Investigating physiological markers of heat stress in response to water temperature in kākahi (*Echyridella menziesii*) to provide insight into their vulnerability to anthropogenic climate change

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Abstract

Anthropogenic climate change (ACC) is expected to increase the water temperatures of aquatic ecosystems, which can cause heat stress in aquatic animals. Kākahi (*Echyridella menziesii*) are a species of freshwater mussel that are endemic to Aotearoa New Zealand. Kākahi are an ecological and cultural keystone species in Aotearoa New Zealand's aquatic ecosystems. Little is known about the thermal physiology of this species and, therefore, their vulnerability to increasing water temperatures under ACC. Increasing our knowledge of kākahi thermal physiology and their vulnerability to projected future water temperatures is important for the conservation of the species and the ongoing health of the ecosystems of which they are part.

For bivalve species (Class: Bivalvia), measuring the concentrations of heat stress biomarkers in the circulatory fluid is a common method of assessing vulnerability to elevated water temperatures. This has not been attempted in kākahi, leaving a gap in our understanding of how different water temperatures affect their metabolism and physiological performance.

In this thesis, I measured the concentrations of three known physiological markers of heat stress (lactate dehydrogenase, alanine aminotransferase, and aspartate aminotransferase) in the haemolymph of kākahi exposed to different water temperatures. This method was used in separate field and laboratory studies to measure potential heat stress at current (2022) summer water temperatures (field) and at projected water temperatures under different warming scenarios (laboratory). In the field study, I found that current summer water temperatures in 2022 did not cause an increase in heat stress biomarker concentrations in kākahi haemolymph. In the lab study, I found no significant increases in heat stress biomarker concentrations in kākahi exposed to 26˚C or 32˚C for seven days. The results suggest that kākahi may be resilient to increasing water temperatures under ACC.

Additionally, I extracted DNA from kākahi gill tissue and used primers designed from the consensus sequences of other molluscs to attempt to amplify the heat shock protein 70 (HSP70) gene. This additional piece of research aimed to provide important baseline information to enable future studies to measure changes in HSP70 expression in response to elevated water temperature. The designed primers were unfortunately unsuccessful at targeting the desired gene. However, this work provided important knowledge that will help refine the process for future attempts in identifying the HSP70 gene in kākahi.

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Chapter 1: General Introduction

1.1 Anthropogenic climate change and physiological heat stress

The increasing concentration of greenhouse gases emitted into the atmosphere due to human activity has caused a phenomenon called anthropogenic climate change (ACC) (IPCC, 2021). Greenhouse gases are gases that absorb and emit infrared radiation (Schneider, 1989). Carbon dioxide is the greenhouse gas that has the most profound impact on the climate. However, other greenhouse gases contribute, including methane, nitrous oxide, and hydrofluorocarbons (Gillingham & Stock, 2018). As the atmosphere absorbs greenhouse gases produced by human activity, particularly the burning of fossil fuels, higher levels of solar radiation remain in the biosphere. This increases the temperature of the aquatic and terrestrial mediums that all life functions within.

All animals have a set range of temperatures where physiological performance and function can be maintained (Pörtner, 2002). If ACC causes the optimal temperature range of an animal to be exceeded, physiological heat stress can occur. Heat stress can be defined as an imbalance between heat gain and loss that causes internal body temperature to surpass the thermal optimum for the animal (Aggarwal & Upadhyay, 2013). This poses challenges for cellular metabolism and the homeostasis of physiological systems. Further, the health of whole ecosystems may be affected if this heat stress affects the animal's ability to carry out ecological roles (Woodward et al., 2010).

The impacts of increasing water temperatures on aquatic animals and environments, specifically freshwater ecosystems, are the focus of this thesis. This will be investigated in the context of one study species: the kākahi (*Echyridella menziesii*) or New Zealand freshwater mussel. This research investigates the impacts of increasing water temperatures on kākahi physiology and, therefore, the health of the freshwater ecosystems they inhabit.

Previous research into the impacts of ACC on aquatic animals and their environments has focused largely on the ocean. This is likely due to the large scale of marine environments and human reliance on these ecosystems for food production and gathering. Oceans are known to absorb 93% of the heat generated by green-house gas emissions (Brewer, 2019). The absorption of heat by the ocean has resulted in water temperature increases that have paralleled air temperatures. Freshwater ecosystems have been a much smaller focus of the Intergovernmental Panel on Climate Change (IPCC). However, freshwater environments also act as carbon sinks, absorbing carbon dioxide from the atmosphere (Hoegh-Guldberg et al., 2014). Water temperatures in freshwater environments are increasing at similar rates to marine environments due to ACC (Hoegh-Guldberg et al., 2014). Freshwater animals are therefore also at risk of physiological heat stress, which may impact ecosystem health.

1.2 ACC is changing aquatic environments in several ways

An increase in environmental temperature is not the only change expected to occur in aquatic environments due to ACC. In many aquatic systems, both marine and freshwater, the average concentration of dissolved oxygen is decreasing (Matear et al., 2000). This process is closely related to the increase in average water temperature. Decreased oxygen concentration puts animals at risk of hypoxia (Breitburg et al., 2018). Another impact of ACC is the acidification of the aquatic environment. As oceans and freshwater systems absorb carbon dioxide, there is an increase in carbonic acid production, therefore an increased hydrogen ion concentration leading to the weak acidification of the water (Jeffrey et al., 2017). Research into ocean, rather than freshwater acidification dominates the literature, partly because the partial pressure of carbon dioxide is highly variable in freshwater (Hasler et al., 2016). However, there is evidence that acidification is occurring in both marine and freshwater environments, which puts animals at risk of hypercapnia and acidosis (Michaelidis et al., 2005; Jeffrey et al., 2017) .

ACC can affect water salinity, presenting another challenge to aquatic systems. Decreased salinity is mainly caused by the increased melting of polar ice, glaciers, and sea ice, and increased rainfall, driven by ACC (Callaway et al., 2007). Increased salinity is also possible in areas that experience lower rainfall due to ACC, if evaporation exceeds precipitation. Changes in salinity can cause osmotic stress in animals, which can have severe metabolic consequences (Ordóñez-Grande et al., 2020). However, while ocean systems are at high risk of salinity changes, it is expected that freshwater systems will not be affected to the same extent (Bertrand et al., 2017).

ACC is also predicted to increase aquatic animals' exposure to ultraviolet (UV) radiation. Specifically, some aquatic animals may be at risk of increased exposure to UV-B radiation which is highly damaging to cells (Yin et al., 2019). Exposure to UV-B is predicted to increase due to feedback loops between solar radiation and other climatic factors like cloud cover and ozone levels (Erickson et al., 2015)

1.3 The decline of freshwater mussels and the possible role of ACC

Freshwater mussels (Order: Unionida) are an animal group with one of the highest rates of extinction and imperilment on Earth (Haag & Williams, 2014). Freshwater mussels are a keystone species that play a crucial role in the health of freshwater ecosystems (Vaughn, 2018). Many anthropogenic factors have contributed to freshwater mussel decline in recent years, including habitat modification, pollution, and climate change (Bolotov et al., 2020).

One aspect of ACC that may contribute to the decline of freshwater mussels is physiological heat stress (Ganser et al., 2015). Freshwater ectotherms (including mussels) may be at high risk of heat stress due to their inability to maintain a body temperature different to that of their environment (Seebacher, 2009). Freshwater mussels have the additional risk factor of having limited mobility, and therefore have a reduced ability to move to cooler areas, unlike more mobile species. As climate change progresses, many species of freshwater mussel around the world are expected to be living close to their thermal limits (Pandolfo et al., 2010). The heat stress that is associated with experiencing

temperatures close to a species' upper thermal limit can disrupt metabolic function, cause cellular and tissue damage, and impact long-term survival through suppression of the immune system and reduced reproductive capacity (Richter et al., 2010). In addition to the direct impacts of heat stress, vulnerability to climate change may also be affected by changes in food web structure, habitat, or the persistence of other species that mussels rely on for food or reproduction (Gilman et al., 2010). How vulnerable a mussel species is to heat stress, as well as potential indirect impacts, is crucial for understanding how they might be affected by ACC.

Like other freshwater mussel species, kākahi are in decline (Rainforth, 2008). These invertebrates are an ecologically and culturally important species in Aotearoa New Zealand. Like other freshwater mussels, they perform the crucial roles of water filtration, nutrient cycling, and sediment bioturbation in streams, rivers, and lakes (Naish, 2021). They are also a taonga (treasured) species for Māori, the indigenous people of Aotearoa New Zealand. For these reasons, their conservation should be a priority, and is the motivation behind this research.

Very little research has been conducted on the physiology of kākahi, particularly their thermal physiology. While kākahi have been identified to be likely at risk due to ACC (NIWA, 2020), we do not know how vulnerable they are to increasing water temperatures. Increasing our understanding of kākahi physiology and their vulnerability to climate change is the purpose of the research presented in this thesis. The overarching goal is to increase knowledge of relevance to conservation planning for kākahi, to support conservation efforts for this important species. The combination of new physiological knowledge, with existing ecological knowledge and Mātauranga Māori, may enhance conservation outcomes for kākahi and the freshwater ecosystems they inhabit.

The overall aims for this study are presented at the end of the Chapter 2 literature review.

1.4 The use of physiological biomarkers to determine vulnerability to climate change

In the field of climate change physiology that has been rapidly expanding over the last 20 years, a common method of predicting an animal's possible vulnerability to ACC is by measuring the concentrations of various stress biomarkers under different environmental conditions (Park et al., 2009; Zhou et al., 2019; Yazhou et al., 2020). Biomarkers can include enzymes involved in metabolism, redox stress and cell protection, fuel substrates like glycogen, or genes that code for proteins that are essential in the cellular stress response. Samples can be collected from animal tissue, blood and other circulatory fluid, or urine, after exposure to the environmental factor of interest. The concentrations of stress biomarkers in the sample can be used to monitor the physiological response of animals to different types of environmental change. The biomarkers measured in this thesis were chosen to provide insight into how the cellular metabolism of kākahi may be affected by different water temperatures. The concentrations of stress biomarkers under specific environmental conditions can then be used to predict the physiological and ecological implications of an animal living in those conditions in the future.

1.5 Chapter outline

This section explains the structure of the thesis, including what will be included in each chapter.

Chapter 2: Literature review

This chapter provides an in-depth review of the literature that is relevant to this research. First, the phenomenon of anthropogenic climate change and how it is causing changes to aquatic environments. Second, the impacts of these changes on aquatic animal physiology, and ecosystem health. Third, an overview of bivalve mollusc biology, physiology, and specific impacts of ACC on these animals. Fourth, a discussion of the freshwater mussels, how ACC may be contributing to their decline, and how biomarkers may be used to measure vulnerability to ACC in these animals. Finally, a review of kākahi is presented, including their ecological and cultural importance, and a discussion of the unknown role of ACC in their decline. The chapter will conclude with the aims and hypothesis for this research.

Chapter 3: Field sampling kākahi for potential markers of heat stress

Field sampling kākahi was a major component of this research. This chapter explains the processes involved in the field study, including sampling sites, protocols, and what was found in the samples taken. The aim of the field study was to measure known markers of heat stress in kākahi at the peak of summer and during cooler autumn/winter water temperatures at five locations. Haemolymph sampling and enzyme assays were used to measure the concentrations of biomarkers associated with heat stress.

Chapter 4: Laboratory experiment to investigate potential heat stress in kākahi under projected water temperatures

The second major component of this research was to measure the concentrations of the same heat stress markers from Chapter 3 in kākahi exposed to temperatures that aimed to simulate projected warming scenarios under ACC. This chapter describes the experiment run in the laboratory with captive kākahi. It includes the pilot study, which was needed to validate methods for maintaining kākahi in tanks, culturing algae to feed to the kākahi and sampling strategies for the main temperature experiment. The latter involved haemolymph sampling after temperature exposure and subsequent measuring of biomarkers associated with heat stress.

Chapter 5: Identification of heat shock protein 70 in kākahi tissue

The last experimental chapter of this thesis describes an additional piece of research that investigated the presence of the heat shock protein 70 gene in kākahi tissue. This chapter describes the processes of designing primers, DNA extraction, PCR, gel electrophoresis, and sequencing. This research was conducted outside the main aims investigated in chapters 3 and 4. Instead, the aim of this experiment was to provide baseline information that would be required for future research into the roles of gene expression in climate change vulnerability in kākahi.

Chapter 6: General discussion and conclusion

This chapter synthesizes the findings from the previous chapters and situates them within the literature. Conclusions are made about how the results from each chapter support or

do not support the hypothesis, and whether the aims were achieved. Limitations of the research are discussed, and suggestions for future research are given.

Chapter 2: Literature Review

2.1 What is anthropogenic climate change?

The Earth has experienced dramatic changes in the climate multiple times in its history (Lister & Stuart, 2008). For example, at the end of the Early Triassic period, specifically at the Smithian-Spathian boundary, there is evidence of major climate warming (Chen et al., 2013). It has been suggested that rising atmospheric carbon dioxide in this period was responsible for the climate warming and species extinction event that followed (Galfetti et al., 2007). Although the planet has experienced climate change before, anthropogenic climate change is a unique threat because it is the first time that the activities of one species (*Homo sapiens*) has caused such pronounced and rapid warming of the climate.

Anthropogenic climate change (ACC) is the overarching term for the multitude of environmental changes occurring due to increasing emissions of greenhouse gases caused by human activity. These changes include global warming, increased rainfall, and an increase in the frequency of extreme climate events (IPCC, 2018). This thesis focuses on the impacts of global warming on kākahi, a species of freshwater mussel that is native to Aotearoa New Zealand. Global warming refers to the increase in average global temperature that has been occurring since the start of the industrial revolution (Jonsson, 2012). Global warming is driven by the increasing concentration of greenhouse gases in the Earth's atmosphere. The greenhouse effect is a natural and important process for life to be successful on Earth. Briefly, solar radiation passes relatively easily through the atmosphere reaching the Earth's surface, heating it in the process. Some energy is reflected from the Earth's surface as infra-red emissions. Most of the infra-red emissions are absorbed by atmospheric carbon dioxide and water vapour (Anderson et al., 2016).

The greenhouse effect allows the Earth to exist at temperatures that have allowed life to thrive for millions of years. Without it, the average surface temperature of the Earth would be approximately -21˚C (Anderson et al., 2016). However, when humans began burning fossil fuels in the industrial era, the amount of greenhouse gases, particularly carbon dioxide (CO_2), emitted into the atmosphere started increasing. A higher concentration of CO_2 intensifies the natural greenhouse effect. The atmosphere absorbs more energy, trapping heat and subsequently warming the planet. It is well established that the gradual increase in greenhouse gas emissions associated with human activity, particularly burning fossil fuels, has been accompanied by an increase in average global temperatures (IPCC, 2021). This relationship between atmospheric carbon dioxide and global temperature, which drives ACC is known as the Callendar Effect (Figure 1).

