal production and speed up the rate of larval development. The physiological response of geoduck when subjected to transport stress contributes a better understanding of this part of the production chain and will be instrumental in developing future transport methods from hatchery to growout. With the increase in sea temperatures and marine heatwaves, it was important characterize the behavioural and physiological responses to such events. This research can be applied to commercial and future hatchery, transport and grow out operations for the purpose of improving geoduck condition and health. *Panopea zelandica* remains poorly represented in the literature and considering its economic and cultural significance, research on the development of this species during all stages of production should be continued. It is envisioned that more procedures and strategies will be developed in the coming years to facilitate the establishment of geoduck aquaculture in New Zealand

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