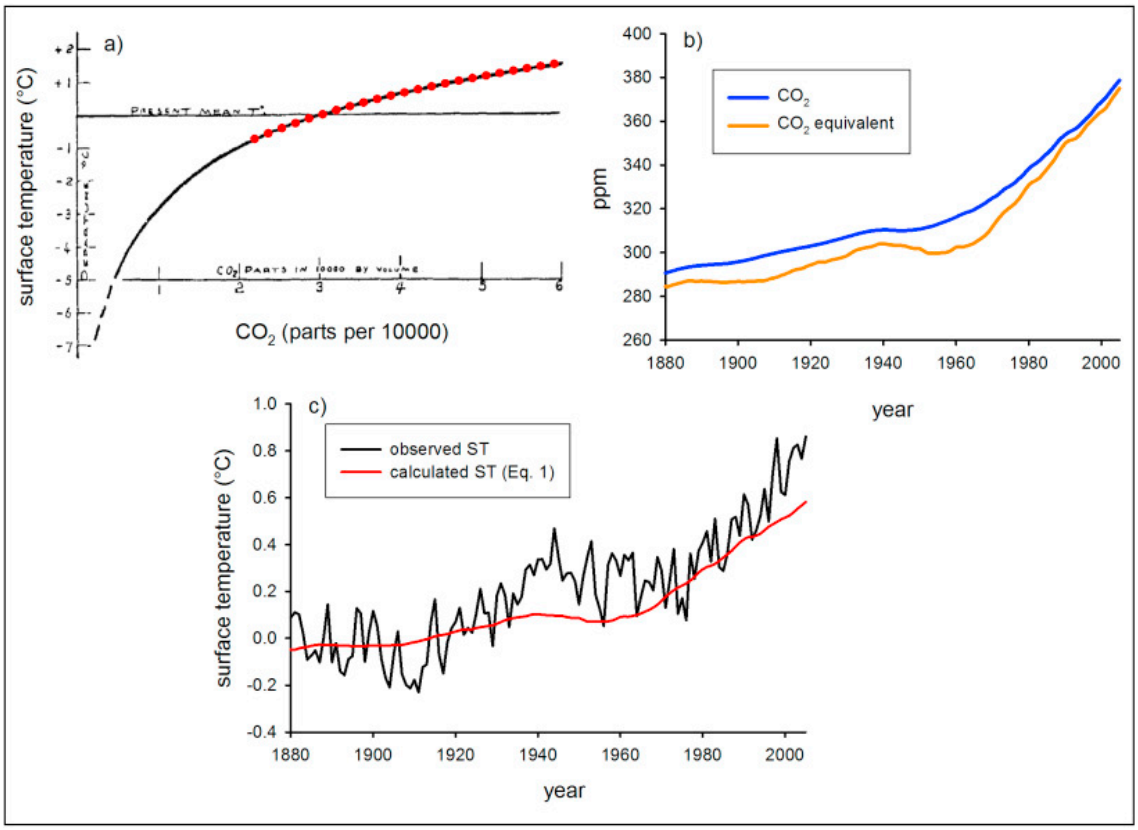

Figure 1: Callendar's model that demonstrates the link between global annual mean surface air temperature and atmospheric CO_2 levels. Graph (a) is from a paper written by Guy Stewart Callendar in 1938. It plots the positive relationship between the partial pressure of carbon dioxide (pCO_2) and surface temperature (˚C). Graph (b) plots the annual global average of parts per million (ppm) of CO_2 between 1880 and 2000. Graph (c) plots the observed and calculated surface temperature (˚C) between 1880 and 2000 (Anderson et al., 2016).

Due to the relationship between greenhouse gas emissions and global temperature (Figure 1), global surface temperature has increased by between 0.8˚C and 1.3˚C above pre-industrial levels, with a best estimate of 1.07˚C (IPCC, 2021). The rate of greenhouse gas emissions from human activity and global temperature rise are still increasing. The level of temperature increase that occurs over the remaining decades of the 21st century will depend on whether sufficient action is taken to reduce global greenhouse gas emissions (Figure 2).

If international climate targets set at the 2015 United Nations Climate Change Conference are met, the predicted catastrophic impacts associated with global warming above 2˚C might be avoided (Leggett, 2020; Savaresi, 2016). This situation is known as Representative Concentration Pathway 2.6 (RCP2.6) by the Intergovernmental Panel on Climate Change (IPCC) (Figure 2) (Chaturvedi et al., 2012). The most severe pathway is called RCP8.5 (Figure 2), in which global greenhouse gas emissions continue to increase throughout the coming decades, causing global warming of up to 4.8˚C above pre-industrial levels (Chaturvedi et al., 2012). This is believed to be untenable for some aspects of human activity and cause severe damage to ecosystems.

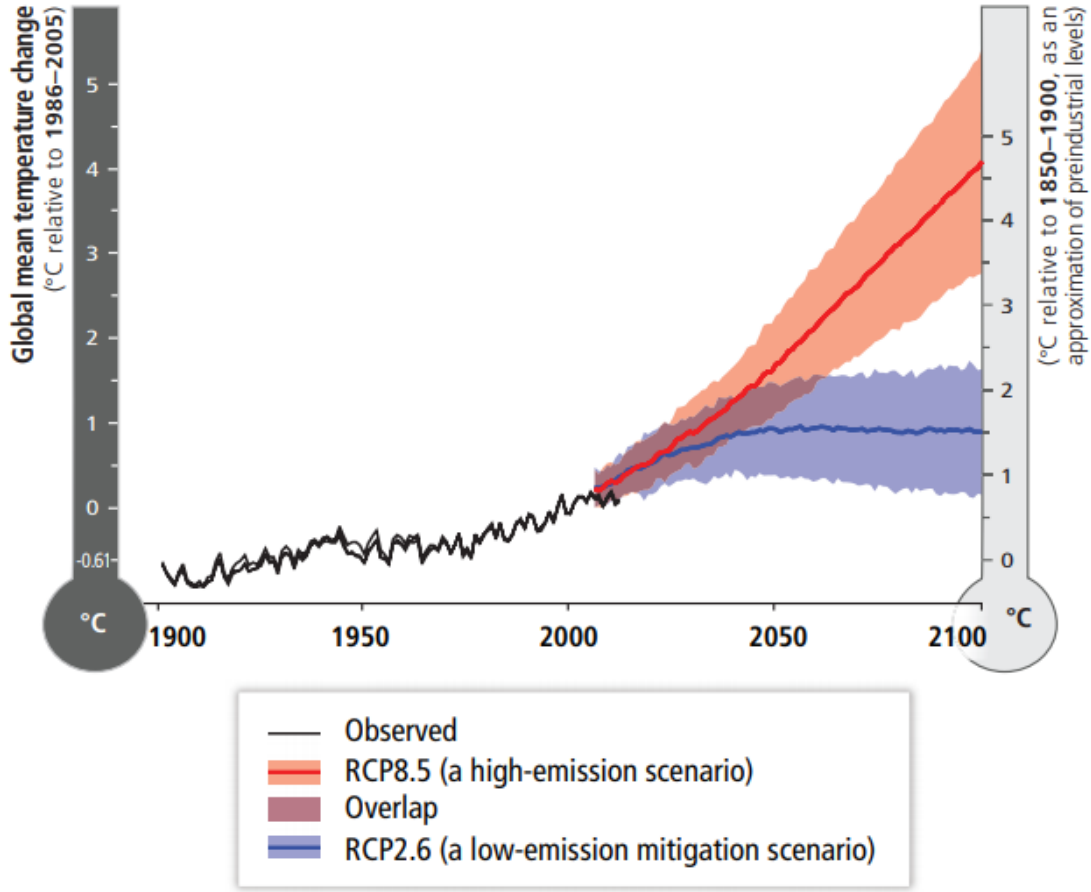

Figure 2: The IPCC's predicted increases in global mean temperature by 2100, relative to pre-industrial levels. The blue and red lines and shading represent different warming scenarios that depend on levels of greenhouse gas emissions over this period. The blue line represents the predicted global mean temperature in an optimistic low-emission situation of a 1.5˚C increase from pre-industrial levels. This is known as Representative Concentration Pathway 2.6 (RCP2.6). The red line represents the predicted increase in the conservative high-emission situation of a 4.8˚C increase from pre-industrial levels (RCP8.5). Some overlap is expected in these situations, represented by the purple shading (Field & Barros, 2014).

The extent of warming that occurs between now and 2100 will depend on the actions taken by national and international governing bodies throughout the world. However, even the low-emission scenario of 1.5-2˚C of warming (RCP2.6) poses significant challenges for animal physiology (Bellard et al., 2012). If no action is taken to reduce emissions, the extinction of thousands of species is likely (Cahill et al., 2013). This thesis is concerned with the impacts of global warming on aquatic ecosystems, specifically increased water temperatures. However, as well as increases in water temperature and an increased frequency of heat waves, aquatic systems face changes in gas compositions, pH, salinity, and UV exposure (IPCC, 2019). The next section considers these impacts of ACC on aquatic ecosystems, including the physiology of aquatic animals and the health of the wider ecosystem.

2.2 Impacts of ACC on aquatic animal physiology

Aquatic ecosystems, including freshwater, marine and estuarine, house a significant percentage of the world's animal biodiversity (Geist & Hawkins, 2016). All are experiencing change to their physical environments due to ACC. This thesis focuses on the increase in water temperature. Unlike terrestrial ecosystems, aquatic systems also face changes in pH, dissolved oxygen, nutrient composition, and salinity (IPCC, 2014). This section focuses on the impacts of increased water temperature on aquatic animal physiology and ecosystem health. However, in a later section on bivalve physiology, the impacts of the other physical changes will be discussed briefly. This is because the combination of these changes can cause compounded stress for aquatic animals. Bivalve molluscs are used as the example because kākahi, the species of interest of this thesis, belongs to this class of aquatic animals. When considering the damage ACC may cause, it is important to understand how higher temperatures impact animal physiology and ecosystems, but to also consider how this may interact with other forms of environmental stress (Huo et al., 2019).

2.2.1 Thermal physiology of aquatic animals under ACC

Water temperatures in ocean and freshwater systems are paralleling increases in air temperature due to their ability to absorb significant amounts of heat (Hoegh-Guldberg et al., 2014). Aquatic systems act as a significant thermal buffer, absorbing the excess heat from the atmosphere that is trapped by the increasing concentrations of greenhouse gases. The ocean alone absorbs approximately 93% of green-house generated heat (Brewer, 2019). As the ocean and other water bodies absorb greater quantities of heat associated with green-house gas emissions, a greater thermal burden is placed on aquatic organisms that must deal with higher water temperatures.

The extent to which aquatic animals are affected by increasing temperatures depends on two major elements of their thermal physiology: the thermal window and the thermoregulatory strategy. The thermal window is the range of temperatures at which an animal can maintain physiological function (Campbell et al., 2010). The thermal window is book-ended by upper and lower critical thermal limits (Häder & Barnes, 2019). Thermoregulatory strategy refers to being endothermic or ectothermic. Endothermic animals can maintain a relatively stable body temperature that is different to the environmental temperature, due to the production of metabolic heat. Conversely, ectothermic animals are unable to produce their own heat, and thermoregulate solely by exchanging heat between their body and the environment (Buckley et al., 2012). It is, therefore, more difficult for ectotherms to maintain an internal temperature different to that of the environment. Thermoregulatory strategy is important when considering the impacts of increasing temperature on aquatic animals because the physiological performance of ectotherms is highly constrained by environmental temperature. The standard method used to conceptualise the relationship between temperature and performance under ACC is a thermal performance curve (Figure 3).

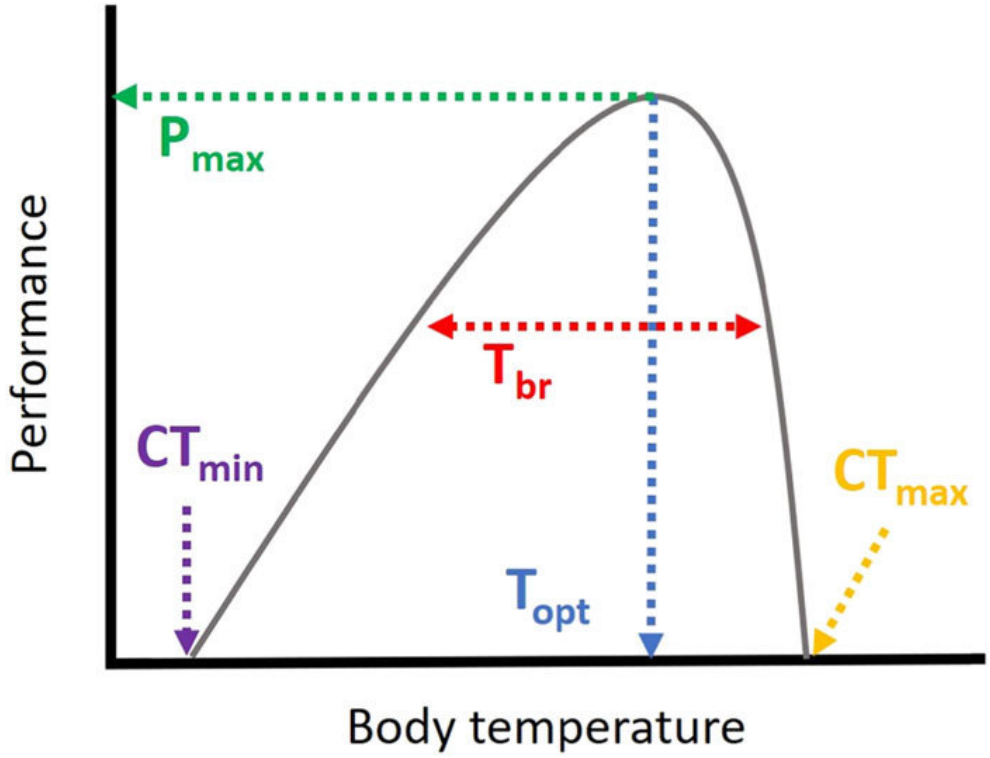

Figure 3: A standard thermal performance curve which shows the relationship between body temperature and physiological performance. The five key thermal values are: Critical thermal minimum (CTmin), maximal performance (Pmax), performance breadth (Tbr), thermal optimum (Topt), and critical thermal maximum (CTmax) (Logan et al., 2020).

There are some key terms in thermal physiology used to explain an animal's thermal window. The critical thermal maximum (CTmax) is the highest body temperature that the animal can experience while maintaining physiological function (Lutterschmidt & Hutchison, 1997). Body temperatures above this value would result in acute thermal death. The critical thermal minimum (CTmin) is the lowest body temperature that the animal can experience and maintain physiological function. In short, thermal limits are the extreme temperatures that if exceeded, almost immediate cessation of enzymatic reactions and ultimately death would result (Bennett et al., 2021). Large scale thermal death events believed to be associated with ACC have been documented in several parts of the world. In June 2021, a marine heat wave in British Columbia, Canada, caused a massive thermal death event where an estimated one billion shellfish died (Williams, 2021). Closer to home, kilometres of dead shellfish have washed up at several Horowhenua beaches in Aotearoa New Zealand almost annually over the last 5 years and are believed to be associated with warmer ocean temperatures (Moore, 2021). While thermal death events are one consequence associated with increasing temperatures, sub-lethal heat stress also has negative effects on the physiological performance and fitness of aquatic animals (Penick et al., 2017).

If increasing temperatures associated with ACC do not cause thermal death, the physiological performance and fitness of an animal can still be reduced. This is due to the

impacts of sub-lethal heat stress on metabolism, which will be discussed in the next section. Physiological performance and fitness are directly linked (Ramajo et al., 2020). Efficient performance of each physiological system is vital for high fitness (Bozinovic et al., 2011). To have high fitness, an animal must not only survive, but must successfully reproduce and, thereby, pass on their genetic material (Grafen, 2015). Thermal breadth (Tbr) (Figure 3) represents the range of body temperatures where varying levels of physiological function can occur. Each animal has an optimal temperature where physiological performance is highest (Pmax), known as thermal optimum (Topt). At the wider points of the thermal breadth, performance and fitness are significantly reduced, which has implications for individual physiology, population conservation, and ecosystem health. The next sections will discuss the direct impacts of increasing temperatures on aquatic animal metabolism and the indirect impacts on population conservation and ecosystem health.

2.2.2 Direct impacts of ACC on aquatic animal metabolism

Metabolism in animals is highly sensitive to temperature, due to the temperature specificity of the enzymes that drive biochemical reactions. The positive relationship, though with limits, between temperature and reaction rates means that increasing environmental temperature causes an increase in the metabolic rate of many aquatic animal species (Riemer et al., 2018). Rapidly occurring biochemical reactions require oxygen to be consumed at a higher rate (Yazhou et al., 2020), hence the increase in metabolic rate.

This can be stressful for aquatic animals because it can disrupt energy balance (Sokolova et al., 2012). A higher metabolic rate requires a higher energy intake. If increasing energy intake to fuel a higher metabolic rate is impaired, e.g. due to limited mobility or food supply, energy will be directed away from non-essential functions such as growth and reproduction to maintain homeostasis (Sokolova et al., 2012). If the metabolic rate remains elevated, as is expected with ACC, there is a risk of prolonged metabolic disturbance. This may impact the long-term survival and fitness of the individual and wider population.

Metabolic stress due to climate change may also present as metabolic depression, which is a plateau and subsequent decrease in metabolic rate (Anestis et al., 2007). Metabolic depression is a concern for physiological function as it reduces an animal's activity levels, restricting their ability to increase food intake or put energy into growth and reproduction. Metabolic depression can be indicated in an animal by the increased concentrations of several metabolic enzymes including lactate dehydrogenase (LDH), pyruvate kinase (PK), and hexokinase (HK). Higher concentrations of these enzymes indicate a shift to anaerobic metabolism and the activation of 'energy-saving mode'. The roles of these enzymes in metabolism and their usefulness as markers of heat stress will be discussed extensively in a later section.

The impacts of prolonged metabolic depression are largely about the allocation of energy within the body. Protective mechanisms that are a necessary response to thermal stress such as heat shock protein upregulation are energy dependent (Sørensen et al., 2003). Therefore, metabolic depression reduces the energy available for protection against cell

and tissue damage (Anestis et al., 2007). This has implications for the individual animal as well as the wider ecosystem, which will be discussed in the next section.

2.2.3 The links between physiological performance, population conservation and ecosystem health

High physiological performance, and therefore fitness, are crucial for sustaining an animal population and for maintaining the health of the ecosystem they live in. Successful propagation of the population requires high physiological performance. As mentioned in the previous section, during heat stress, energy is prioritised to functions that best ensure survival of the individual, whereas some non-critical functions such as reproduction are reduced. If the heat stress event is prolonged, as is expected with ACC, the reproductive capacity of an animal population may remain low for extended periods (Pörtner & Farrell, 2008). Low rates of reproduction reduce the propagation of the population and increases extinction risk. Decreasing population sizes also has flow-on impacts on ecosystem health.

Each population of animals carries out important roles in the aquatic ecosystem they are part of. These roles could include nutrient cycling, water filtration, and food chain dynamics (predators and prey). Due to the relationships between temperature, performance, and fitness, increases in temperature impact not only individual and population physiological performance but their ability to contribute to ecosystem health (Gilman et al., 2010). Considering the bioenergetics of heat stress at the ecosystem level is a method of connecting physiological and ecological health. A framework has been developed called the framework of adverse outcome pathways (Goodchild et al., 2019). Adverse outcome pathways (AOPs) aim to link the impacts of multiple stressors across levels of biological organisation from cellular energetics through to the population and ecosystem levels. When tested using case studies, this generalised bioenergetic AOP showed correlations between how cellular energy is allocated during stress, metabolic rate, and animal growth (Goodchild et al., 2019). These frameworks are a way of explaining the connections between performance and health from the molecular and cellular levels, all the way through to the ecosystem level.

For animals to perform their ecosystem roles optimally, physiological performance must be high. As explained earlier, this relies on energy homeostasis of the body (Sokolova et al., 2012). If energy is being redirected towards heat protection, less energy will be invested into ecosystem roles. Using bivalve molluscs as an example, a key ecosystem role performed by these animals is water filtration. Heat stress can cause filtration rates of the individual animals and population to decrease (May et al., 2021). This reduction in physiological performance (lower filtration rates) may reduce the quality of the water. This may negatively impact the other organisms within the ecosystem and, thus, ecosystem health. Poorer water quality may promote the growth of cyanobacteria, which increases the occurrence of hypoxic zones (Griffith & Gobler, 2020). Hypoxic water reduces the ability of animals such as fish and invertebrates to respire, further compromising physiological health and performance (Altieri & Gedan, 2015). Aquatic ecosystems exist in a balance, and the occurrence of one environmental stressor (like

increased temperature) can tip this balance, creating a cascade of physiological and ecological impacts that ultimately reduce ecosystem health. Therefore, it is crucial to understand the physiological implications of heat stress due to ACC, as well as how these are closely linked to ecological function and ecosystem health.

2.2.4 Heat stress disrupts homeostasis and causes physiological damage in aquatic animal cells

During both acute and chronic heat stress, where an animal experiences temperatures above their thermal optimum, cellular homeostasis is disrupted and damage can occur (Richter et al., 2010). Cellular homeostasis requires the stability of proteins and lipids so that individual reactions can occur, organelles can function and, therefore, overall cellular function can be maintained. One of the key elements of cellular homeostasis that heat stress can disrupt is proteostasis. Proteostasis is achieved by maintaining a balance of protein synthesis, folding, trafficking, and degradation (Dokladny et al., 2015). High temperature can disrupt this balance, changing the rates of protein synthesis and degradation.

Proteins are temperature sensitive molecules, and heat stress can cause misfolding or unfolding of the 3D structure, rendering it non-functional (Richter et al., 2010). Degradation of these proteins is also disrupted, leading to an accumulation of misfolded proteins in the cytoplasm (Park et al., 2018). These can entangle and form aggregations of non-specific proteins that are difficult to degrade (Richter et al., 2010). Protein aggregation can disrupt the structural integrity of the cell via changes to the cytoskeletal network (Ahmed et al., 2015).

Lipids, which are also crucial for cellular homeostasis, can become compromised during heat stress. High temperature disrupts the phospholipid profile of the plasma membrane which can trigger heat injury and cell death (Hochachka & Somero, 2002).

Damage to the plasma and organelle membranes also impacts cellular metabolism as the flux of ions shifts away from homeostatic rates of transport. Increased membrane permeability due to heat stress increases the leakage of ions out of the cell, reducing the transmembrane gradients and disrupting ion homeostasis. This is problematic for nutrient and energy transport as adenosine triphosphate (ATP) generation relies on several ion gradients to transfer ions and nutrients across organelle membranes (Dai et al., 2019).

All of these types of cellular disturbance and damage during heat stress increase the total amount of tissue damage an animal is subjected to. One way that increased cellular and tissue damage can be measured in aquatic animals is through the concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the circulatory fluid. Cellular separation can occur during heat stress, due to the damage that can be inflicted on cellular structures. As a result of this damage and separation, more cells may enter the circulatory fluid (Park et al., 2009). This triggers an increase in the activation of ALT and AST. Elevated concentrations of ALT and AST are thought to be important for the degradation of foreign proteins, lipids, and carbohydrates during stress (Fritts et al., 2015a).

Heat stress can also disrupt homeostasis and cause damage via the increased occurrence of oxidative stress. Oxidative stress is defined as an imbalance between the production of

oxidants and antioxidants, in favour of oxidants, which leads to a disruption in redox signalling and causes molecular damage (Sies et al., 2017). The redox reactions that produce pro-oxidant molecules are upregulated in situations of cellular stress, such as high environmental temperature.

Oxidation reactions are a normal and critical part of cellular metabolism. In non-stressful conditions, the harmful pro-oxidant substances, called reactive oxygen species (ROS), are neutralised by antioxidant enzymes (Verlecar et al., 2007). However, when a stressor such as heat causes an increase in ROS production, the neutralisation capacity of these enzymes can be exceeded. ROS production increases at higher environmental temperatures because metabolic rate increases to supply more energy, maintain homeostasis, and upregulate the stress response. This is driven by an increase in oxidative phosphorylation. Because oxidative phosphorylation is a major source of ROS, as ATP production increases, so does the concentration of ROS (Di Meo et al., 2016).

The resulting pool of un-neutralised pro-oxidant molecules can wreak havoc on the essential structural elements of the cell, resulting in severe damage (Richter et al., 2010). This damage can include protein oxidation, lipid peroxidation, and direct injury to nucleic acids. These impacts can cause dysfunction of several organelles and if severe, degradation or death of the cell by autophagy, apoptosis or necrosis (Sies et al., 2017). High rates of cell damage and death impairs the normal physiological function of animal cells and the tissues and organs they make up.

2.2.5 Physiological responses to heat stress and the ability to adapt to changing environmental temperature

All animals share a highly conserved heat shock response, an innate mechanism that protects cells from heat-induced damage and reduces cell death (Ahmed et al., 2020). This mechanism is driven by the upregulation of heat shock proteins (HSP), which are molecular chaperones. The increased concentration of heat shock proteins in aquatic animal cells is used as a key marker of the presence of heat stress when an organism is exposed to elevated water temperatures. The physiology of the heat shock response is conserved throughout phylogenetic lineages, but there is variation in some of its characteristics, due to the thermal adaptation of different species. This includes the environmental temperature at which HSP are upregulated, the temperature where maximal HSP synthesis occurs, and the upper thermal limit of HSP synthesis (Hochachka & Somero, 2002).

Once heat stress is detected, HSP are recruited to target the misfolded proteins that accumulate in the cytosol. Working as nuclear and cytosolic chaperones, HSP recognise and bind to proteins that are not in their correct formation. When HSP bind to the exposed hydrophobic regions of misfolded or unfolded proteins, they facilitate the folding of the protein into its correct conformation (Matambo et al., 2004). Their capacity to bind to misfolded proteins is regulated through allosteric mechanisms that use ATP binding and hydrolysis (Yebra-Pimentel et al., 2019). The refolding of proteins into their correct conformation by HSP prevents nonspecific proteins interacting with each other and forming aggregations (Feder & Hofmann, 1999). HSP also increase the activity of protein

degradation pathways which helps to maintain proteostasis during heat stress (Somero, 2020).

The other major part of the heat shock response is the upregulation of the antioxidant system. The role of this system during heat stress is to combat heat-induced oxidative damage. It includes non-enzymatic components and a suite of antioxidant enzymes (Lu et al., 2010). The components that have been researched in the greatest depth include glutathione (GSH) which is a non-enzymatic antioxidant. Others are superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GSH-Px), which are antioxidant enzymes (Habashy et al., 2019). The non-enzymatic antioxidants work closely with the enzymes to eliminate ROS inside cells. For example, GSH donates an electron to GPx which facilitates the reduction of hydrogen peroxide to water (Habashy et al., 2019). Antioxidants act as ROS scavengers in a cell experiencing oxidative stress. Their actions make up an extensive network and each antioxidant pathway carries out a different role in minimising ROS-induced damage.

An aquatic animal can be thermally sensitive or resilient, which is partially determined by the ability of their heat shock and antioxidant systems to protect cells from heat-induced damage (Liu et al., 2018). If an animal has a wide thermotolerance range, they can tolerate temperatures above their Topt without needing to significantly upregulate heat shock proteins or antioxidant enzymes. Thermally resilient species tend to have higher basal levels of heat shock protein transcription and less marked upregulation as temperature increases (Gleason & Burton, 2015). Mild to moderate heat stress can be tolerated by the basal pool of HSP and, therefore, these animals use less ATP for upregulation. In contrast, thermally sensitive species may have low basal levels of heat shock proteins and antioxidant enzymes. A smaller increase in temperature may elicit a more marked heat shock response, which in turn requires more ATP. Therefore, thermally sensitive aquatic animals are at risk of metabolic disruption as energy may need to be directed away from the housekeeping functions of the cell, towards heat stress responses (Payton et al., 2016).

2.3 Bivalve mollusc biology and physiology

The focus of this chapter will be on a specific group of aquatic animals, the bivalve molluscs (Phylum: Mollusca, Class: Bivalvia). Kākahi, the species of interest for this thesis, belongs to this group. An overview of the biology of this group will be discussed, including a description of each major physiological system. This will be followed by the impacts of climate change on bivalve-specific physiology.

Bivalves are a species-rich group that live in a wide variety of aquatic environments, both marine and freshwater. Within these habitats, bivalves are often considered keystone species due to their key roles in the functioning of their aquatic ecosystems (Gallardi, 2014). Bivalves are often referred to as eco-engineers because of their important role in the modification of their physical environment (Donadi et al., 2014). They modify the surrounding substrate and increase the nutrient quality of the water by cycling nutrients through their water filtration system. Their biological activities directly influence the composition of organisms in their habitat. This has flow on effects for several important

ecosystem characteristics such as the oxygen content of the water and the transfer of nutrients through the trophic levels (Karlson et al., 2020).

2.3.1 The bivalve body plan and defining characteristics

The bivalve body plan is unique among the molluscs. The distinguishing feature of bivalves is the laterally compressed body that is housed within two shell valves (Sharma et al., 2012). Through the evolution of two shell valves, bivalves became the only group of molluscs that have been successful in covering their entire soft body with a hard shell (Wada et al., 2020).

The paired shell valves have several important functional and structural roles. They give the animal protection from predators and assist burrowing bivalves by keeping substrate out of the body (Gosling, 2003). The major structural role of the valves is to provide a skeleton-like surface for muscle attachment (Nasr, 2019). Because bivalves require water to flow in and out of their shell for many physiological processes, they have a ligament and either one or two adductor muscles that attach to the valves. These structures work together to control the open-close system of the shell valves. Unlike other molluscs, bivalves lack a distinct head. The internal anatomy of a bivalve is specialised to enable them to adopt a range of lifestyles and thrive in many aquatic habitats (Figure 4).

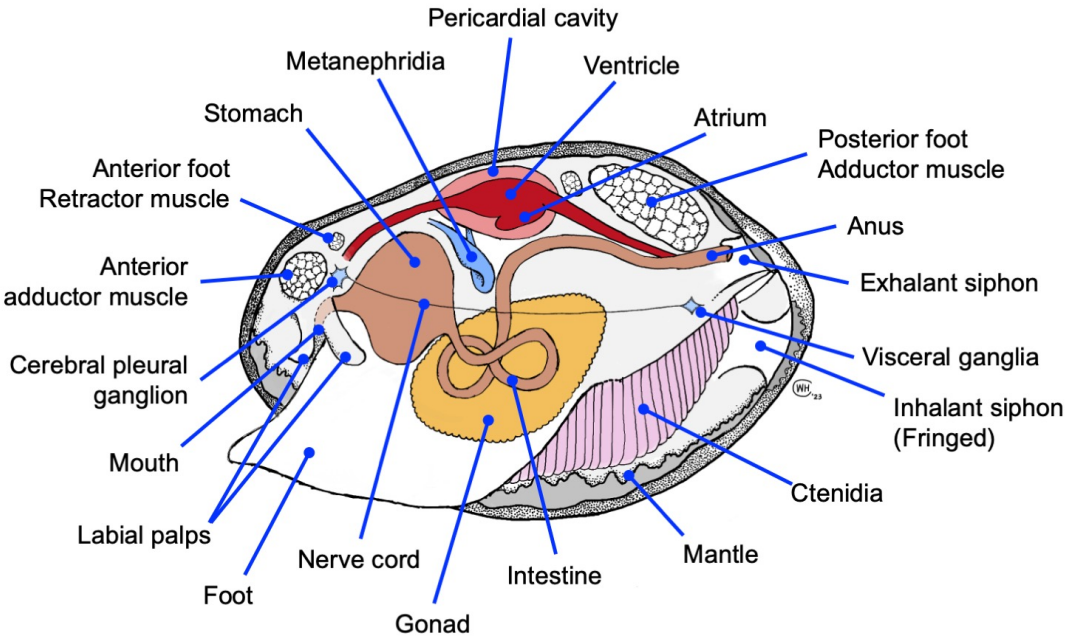

Figure 4: Internal anatomy of a bivalve, showing the major internal organs. There are differences in the internal anatomy of different bivalves, such as byssus threads in some species, the variable size of the foot, and the enlargement of siphons in burrowing bivalves. However, the major organs and structures such as gills (ctenidia), mantle, heart within the pericardial cavity, gonad, nerve cords, digestive and excretory organs, and adductor muscles are similar throughout the group (Illustration by W-H. Chua based on UCMP, 2001 and original photos of kākahi).

The internal organs of a bivalve are enclosed within the mantle that is made up of two lobes of thin, membranous tissue (Dessai, 2012). The mantle has a wide range of functions which differ slightly between bivalve species. However, the three-fold structure of the mantle is conserved throughout the group. The outer mantle fold is responsible for secretion of the hard-shell valves. The middle fold is involved in sensing the external environment. This is often achieved through sensory projections such as tentacles with embedded chemoreceptors (Gosling, 2003). The inner fold of the mantle is more muscular and is crucial for the control of water in and out of the body cavity (Gosling, 2003).

2.3.2 Respiration and feeding

The gills are a critical structure that have been modified for the dual role of feeding and respiration during the evolution of Bivalvia. The enlargement of the gills has achieved a vast surface area through the lengthening and folding of individual gill filaments and the presence of cilia (Gosling, 2003). Many cilia are attached to a single cirrus and these structures are located on the lateral and frontal surfaces of the gill filaments (Galbraith et al., 2009). A unique feature of bivalve gills is the feather-like latero-frontal cilia that are situated at the tops of the blood sinuses of the lamellae, which are complexes of individual gill filaments (Figure 5).

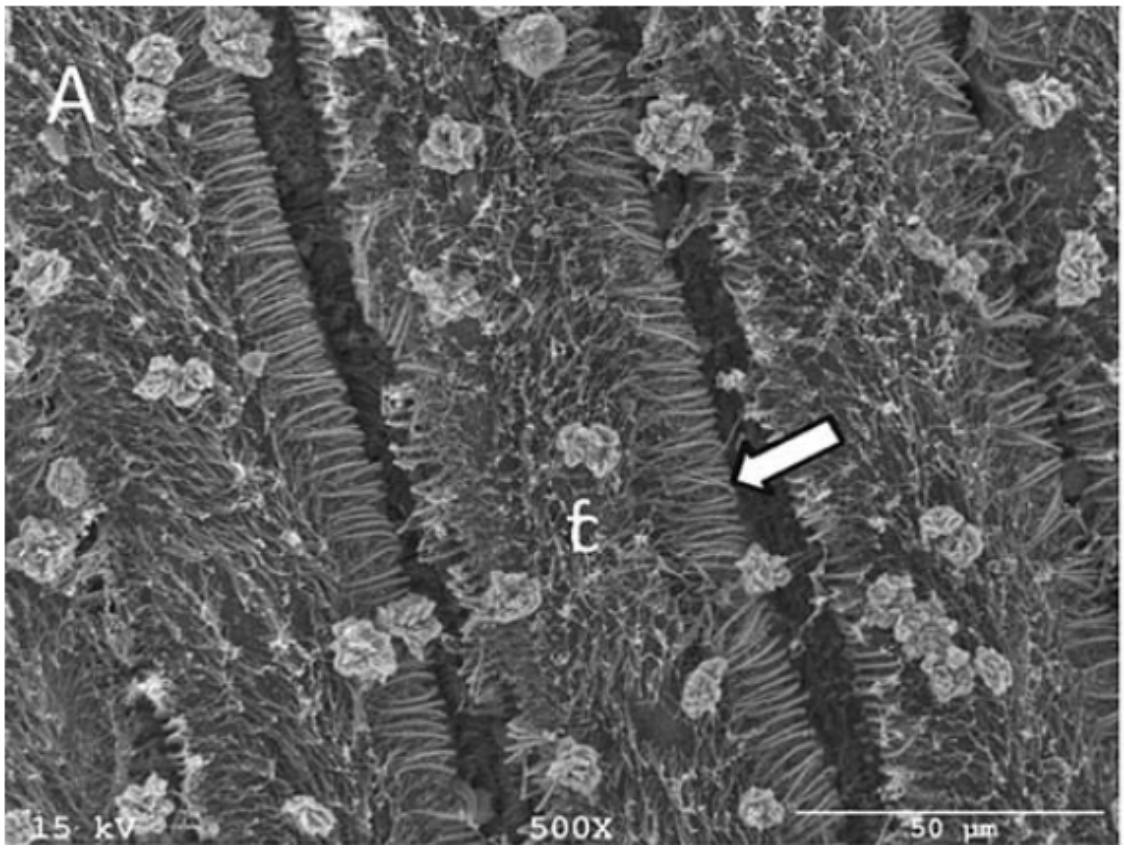

Figure 5: Scanning electron micrograph of the gill of *Actinonaias ligamentina*, a species of freshwater mussel. The white arrow shows the location of the latero-frontal cilia on a lateral cirrus (cirral plate). The fc symbol shows the location of the frontal cilia on the frontal cirrus (Galbraith et al., 2009).

Bivalves that are suspension feeders have slightly different gill morphology to those that are deposit feeders (Jasna et al., 2010). Suspension feeding involves consuming food particles that are suspended in water. In contrast, deposit feeding involves consuming food particles associated with sedimentary deposits (Ward & Shumway, 2004). In bivalves with extendable siphons (including kākahi), muscular pumping and cilial beating of the gill sucks water into the mollusc through the inhalant siphon, which protrudes out of the shell. Bivalves without siphons, such as oysters, allow water to flow in through the inhalant chamber of the mantle, which is lined with small blood vessels, and over the gills. In most bivalves, oxygen is taken up as well as food particles as water passes through the gills (Järnegren & Altin, 2006). The rate of inhalant water flow is controlled by contraction of the longitudinal and water tube muscles of the gill and the beat of the lateral cilia (Gainey & Greenberg, 2003).

The transfer of respiratory gases across the gill surface is optimised due to the large surface area of the gill epithelia and diffuse supply of haemolymph via the afferent gill vein (Gosling, 2003). Transfer of oxygen and other gases from extracellular water to the haemolymph occurs across the gill epithelium by passive diffusion down a partial pressure gradient. Likewise, carbon dioxide from metabolic activities accumulate in the haemolymph and diffuses from the haemolymph into the external water via the protruding exhalant siphon or exhalant chamber of the mantle (Gosling, 2003).

2.3.3 Digestive and excretory systems

The digestive system of bivalves moves food particles from the gills in a thin layer of mucus to the labial palps, which are responsible for discriminating between particles to be ingested and those to be rejected. Rejected particles, including sediment, end up as pseudofaeces and are expelled via the exhalant siphon (Jasna et al., 2010). Particles selected for digestion then begin their journey through the gut attached to a mucus string. The gut of a bivalve has four key components; the oesophagus, stomach, digestive gland, and intestine (Penry, 2000). The mucus string containing the particles for digestion travels down the oesophagus into the stomach. Mechanical and chemical digestion begins in the stomach, aided by the secretion of enzymes from the crystalline style, a solid gel-like structure. The stomach contents travel through either the digestive gland, which is a slower digestive process, or through the intestine for rapid digestion (Penry, 2000). Nutrients absorbed in the digestive gland pass directly into the haemolymph, while there is further absorption in the intestine. Waste products from both the glandular and intestinal pathways pass through the intestine to the anus, and faeces are expelled through the exhalant siphon or chamber (Gosling, 2003).

Ammonia is the primary nitrogenous waste product of bivalve metabolism (Lauritsen & Mozley, 1989). Excretion is mediated by two main organs, the paired kidneys (nephridia) and pericardial glands (Gosling, 2003). Waste products travel in the haemolymph back to the heart, and into the pericardium which is the fluid filled sac that surrounds the heart. Pericardial glands line the auricles of the heart or the pericardium depending on the species. These glands act as an additional location of ultrafiltration and is where urine formation begins. The pericardial fluid then moves out of the pericardial glands into the kidney lumen via the renopericardial duct (Morse, 1987). In the kidneys, reabsorption and

secretion of some solutes occurs to modify the urine which then enters the mantle cavity and is lost to the external environment by passive diffusion across the gill epithelium (Thomsen et al., 2016).

2.3.4 Circulatory system

Like most other molluscs, bivalves have an open circulatory system that is driven by hydrostatic pressure (Eriko & Yoshiteru, 2019). It is referred to as open because the haemolymph, which is the circulatory fluid, bathes the tissues directly. The circulatory system includes the heart, which is enclosed in the pericardium, and arteries which branch into smaller vessels and eventually empty in spaces called sinuses to bathe the tissues (Gosling, 2003). The venous circulation removes haemolymph from these sinuses and carries it back to the heart.

2.3.5 Reproductive biology

The rudimentary reproductive system of bivalves is relatively simple, but the diversity of sexual strategies is vast throughout the class. Being dioecious (having separate males and females) is likely the ancestral state of the phylum Mollusca and this has been conserved in most bivalve groups including freshwater mussels, though some species of scallops, oysters, and clams are exceptions (Collin, 2013). The bivalve reproductive system consists of paired gonads composed of branching tubules. Gametes are released from the epithelial cells of these tubules (Gosling, 2003). Most bivalve species release their gametes into the surrounding environment with fertilisation occurring externally. Reproductive biology will be discussed in more depth in a later section, in the context of freshwater mussels, which have a unique method of reproduction.

2.3.6 Locomotion

Most bivalves, including kākahi, use burrowing as a form of locomotion. Many species burrow either in the sand of the ocean floor or the silt of river and stream beds. Within this lifestyle, there is great variation in the depths that bivalves can burrow to. Burrowing is possible due to bivalves possessing a laterally compressed shell and large muscular foot. Using these two key structures, bivalves burrow using a two-stage anchoring system (Figure 6)

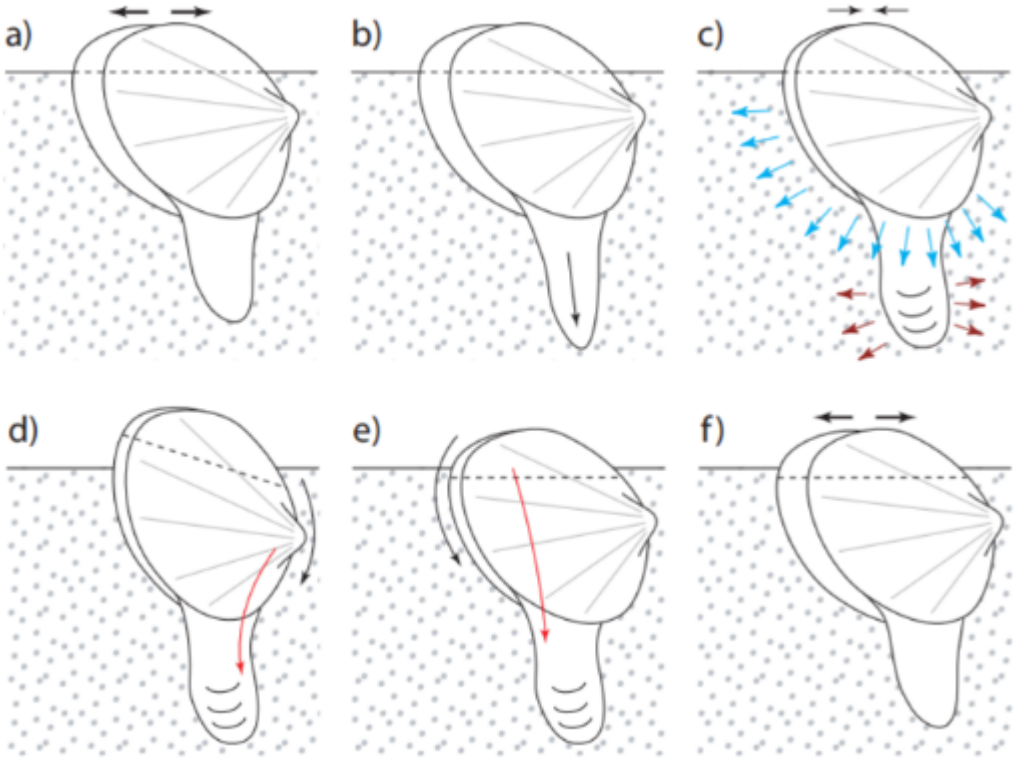

Figure 6: The burrowing process of a clam starting with (a) and finishing at (f). In (a), the shell valves are open to anchor the clam in the sediment, allowing the muscular foot to extend further down (b). In (c) the shell closes slightly, expelling water and reducing resistance. Then the foot extends further creating a new anchor (d) and muscular effort pulls the shell down further (e). In (f) the clam has moved down and opens its shell further to start another cycle (Koller-Hodac et al., 2010).

Several major muscles are involved in the burrowing process. The adductor muscles relax to open the shell which anchors the bivalve in the sediment. The muscular foot then stretches down further into the sediment, providing the next anchoring point. The adductor muscles then contract, partly closing the shell which results in water expulsion from the body cavity. The water being expelled makes the surrounding sediment more liquified, reducing the resistance for the burrowing bivalve (Peck et al., 2004).

Blood from the body cavity moves downwards into the foot, allowing it to successfully anchor the animal (Figure 6). The anterior retractor muscle rotates the shell by pulling the front side of the animal down towards the foot (Figure 6). Simultaneously, the posterior retractor muscle pulls the shell back to the original straight position (Figure 4). This dual rotational action results in net downward movement of the bivalve into the sediment. The adductor muscles then relax, opening the shell so the cycle can begin again (Koller-Hodac et al., 2010).

In a small number of bivalve species, free-swimming has developed as a locomotive strategy. However, this is a very energy expensive strategy, and burrowing has remained the primary locomotive strategy of the bivalves (Cheng & DeMont, 1996). On the other end of the spectrum, some bivalves are sedentary, spending their adult life attached to a

hard substrate. Marine mussels (Family: Mytilidae) and freshwater zebra mussels (*Dreissena polymorpha*) attach via threads called byssus, which are protein fibres secreted by the foot (Carrington, 2002). Byssus threads are also secreted by pen shells such as the noble pen shell (*Pinna nobilis*) which attach to substrate in seagrass meadows or even bare sand (Haberle et al., 2020).

2.3.7 The nervous system

The nervous system of bivalves is relatively simple. It is bilaterally symmetrical, consisting of three sets of paired ganglia; the cerebropleural, visceral, and pedal ganglia (Carroll & Catapane, 2007). These paired ganglia are connected to each other and to other ganglia by commissures, which are locations of tissue connection (Figure 7). This is considered a tetraneural nervous system, because there are four (two pairs) main longitudinal neurite cords (Yurchenko et al., 2019). Several pairs of nerves branch off these ganglia to innervate the tissues.

The three major pairs of ganglia are linked together in a network via connectives, which are nerve cords (Figure 7). From each set of ganglia, nerves branch off to innervate the tissues. The cerebropleural ganglia innervate the labial palps, anterior adductor muscle, anterior region of the mantle, and the sensory organs. The visceral ganglia innervate the gills, posterior adductor muscle, siphons, heart, and the posterior region of the mantle. The pedal ganglia innervates the foot (Kotsyuba et al., 2020).

A variety of signalling molecules are involved in the nervous system of bivalves. There is variation in which neurotransmitters and neuropeptides are present in each set of ganglia. For example, H-Phe-Met-Arg-Phe-NH2 (FMRFamide), choline acetyltransferase (ChAT), γ-aminobutyric acid (GABA) and tyrosine hydroxylase (TH) are found in neurons in all three ganglia (Kotsyuba et al., 2020). In contrast, serotonin producing neurons are only found in the cerebropleural and pedal ganglia. There is much more to be understood about the signalling pathways involved in the bivalve nervous system. However, the general structure and function of the nervous system is well understood, as well as the range of neurotransmitters involved in neurophysiological functions.

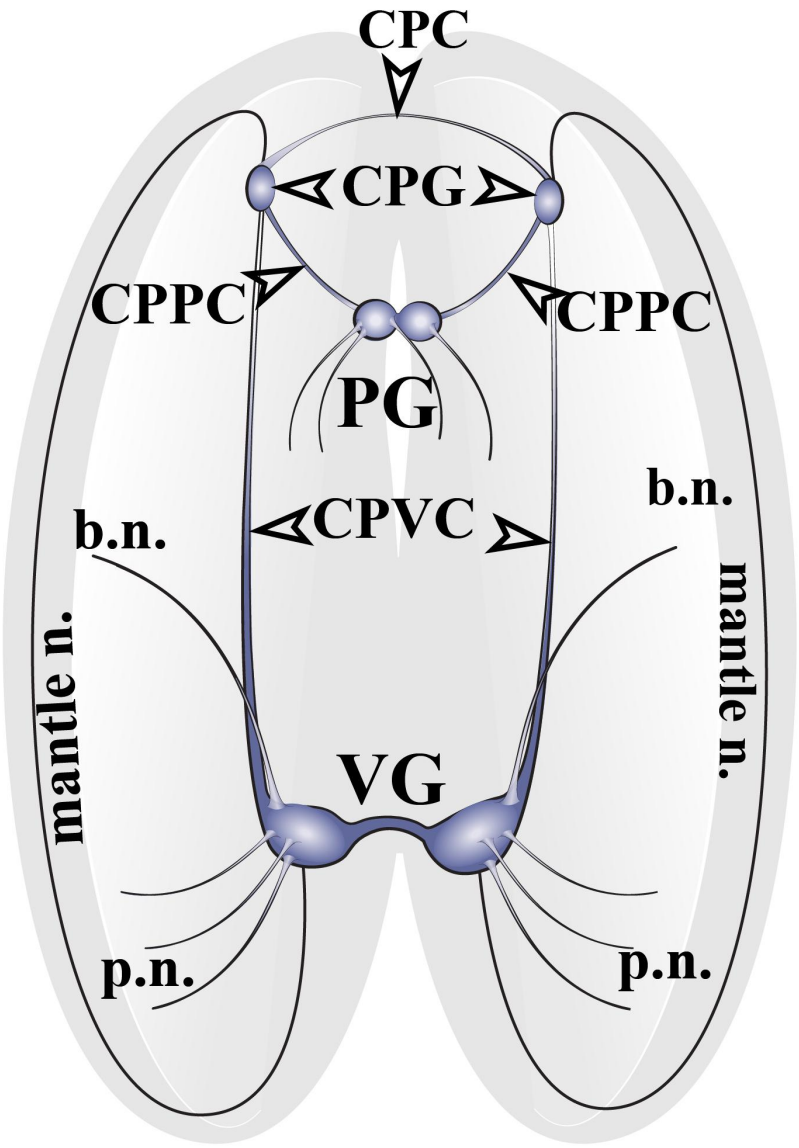

Figure 7: The structural layout of the nervous system of a marine mussel species (*Crenomytilus grayanus*). Paired cerebropleural ganglia (CPG) are located at the anterior end of the mussel and are connected to each other by cerebropleural commissures (CPC). These are also connected to the pedal ganglia (PG) via cerebral-pleural-pedal connectives (CPPC). The paired visceral ganglia (VG) are located towards the posterior end of the mussel and are connected to each other by a visceral commissure. The visceral, cerebral, and pedal ganglia are connected via the cerebral-pleural visceral connectives (CPVC). The pallial nerves (p.n.) branch from the visceral ganglia and innervate the posterior end of the mantle. The mantle nerves branch off both the CPG and VG, to innervate the anterior and posterior areas of the mantle. Branchial nerves (b.n.) branch from the VG and innervate the gills (Kotsyuba et al., 2020).

2.4 Bivalve thermal physiology and the impacts of increased temperatures due to ACC

As discussed earlier, higher water temperatures can cause an increase in metabolic rate. Due to the limited mobility of many bivalve species and their mode of feeding (filter and

suspension feeding), increasing energy intake to support a higher metabolic rate presents greater energetic and metabolic challenges to bivalves. Once a threshold temperature is reached, or if energy intake cannot support a higher metabolic rate, this rate plateaus, then decreases via metabolic depression. This threshold temperature differs between bivalve species due to several factors, such as how much temperature fluctuation their habitat experiences, thermal limit differences, and species differences in thermal plasticity and acclimatisation potential.

In bivalves, metabolic depression is usually associated with shell valve closure, therefore limiting respiration, locomotion, and feeding (Anestis et al., 2007). This process is closely linked to hypoxia which is another key environmental stressor that will be discussed in a later section. Furthermore, a major physiological consequence of metabolic depression in bivalves is oxidative stress.

Oxidative stress may be a very common consequence of many types of environmental stress in bivalves. When the bivalve is stressed by high temperatures and other stressors, metabolism is disrupted and mitochondrial oxidative capacity reduces, increasing the production of reactive oxygen species (ROS) (Dimitriadis et al., 2012). If ROS are being produced at a higher rate than the antioxidant enzymes that detoxify them, a redox imbalance occurs, causing oxidative stress. This has severe implications for physiological function (Figure 8).

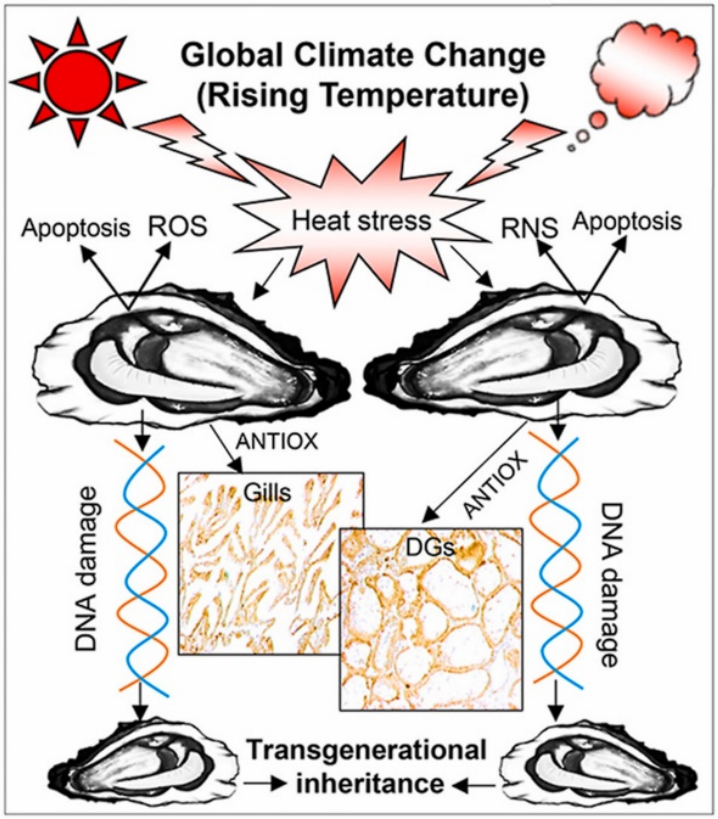

Figure 8: Effects of heat stress from rising water temperatures on redox status in the American oyster (*Crassostrea virginica*). Heat stress causes an increase in ROS production which activates apoptotic pathways, leading to cell death. ROS also causes damage to DNA. If the antioxidant enzymes cannot balance the redox status, DNA will be damaged which can affect subsequent generations (transgenerational inheritance) (Rahman & Rahman, 2020).

A major physiological consequence of oxidative stress is an increase in apoptosis (Figure 8). Increased levels of apoptotic cell death disrupt tissue function, affecting normal physiological processes. Oxidative stress does not always result in cell death, but sub-lethal effects such as cell membrane damage, protein oxidation, lipid peroxidation, and DNA damage also restrict normal function at the cellular, tissue, organ and whole body level (Rahman & Rahman, 2020). For example, cellular damage in the bivalve digestive gland can cause hypertrophy and disorganisation of the epithelial lining. This damage disrupts the normal function of the digestive gland, which is an important metabolic organ, responsible for detoxification and digestion (Kumar et al., 2011).

Upregulating a suite of heat shock proteins is an important process that bivalves use to deal with thermal stress. But it comes with its own set of costs to the animal. It is a very energy expensive process because the synthesis of new protein and their chaperoning activities uses large amounts of ATP (Anestis et al., 2007). If metabolic depression occurs, ATP production is compromised and the continued synthesis and activity of HSP will be unsustainable. The success of the heat shock response is also species dependent. Bivalves that are more thermally tolerant can mount a stronger heat shock response that does not compromise other physiological processes. In contrast, thermally sensitive species mount a weaker response and may be unable to maintain physiological performance (Payton et al., 2016).

2.5 Other impacts of ACC on bivalve physiology

While heat stress due to increasing water temperatures is the focus of this literature review, it is important to acknowledge the other effects of ACC on bivalve physiology and ecosystem health. As well as heat stress, ACC puts bivalves at risk of acidosis due to acidification of the water, salinity changes and osmotic stress, hypoxia due to reduced oxygen concentrations, and stress from increased UV radiation. Importantly, these other changes are likely to interact with higher temperatures, compounding the impacts of heat stress on bivalve physiology and aquatic ecosystem health.

2.5.1 Hypoxia and temperature-dependent oxygen limitation

A major impact of ACC is a decrease in the average dissolved oxygen concentration. Over the last fifty years open ocean oxygen concentrations have decreased on average by 0.1 to >0.3 μmol kg^{-1} yr^{-1} (Pörtner et al., 2014). This has created zones in ocean and freshwater systems that have become hypoxic. Both marine and freshwater systems are at risk of expanding hypoxic zones, both from increasing global temperatures and nutrient contamination from human activity (Breitburg et al., 2018). Changes in the level of oxygen at different water depths (stratification) are being influenced by warming temperatures. Stratification restricts the exchange of gas between different water layers, resulting in hypoxia in different layers or zones of the water column (Pörtner et al., 2014).

Hypoxia has severe consequences for bivalve physiology. Bivalves respond to low oxygen availability by decreasing metabolic rate, which reduces oxygen consumption in biochemical pathways. This is partly achieved by down-regulating the transcription and

translation of key metabolic enzymes (Hermes-Lima et al., 2015). In particular, enzymes involved in the Kreb's cycle are downregulated and production of ATP is reduced (Nogueira et al., 2017). Hypoxia can also induce oxidative stress, which can compromise immunity by reducing haemocyte number and viability (Nogueira et al., 2017). This leaves bivalves susceptible to pathogen infection, and lowers survival rates (Nam et al., 2020).

The combination of heat stress and hypoxia can cause a phenomenon called temperature-dependent oxygen limitation. The thermal window of a bivalve mirrors the range of oxygen consumption where they can maintain aerobic metabolism. Therefore, temperature and oxygen levels determine their aerobic scope (Pörtner, 2010). As previously discussed, as water temperatures increase, so do the metabolic rates of bivalves. A higher metabolic rate requires a greater rate of oxygen consumption to match oxygen demand and delivery to cells throughout the body (Pörtner, 2012). However, if the bivalve is also experiencing hypoxia, their ability to increase oxygen supply to fuel aerobic metabolism is diminished. Heat stress alone can cause systemic hypoxia in mussels, due to the imbalance between oxygen supply and demand above a threshold temperature (Boutet et al., 2022). However, the presence of hypoxic water can exacerbate this process, causing further disruption to metabolism and normal physiological functioning.

The ventilatory and circulatory capacity of bivalves is not very efficient due to their open haemolymph system (Eriko & Yoshiteru, 2019). Therefore, they are susceptible to a loss of aerobic scope during hypoxic and thermal stress. Aerobic scope is the ability to supply enough oxygen to the tissues to maintain aerobic metabolism (Farrell, 2016). If the stressors remain, there may be a switch to anaerobic metabolism as metabolic depression occurs to conserve oxygen and energy (Pörtner et al., 2006). If this condition becomes chronic, as is expected with ACC, it may prevent growth, reproduction and survival due to loss of metabolic function (Pörtner, 2010).

2.5.2 Decreasing pH and risk of hypercapnia and acidosis

As carbon dioxide emissions have increased over recent decades, approximately one third of the CO_2 has been absorbed by the ocean (Matear & Lenton, 2014). As CO_2 dissolves in the seawater, it undergoes a chemical reaction that reduces the concentration of carbonate ions and increases the concentration of hydrogen ions. This shift in composition towards having more acidic ions decreases the pH of the sea water, termed ocean acidification (Matear & Lenton, 2014). Freshwater systems such as rivers, streams, and lakes also absorb carbon dioxide from the atmosphere. Due to increasing carbon dioxide emissions, this is causing an increase in carbonic acid production, the release of hydrogen ions and weak acidification of freshwater systems (Jeffrey et al., 2017).

The two stressors that bivalves can experience as water pH decreases are hypercapnia and acidosis (Michaelidis et al., 2005). Accumulation of carbon dioxide can occur in the haemolymph via diffusion across the gills. Decreased haemolymph pH is called extracellular acidosis (Michaelidis et al., 2005). Intracellular acidosis also occurs, but bivalves seem to be better at regulating intracellular pH without negatively affecting physiological function, though this is species specific (Haider et al., 2016). As

hypercapnia and acidosis occurs, a common response among bivalves is the lowering of respiration rate and onset of metabolic depression (Michaelidis et al., 2005). In addition to a decrease in haemolymph pH, levels of calcium ions (Ca^{2+}) also decrease.

Bivalves are particularly vulnerable to pH stress due to their method of responding to extracellular acidosis. This response involves partially breaking down the shell which is made of calcium carbonate. Breaking down the inner shell surface releases bicarbonate ions into the haemolymph, buffering the pH (Zhao et al., 2017). This process of using bicarbonate ions from the shell to buffer haemolymph pH has also been observed in freshwater bivalves (Pynnonen, 1990). Over time, the shells may become brittle and less protective, leaving the animal at risk of shell and tissue damage, parasite invasion, and predation.

2.5.3 Changes in salinity and osmotic stress

Effective osmoregulation is essential for bivalves to live in water with varying salinities. Bivalves have adapted to survive in a variety of aquatic environments, each with very different water salinities. However, climate change is altering the salinity of these environments, particularly marine and estuarine habitats. This puts the bivalves that live in them at risk of osmotic stress, which inhibits normal physiological function.

For marine bivalves, decreased water salinity is likely to be a consequence of climate change. Warmer temperatures are causing sea ice and glaciers to melt at a faster than normal rate. Precipitation is also expected to increase in some areas such as the tropics. These phenomena are decreasing the salinity of the ocean (IPCC, 2019). A decrease in water salinity puts bivalves at risk of hypoosmotic stress, where internal osmolality drops below normal levels. Increasing salinity is also a possibility in some aquatic systems such as estuaries that experience lower rainfall, or in freshwater systems where evaporation exceeds precipitation. Increased salinity can also cause osmotic stress for bivalves, known as hyperosmotic stress, because internal osmolality is higher than normal.

All bivalves are osmoconformers so are unable to regulate their extracellular fluid at a different osmolality to that of the surrounding water in their environment (Wang et al., 2011). This makes them vulnerable to salinity changes, as their main response is to shut their shell valves to reduce the amount of hyperosmotic or hypoosmotic water that comes into contact with their haemolymph (Pourmozaffar et al., 2020).

During the period of osmotic stress, bivalves can regulate their intracellular osmolality by transporting potassium, chloride, and sodium ions and by increasing or decreasing the free amino acid pool within cells (Sokolov & Sokolova, 2019). This allows them to prevent cell shrinkage or swelling and ensure survival of the acute stress. However, osmotic stress has many implications for metabolic function, due to the importance of closing the shell valves. It also compromises bivalve immunity and results in oxidative stress.

When bivalves are exposed to water salinities outside their tolerable range, they respond by immediately shutting their shell valves. They open them periodically throughout the stress event to respire and prevent mortality. This response results in metabolic depression, with filtration rate, respiration rate and energy intake all decreasing

(Pourmozaffar et al., 2020). The small amount of energy they ingest during this period is not very useful as the efficiency of absorption also decreases during metabolic depression (Wang et al., 2011).

This creates problems for intracellular osmoregulation as the control of cell volume via ion and amino acid transport requires ATP (Pourmozaffar et al., 2020). As a result, osmotic stress puts a heavy strain on bivalve energy balance which has implications for tissue and shell growth, immunity, and long-term survival.

The entire immune response of bivalves is mediated by haemocytes and the effectiveness of their function is impaired by osmotic stress. Total haemocyte count in the haemolymph has been shown to increase in bivalves during hypoosmotic stress (Matozzo et al., 2007). This is likely due to haemocytes being mobilised from the tissues into the haemolymph to assist in the stress response. However, both hyperosmotic and hypoosmotic stress lowers the phagocytosis capacity of bivalve haemocytes, leaving them vulnerable to pathogen infection and disease related mortality (Matozzo et al., 2007).

2.5.4 Exposure to UV radiation

UV radiation can negatively affect animal physiology when exposure rates are too high. The natural systems of Earth have developed in a way that protects animals from deadly levels of UV radiation. However, climate change is shifting this balance, putting animals, including bivalves, at risk of increased UV exposure. Specifically, climate change is putting bivalves at risk of increased exposure to UV-B radiation (Erickson et al., 2015), which is the type of UV that can cause severe damage to animal cells (Yin et al., 2019). The processes controlling UV-B exposure are complex and not limited to ACC related factors. However, increasing levels of greenhouse gas emissions are restricting the recovery of the ozone layer. Ozone depletion due to ACC is one factor contributing to the increasing intensity of UV-B exposure (Erickson et al., 2015). ACC also affects the cloud cover and surface reflectivity around the Earth. In mid-latitude areas, cloud cover is decreasing, promoting an increase in surface UV radiation (Bais et al., 2019).

Increased exposure to UV radiation can initiate toxicity in bivalves. This is because UV radiation can activate photo-active toxins that can accumulate in bivalve tissues during water filtration (Ahrens et al., 2002). Increasing UV radiation is also a concern because it can activate the apoptosis of bivalve haemocytes. UV exposure causes stress to several hemocyte organelles, including mitochondria, lysosomes, and endoplasmic reticulum (Gervais et al., 2015). This organelle stress leads to the release of calcium into the cytoplasm, which can cause dysregulation and activate apoptosis of the haemocyte. UV exposure also causes disruption to the cytoplasmic membrane which can lead to cell damage and apoptosis activation (Romero et al., 2011).

This section of the literature review aims to illustrate that ACC has many impacts on bivalve physiology, rather than heat stress alone. It is a multifactorial and complex process, and therefore, the impacts on physiology are dynamic and interlinked. However, the impacts of global warming as an individual stressor on bivalve physiology are important to understand. It is one piece of the bigger puzzle of how ACC may impact the physiology of a given bivalve species. Due to the scope and focus of this thesis, from this

point onwards, I will focus on the impacts of heat stress on freshwater mussels (order Unionida), which is the group that the kākahi belong to.

2.6 The freshwater mussels

Kākahi, the New Zealand freshwater mussel, belongs to the family Unionidae which is the largest of six freshwater mussel families. There are approximately 958 known species of freshwater mussels worldwide. These species are distributed throughout North, Central and South America, the Afrotropics, North Eurasia, East Asia and Australasia (Graf & Cummings, 2021). Throughout these regions, different species have found niches within the sediment and water of rivers, streams, and lakes. In all of these aquatic ecosystems, freshwater mussels are considered keystone species due to their vital ecological roles. Freshwater mussels are eco-engineers, as they modify the environment through burrowing in the sediment and filtering the water (Cyr et al., 2017). These ecological roles can directly influence the nutrient and organism compositions of their habitats, which are critical components of ecosystem health.

Unfortunately, freshwater mussel species around the world are highly susceptible to anthropogenic activities. As mentioned in Chapter 1, freshwater mussels are a group with one of the highest rates of extinction and imperilment on Earth (Haag & Williams, 2014). This could be attributed to many anthropogenic activities including habitat modification, pollution, and climate change (Bolotov et al., 2020).

2.6.1 ACC and freshwater mussels: thermal resilience and thermal sensitivity

ACC is a major threat to freshwater mussel survival, as many studies have found projected temperatures to cause heat stress in a wide range of species (Falfushynska et al., 2014; Ferreira-Rodríguez et al., 2018; Payton et al., 2016). What is clear from previous studies is that some freshwater mussel species are more vulnerable to climate change than others. Vulnerability to climate change is determined by the extent of change occurring in the external environment (external factors) and the physiology of the animal (internal factors) (Williams et al., 2016). When considering physiological sensitivity to climate change, some species are considered to be ‘thermally resilient’, while others are ‘thermally sensitive’ (Payton et al., 2016). It is the latter group that are most vulnerable to increasing temperatures a result of ACC. These terms do not have a universal definition, but one method of determining whether a species is thermally resilient or sensitive is by measuring the concentrations of heat stress biomarkers at different water temperatures.

Due to evolutionary and life histories, habitat variations, and species-dependent thermal physiology, different freshwater mussel species may have very different physiological responses to the same water temperature. An extensive number of biomarkers have been used in previous studies, to provide insights into the physiological response of a species to a particular thermal regime, and therefore, their potential resilience or vulnerability to ACC (Falfushynska et al., 2014; Fritts et al., 2015; Payton et al., 2016). The concentrations of these biomarkers when mussels are exposed to predicted future water

temperatures provide valuable knowledge about the specific vulnerability of a freshwater mussel species to ACC. Higher concentrations of certain biomarkers in a species at a particular water temperature indicate that the physiological systems are under more strain compared to another species with lower concentrations at that same temperature. The next section will describe the biomarkers that were measured during this research, as well as some additional markers measured in previous studies. Their roles during heat stress will be discussed, including an explanation of what a higher or lower concentration may indicate in terms of vulnerability to ACC.

2.6.2 Biomarkers used to indicate thermal resilience or sensitivity under ACC

A variety of biomarkers have been used widely to measure physiological stress in various animals in response to environmental change. The markers described in this section have been measured in aquatic invertebrates after exposure to increased water temperatures. Each marker has a specific link to thermal metabolism and provides information about how a particular water temperature affects the physiological function of the animal. Measuring a series of different markers in tissue or haemolymph can provide important information about the thermal resilience or sensitivity of a species to different water temperatures. The markers discussed here includes lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, hexokinase, pyruvate kinase, catalase, superoxide dismutase, and caspase-3.

Lactate dehydrogenase (LDH) is a highly conserved tetrameric enzyme that is found in almost all living cells (Kumar et al., 2018). It is an essential enzyme for cellular metabolism, particularly the production of ATP during anaerobic metabolism. In non-stressful conditions, animal cells primarily produce ATP through oxidative phosphorylation (Piccoli et al., 2006). However, when heat stress occurs, metabolic rate increases, and the aerobic energy system is put under pressure. ATP is required at a faster rate, to initiate and maintain stress responses such as heat shock protein and antioxidant upregulation. If a threshold is reached where oxygen intake is not sufficient to produce enough ATP to fuel these processes while maintaining homeostasis, anaerobic production of ATP is required (Valvona et al., 2016). This is achieved primarily through anaerobic glycolysis, which does not require oxygen. During anaerobic glycolysis NADH is oxidised to NAD+ during the conversion of pyruvate to lactate. This reaction is catalysed by LDH. Anaerobic glycolysis produces 2ATP molecules from one glucose molecule, with lactate as the main by-product (Melkonian & Schury, 2021).

Chronic heat stress disrupts the normal cellular metabolism of freshwater mussel cells. If this is severe or prolonged, the energy requirements of the mussel may be unable to be met solely by aerobic metabolism. LDH is a useful biomarker of metabolic disturbance during heat stress because concentrations increase in the haemolymph and tissues when the mussel increases the amount of energy produced via anaerobic glycolysis. An increase of LDH concentration in the haemolymph of freshwater mussels when exposed to a particular water temperature indicates that the temperature challenges the cellular metabolism of that species (Zhang et al., 2022). Prolonged metabolic disturbance can cause a suite of issues for freshwater mussels, including immune system dysfunction, limited ability to filter feed, and reduced energy availability for reproduction. Therefore,

a sharp elevation in LDH may indicate vulnerability to ACC, given the links between metabolic disturbance and long term physiological and ecological function.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are transaminase enzymes involved in carbohydrate and protein metabolism. ALT catalyses the reversible transamination from pyruvate and glutamate to alanine and α-ketoglutarate (Xu et al., 2017). AST catalyses the conversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate (Washington & Van Hoosier, 2012). Both ALT and AST are commonly measured markers of stress in aquatic invertebrates (Fritts et al., 2015). Elevated concentrations of both of these enzymes have been found in response to several types of environmental stress, including increased water temperature and ocean acidification (Park et al., 2009; Liao et al., 2019). Specifically, elevated concentrations of ALT and AST can indicate an increase in cell and tissue damage. As described in section 2.4, oxidative injury is common during heat stress. This damage can cause an increased inflow of cells into the haemolymph due to the cellular separation that occurs during tissue damage. This inflow of cells can raise the concentrations of ALT and AST in the haemolymph (Park et al., 2009). Therefore, measuring concentrations of ALT and AST in freshwater mussel haemolymph at different temperatures may be useful in determining if tissue damage is occurring.

Hexokinase (HK) and pyruvate kinase (PK) are both important enzymes in the glycolytic pathway. HK initiates the first step of glycolysis by catalysing the glucose to glucose-6-phosphate reaction. At the end of the glycolytic pathway, PK converts phosphoenolpyruvate to pyruvate, which then enters the tricarboxylic acid (TCA) cycle (Xu et al., 2017). Unlike the other biomarkers discussed, concentrations of both HK and PK are likely to decrease during moderate and severe heat stress. During metabolic depression, which can occur during exposure to elevated water temperatures, rates of glycolysis decrease (Payton et al., 2016). The genes that code for these key glycolytic enzymes are regulated at the transcriptional level during environmental stress (Le Moullac et al., 2007). Transcription of HK, PK, and other glycolytic enzymes can be reduced when a reduction in metabolic rate is required. Therefore, measuring the concentrations of HK and PK in mussel tissue or haemolymph may be useful to identify metabolic depression at elevated water temperatures.

The concentrations of antioxidant enzymes such as CAT and SOD can be used as biomarkers of oxidative stress, which is common during heat stress (see 2.4). The roles of these enzymes during heat stress have already been discussed (see 2.2.5). However, it is important to mention them here as specific biomarkers of oxidative stress at elevated water temperatures. In all animals, including mussels, elevated concentrations of CAT and SOD during heat stress are indicative of an increase in ROS inside cells. Upregulation of these enzymes aims to protect the cells from the damage that ROS can cause (Regoli & Giuliani, 2014). The positive relationship between increased levels of oxidative stress and increased concentrations of SOD and CAT makes these enzymes useful markers of oxidative stress at elevated water temperatures.

The final biomarker of heat stress discussed here is caspase-3. Caspase-3 is a protein that is known as the executioner caspase in the apoptosis (programmed cell death) pathway. Measuring the activity of caspase-3 is therefore a useful tool for monitoring the amount of apoptosis occurring. Increased caspase-3 activity has been observed in several bivalve

species in response to heat stress (Nash & Rahman, 2019; Zhou et al., 2019). An increase in apoptosis is common during heat stress when heat-induced oxidative stress exceeds the neutralisation capacity of the antioxidant system. When this occurs, several pro-apoptotic signalling pathways are activated, which converge on the pro-oxidant p53 pathway (Cheng et al., 2015). The activation of this pathway initiates a cascade of caspase activation, including activation of caspase-3, which leads to apoptosis of the cell. High rates of apoptosis are problematic for physiological function, due to the high energy demands of this process and the significant loss of cells (Troyano et al., 2003). Therefore, caspase-3 may be a key marker to indicate the vulnerability of a freshwater species to increased water temperatures.

Due to time and budget constraints, three of the eight described markers were selected to measure in kākahi haemolymph. In both the field study (Chapter 3) and laboratory study (Chapter 4) concentrations of LDH, ALT, and AST were measured in non-lethal samples of kākahi haemolymph.

2.7 Kākahi: the study species

This thesis focuses on a species of freshwater bivalve called kākahi (*Echyridella menziesii*), which is one of three freshwater mussel species endemic to Aotearoa New Zealand. The other two species are *Echyridella onekaka*, which has a very limited distribution and *Echyridella aucklandica.* The three species are genetically distinct, but morphologically similar (Figure 9) (Hu, 2017). *E. menziesii* is the most common and widely distributed of the three kākahi species. This species is found throughout Aotearoa New Zealand's rivers, streams, and lakes in both the North and South Island (Clearwater et al., 2014).

Figure 9: The three identified species of kākahi. All three species share a similar morphology, with minor differences. *E. menziesii* (left) and *E. onekaka* (middle) are particularly similar in size and shape. *E. aucklandica* (right) has a more elongated shell shape (NIWA, 2020b) and often has obvious nodules visible on the shell (Catlin et al., 2017).

2.7.1 Ecological and cultural importance of kākahi

Kākahi can live in a variety of freshwater habitats but there are several characteristics that support a high population density. They prefer areas with stable sediment, but are found in both lotic (rapid water flow) and lentic (still) areas (Clearwater et al., 2014). Kākahi are most common at water depths of 3-15 m and in areas with low slope (Roper & Hickey,

1994). Population density appears to be highest in lentic lakes, with densities of several hundred kākahi per metre squared (m^2) observed in Lake Taupo and other lakes throughout Aotearoa, New Zealand (Cyr et al., 2017). However, densities of kākahi can also be high in lotic environments, particularly in small patches and pools within streams. Population density can be lower in fast flowing rivers and streams due to the risk of shear stress. In these areas the density may be less than one mussel per m^2 (Ferris, 2020).

Kākahi are a long-lived species with some individuals reaching 55 years old and potentially older. However, the average age that *E. menziesii* has been found to live is 12 years (Roper & Hickey, 1994). The age structure of a kākahi population can be estimated by counting the growth rings on the shells of each individual (Roper & Hickey, 1994). A more accurate estimation of age and growth can be attained from counting growth rings on the cut surface of the bivalve shell (Herath, 2018). Body condition can also be measured in individual kākahi using a lethal method, where whole dry tissue and shell weights are calculated (Hickey et al., 1997). A non-lethal method of scoring the condition of kākahi is to rate the extent of shell erosion. A categorical scale of 1 (no erosion) to 5 (severe erosion) is usually used (Roper & Hickey, 1994).

Kākahi perform crucial roles within Aotearoa New Zealand's freshwater ecosystems. Their main activity is to filter the surrounding water. A single adult kākahi can filter one litre of water every hour (Clearwater et al., 2014). Like other freshwater mussels, kākahi excrete waste from their feeding filtration system as faeces and pseudofaeces into the surrounding water (Cyr et al., 2017). Therefore, their activities as primary consumers can influence the nutrient dynamics of their habitats. Furthermore, kākahi are often called eco-engineers, as their burrowing and filtering activities change the sediment composition via bioturbation (Spooner et al., 2012). Bioturbation also increases the sediment oxygen content and releases nutrients from the sediment into the surrounding water (Hu, 2017).

Given the high filtration rates of kākahi in freshwater ecosystems, their nutrient cycling abilities impact phytoplankton densities, algal bloom growth, and invertebrate abundance (number of individuals of a species). The rates of nutrient cycling in kākahi are similar to freshwater mussels in Europe, Asia, and North America (Cyr et al., 2017). The impact of mussel nutrient cycling on the abundance of other organisms depends on mussel density in the stream, river, or lake. At high densities, freshwater mussel species have been found to reduce the amount of phytoplankton and bacteria in small eutrophic systems (Kim et al., 2011).

In lakes where kākahi reach densities of >100 individuals per m^2, the kākahi populations have been found to filter and completely clear the phytoplankton from five to seven centimetres above the substrate every hour (Cyr et al., 2017). However, this only occurs in sheltered lakes, as areas that experience a lot of wind replenish phytoplankton levels rapidly. A case study of kākahi filtration in Lake Tuakitoto in Otago is an example of the incredible filtration capacity of a dense kākahi population, where the population was able to filter a volume of water equivalent to the lake volume every 32 hours, provided the water temperature remained between 19 and 20˚C (Ogilvie, 1994). Lake Tuakitito is shallow (0.7 metres) and thus has a relatively low volume, which was likely a key factor in the notable filtration capacity of this kākahi population. Further, phytoplankton growth was suppressed by kākahi filtration in this lake, demonstrating the relationship between kākahi activity and organism abundance.

Kākahi activity also impacts the growth of algal blooms in freshwater ecosystems. This is because they can store nutrients for a prolonged amount of time, after removing them from the sediment during filtration. The removal and storage limits the nutrients that are bioavailable for phytoplankton to use during algal bloom growth (Moore & Clearwater, 2019). Therefore, high densities of kākahi may be helpful in preventing eutrophication of freshwater systems. However, high levels of cyanobacteria (algal blooms) have been detected in many of the lakes where kākahi are found (Butterworth, 2008). Cyanobacterial growth depends on oxygen availability, sunlight exposure, nutrient balance, and several other factors, in addition to mussel density.

Kākahi are also an important wildlife species in Aotearoa New Zealand due to their cultural significance. They are a traditional kai (food) source for Māori iwi and the shells were once used for cutting hair and the umbilical cords of new-born babies (Rainforth, 2008). Kākahi are considered a taonga (treasured) species and they often feature in both Māori place names and whakataukī (proverbs), further indicating their cultural importance (McEwan et al., 2020). Māori oral records mention kākahi in several contexts including as a kai source, particularly for young children, and as symbols of chief leadership (Whaanga et al., 2018). The links between kākahi and freshwater ecosystem health also has a cultural element. Freshwater itself is taonga for Māori, and the maintenance of each ecosystem's mauri (life force) and mana (prestige) is of vital importance (MfE, 2020). Therefore, conserving kākahi and ecosystem health is not only important from an ecological perspective, but also from a cultural one.

Understanding the importance of kākahi from a mātauranga perspective enables the integration of Western science and indigenous knowledge. Te Ao Māori (Māori worldview) recognises that all living things are dependent on each other and a change in one aspect of the taiao (environment) disrupts the balance (Michel et al., 2019). Combining mātauranga and Western science has resulted in successful kākahi projects, such as the translocation of kākahi from Lake Wairarapa into an artificial lake in Zealandia (Michel et al., 2019).

2.7.2 The kākahi lifecycle

The unioid group that kākahi are part of (Family: Unionidae) are characterised by a unique life cycle, which involves an obligate parasitic larval stage (Figure 10). It is thought that freshwater mussels, including kākahi, co-evolved with host fish, allowing this fascinating process to develop throughout the group (Moore & Clearwater, 2019).

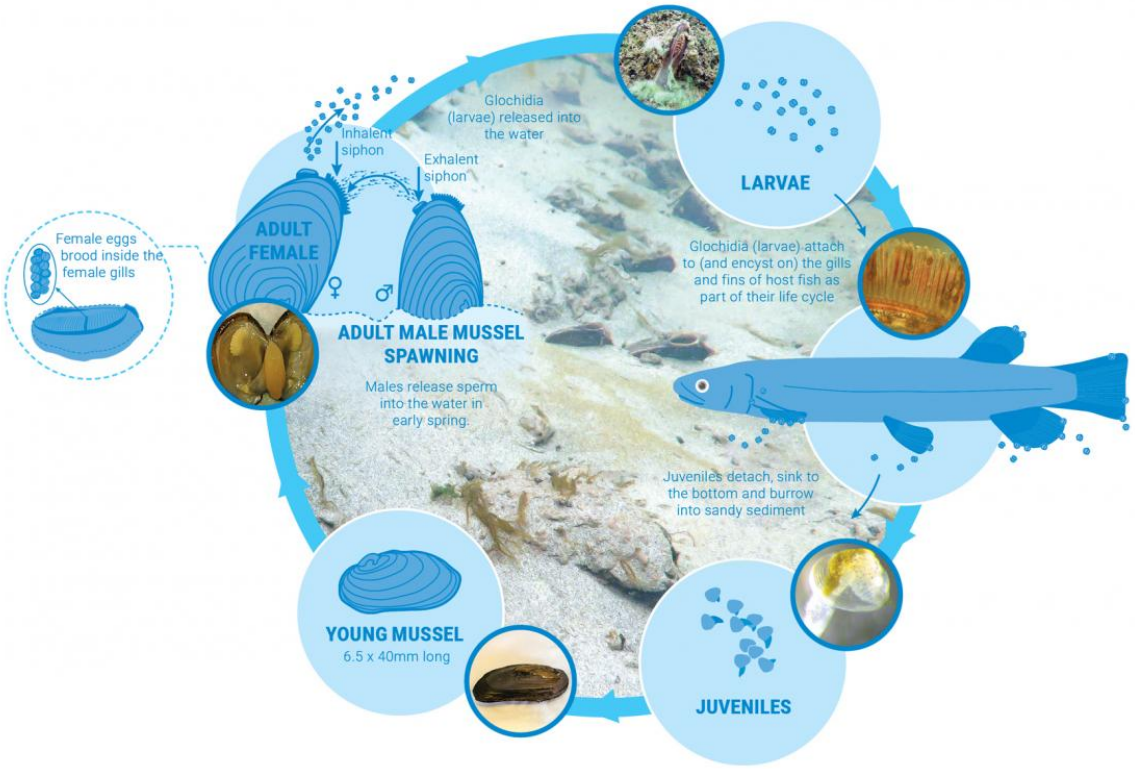

Figure 10: Representation of the kākahi lifecycle. Starting at the bottom of the figure, adult male mussels spawn and release sperm into the water to be taken in by the female through the inhalant siphon. Fertilisation occurs in the females' gills and the fertilised eggs develop into glochidia (larvae). The females release the glochidia into the water where they attach to the gills or fins of a host fish to develop into juveniles. Once juveniles are developed, they detach from the fish, and burrow into the substrate developing into young adult mussels (NIWA, 2020b).

During spawning, male adult kākahi release sperm into the water which is taken in by the female through the inhalant siphon (Figure 10). Within the gills of the female kākahi, fertilisation occurs and the fertilised eggs brood and develop into larvae called glochidia. Once the glochidia are sufficiently developed (to approximately 300 µm long), the female releases them into the water via the exhalent siphon (Figure 10). The glochidia then attach themselves to a fish host and encyst themselves in the epithelial cells of the fish gills, fins, or elsewhere on the body. The length of metamorphosis from glochidia to juvenile varies greatly between freshwater mussel species. In kākahi, this process takes between 12 and 22 days (Clearwater et al., 2014). Once metamorphosis has been completed, the juvenile kākahi detach themselves from the host fish, and are thought to burrow into the sediment, where over the next several years they will grow into adult kākahi.

Success of the encystment phase is dependent on several factors. The host fish must have suitable chemical and nutrient characteristics for the glochidia to develop. Additionally, the glochidia must be resistant to the immune defences of the host fish (Moore & Clearwater, 2019). Different freshwater mussel species have varying levels of host specificity, including between kākahi species (Hanrahan, 2019; Melchior, 2021). *E.*

menziesii are considered a host generalist, as glochidial attachment has been observed in several native fish species and non-native rainbow trout (Moore & Clearwater, 2019). In contrast, *E. aucklandica* appears to be a host specialist, requiring the presence of New Zealand smelt (*Retropinna retropinna*) (Melchior, 2021). Levels of successful recruitment in kākahi are very low when non-native fish such as the brown bullhead (*Ameiurus nebulosus*) are the only available hosts (Moore & Clearwater, 2019).

2.7.3 Decline of the kākahi

Unfortunately, kākahi (*E. menziesii*) are in decline, shown by an observed decrease in population numbers at known kākahi sites in recent years. Evidence for this decline comes from both Western scientific surveys and mātauranga records from iwi. For example, at sites along the Whanganui River, decline in kākahi populations have been observed at 16 out of 22 surveyed sites (Rainforth, 2008). Mātauranga accounts reveal that these sites once had significantly higher numbers of kākahi than are now found.

Decline is also indicated in the lack of juveniles and small adults at several locations around Aotearoa New Zealand. In the Whanganui River, recruitment (indicated by the presence of juveniles) was only observed at 18% of the surveyed sites (Rainforth, 2008). This pattern is observed throughout the country. In several streams in northern Waikato, populations of kākahi are always skewed towards older adults with very few juveniles found (Hare et al., 2019). Recently, in Lake Horowhenua, 900 kākahi were found in a reasonably small area of excavated lakebed. However, the size indicated that they were large adults sized specimens with no juveniles present, also of note is not all specimens were inspected to ascertain if they were in fact live (*pers. comm.* Logan Brown, 2021).

Over the last decade, research has focused on understanding why kākahi populations are declining. This research is urgently needed considering that *E. menziesii* is predicted to decline by a further 50-70% over their next three generations (Grainger et al., 2018). Barriers to native fish hosts are thought to be the main factor causing kākahi decline. Kōaro (*Galaxias brevipinnis*) are a viable host for kākahi glochidia, and their numbers have been decreasing since the arrival of Europeans (Hu, 2017). Other native fish species that could serve as viable hosts are also declining, while species of introduced, exotic fish increase in number (Rowe, 2012). This human-mediated process is called biotic homogenisation and can restrict the recruitment of kākahi juveniles as glochidia metamorphosis is often unsuccessful in exotic fish hosts (Moore & Clearwater, 2019).

Another known threat causing kākahi decline is the modification of river, stream, and lake systems by anthropogenic activities. This can include pollution from agricultural and urban run-off, channel modification, sedimentation, and gravel extraction (Rainforth, 2008).

2.7.4 Are kākahi vulnerable to anthropogenic climate change?

Research into the impacts of ACC on Aotearoa New Zealand's freshwater species has focused largely on fish species and distributions (Robertson, 2016). In contrast, the impacts on freshwater invertebrates are largely unknown (Robertson, 2016). Specifically, little is known about how climate change may contribute to the decline of kākahi presently and in the future. In 2020, The National Institute of Water and Atmospheric Research (NIWA) released a document that assessed the vulnerability of Aotearoa New Zealand's native freshwater fauna to climate change, based on a variety of factors. This document has identified kākahi as a species that may be vulnerable to climate change (Figure 11).

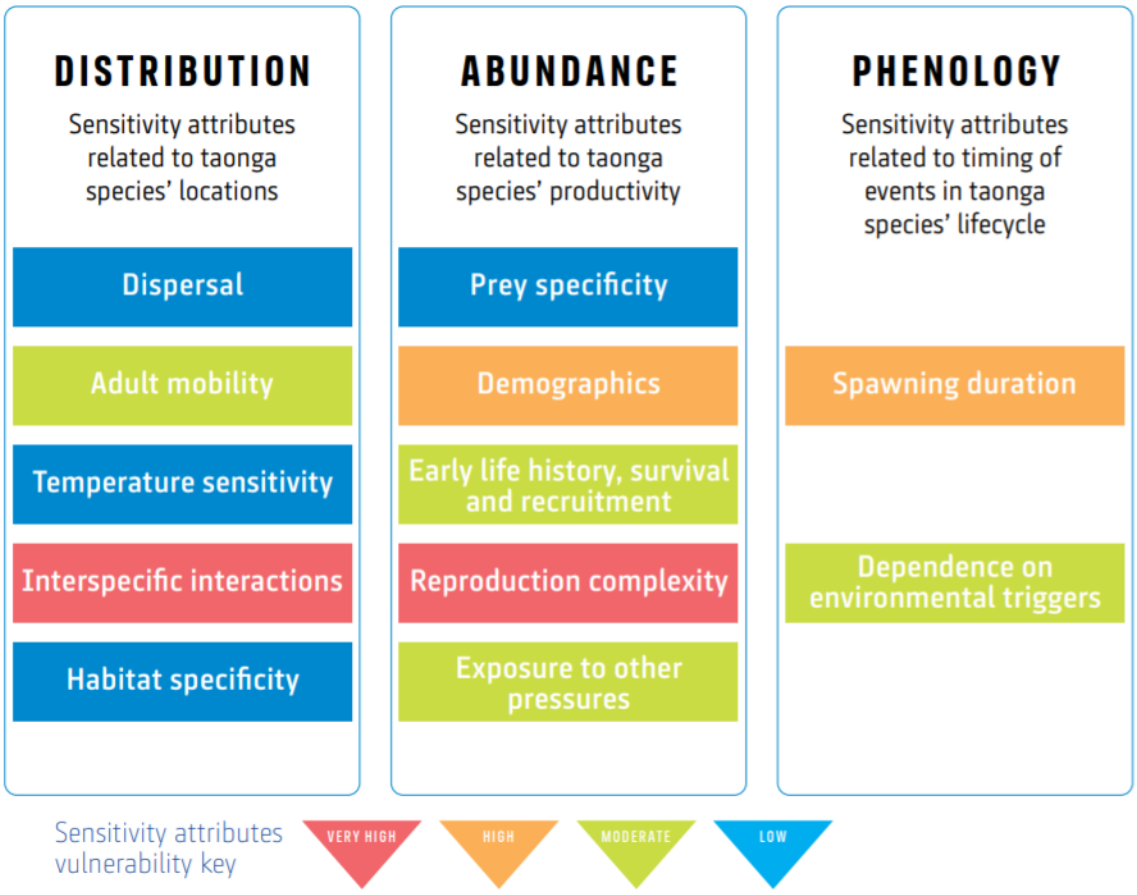

Figure 11: Assessment of kākahi's vulnerability to climate change based on distribution, abundance and phenology. The attributes are ranked for sensitivity from very high (red), high (orange), moderate (green) and low (blue). When all the attributes are considered, kākahi received an overall high sensitivity (orange) rating and were considered vulnerable to climate change (NIWA, 2020a)

The factors that were used to assess kākahi vulnerability to climate change were grouped based on distribution, abundance and phenology (Figure 11). Kākahi were given a low temperature sensitivity score (Figure 11). This was based on the observation that they are found in areas that experience a wide range of water temperatures. Physiological evidence for this score has not been available to date. However, kākahi have been observed to

maintain their filtration ability at 26˚C (*Pers. comm.* Sue Clearwater, 2021) which supports the low temperature sensitivity score in this document. Even so, the thermal tolerance ranges of kākahi are not well-known. Furthermore, it is expected that kākahi in highly degraded habitats will have a narrower temperature tolerance range and higher temperature sensitivity (NIWA, 2020). This vague description of kākahi vulnerability to heat stress highlighted the need for more research into this aspect of their physiology. As far as we know, the research described in this thesis is the first to measure physiological biomarkers of heat stress in kākahi exposed to different water temperatures. The motivation behind the study design was to help fill the knowledge gap of how vulnerable kākahi are to increasing water temperatures. This information will be critical for the ongoing conservation of the species, given that many areas where kākahi reside, are expected to be subject to temperature increases (Figure 12).

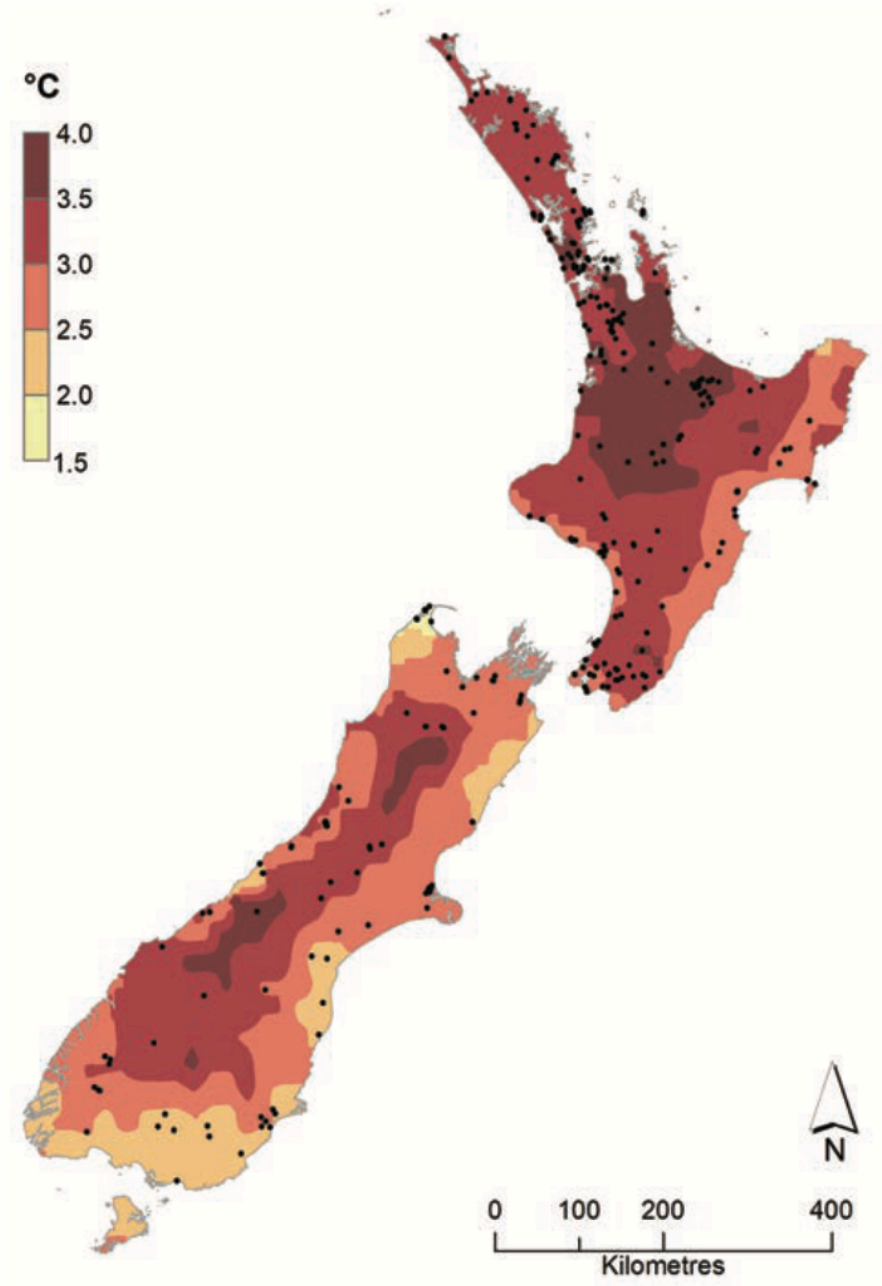

Figure 12: Distribution of kākahi around Aotearoa New Zealand, as of 2020. The black dots represent the presence of kākahi populations. The colour scale represents predicted temperature increase for 2080-2100 based on conservative climate change predictions (RCP 8.5). This shows that in a high emission scenario, many kākahi populations may face temperatures up to 4˚C higher than current temperatures (NIWA, 2020a).

It is likely that kākahi across Aotearoa New Zealand will experience elevated water temperatures over the next few decades (Figure 12). Aotearoa New Zealand's average temperature has increased at a similar rate to the global average increase (Robertson, 2016). Modelling predicts that in a moderate emission scenario, Aotearoa New Zealand will experience approximately 0.9˚C of warming between 1990 and 2040, and a further 1.2˚C warming between 2040 and 2090 (Robertson, 2016). The specific temperatures that

cause heat stress in kākahi and their physiological responses to this has not been researched before. Understanding which temperatures cause heat stress in kākahi and the impacts that this stress has on their physiology was the main aim of my research. This information will encourage water temperature to be considered during kākahi conservation policy discourse. If we know what temperatures cause severe physiological stress, at-risk populations can be easily identified. Appropriate actions can then be taken to conserve these populations, such as translocation into a cooler area.

The conservation of kākahi is crucial for the health of Aotearoa New Zealand's rivers, streams, and lakes. If kākahi are found to be vulnerable to predicted water temperatures this will likely have negative impacts not only for individual physiology, but also for ecosystem health. If a population of mussels are experiencing metabolic disruption and reduced physiological performance due to higher temperatures, they will be redirecting energy towards dealing with the heat stress. Therefore, there will be less energy available for them to continue performing their crucial roles in the ecosystem such as filtration and bioturbation. Water quality may degrade if performance remains low, and other organisms that rely on mussel activity will also be negatively affected.

This research focused solely on temperature as a stressor to consider the vulnerability of kākahi to ACC. The rationale for this decision was partially due to the time frame available to complete the research. In future research, it would be useful to conduct a study that considered the other main environmental changes expected due to ACC including dissolved oxygen content, pH, and nutrient levels. However, the focus on temperature alone is still useful. By keeping all other environmental variables the same in the lab environment, and changing only temperature, we could isolate the stress this one variable caused. This limited the potential ambiguity of the results, as it would be difficult to determine which variable caused the stress, if multiple environmental factors were altered at once. Given that global warming is one of the major effects and concerns associated with ACC, understanding the impacts of this on kākahi physiology is an important first step in understanding their broader vulnerability to ACC.

2.8 Aims and hypotheses

Aims

The first aim of this thesis was to increase the knowledge of kākahi thermal physiology, as this is a poorly understood aspect of the biology of an ecological and cultural keystone species.

The second aim was to provide insight into the vulnerability of kākahi to possible future water temperatures and the implications this may have for persistence of the species, and the health of freshwater ecosystems in Aotearoa New Zealand.

Hypothesis

Kākahi that have been exposed to increased water temperatures will display changes in haemolymph concentrations of biomarkers that are indicative of the metabolic disturbances that occur during heat stress.

Chapter 3: Field sampling kākahi for potential markers of heat stress

3.1 Abstract

Measuring the concentrations of heat stress biomarkers in haemolymph samples taken during different seasons of the year is a method used to investigate the heat stress that wild freshwater mussels may experience at current water temperatures. In the field study presented in this chapter, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) concentrations were measured in haemolymph sampled from kākahi from five field sites during the summer and winter of 2022. Water temperature was measured at each site, and the average temperature across the sites was 20.1˚C (n=5) in summer and 12.7˚C (n=2) in winter. The aim of this study was to determine if summer water temperatures in 2022 caused measurable changes in LDH, ALT, and AST and therefore, potentially indicate heat stress in a selected sample of the local kākahi population.

There were no significant differences in average haemolymph concentrations of LDH, ALT, or AST between summer and winter sampled kākahi, at the two sites able to be sampled in both seasons. Therefore, it is unlikely that current summer water temperatures cause heat stress through metabolic disruption and increased rates of tissue damage in the kākahi populations at these sites. The haemolymph concentrations of the different markers were not related to each other across the sites. These results demonstrate how temperature is just one factor that can influence physiology. In an aquatic system, abiotic factors such as dissolved oxygen concentration, salinity, and pH, and biotic factors like the presence of bacteria and algae, can all impact the physiological function of the animals present. The results from the present study highlight the complex web of abiotic and biotic factors that can influence kākahi physiology in their natural habitats.

3.2 Introduction

Measuring the concentration of heat stress biomarkers from haemolymph samples taken in a field setting is a less common approach to investigating vulnerability to climate change, compared to measuring these markers in a laboratory setting. In bivalves, there are few previously reported studies that measured LDH, ALT, and AST in haemolymph collected in the field. However, average summer temperatures of 27.2˚C have been found to cause a significant increase in haemolymph concentrations of AST and ALT in two species of North American freshwater mussel (*Villosa vibex* and *V. lienosa*) (Fritts et al., 2015b). The elevation in ALT and AST was attributed to increased tissue damage. The increase in AST and ALT was also species specific in this study, with *V. lienosa* suggested to be more thermally vulnerable to increasing summer water temperatures, due to a greater increase in haemolymph concentrations of ALT and AST, compared to *V. vibex* (Fritts et al, 2015b).

Studies that measured LDH, ALT, and AST in bivalves and other aquatic molluscs in response to field summer water temperatures are scarce. However, there have been several studies that measured ALT and AST (but not LDH) in field haemolymph samples

collected from bivalves in response to other stressors, including changes in stream discharge, increased salinity (Jahromi et al., 2020) and before and after translocation (Steinagel et al., 2018). ALT and AST were not significantly elevated in the gulf pearl oyster (*Pinctada radiata*) in response to hypo-salinity (Jahromi et al., 2020). This result was attributed to a lack of tissue damage induced by exposure to low salinity. ALT and AST were also not significantly elevated in the haemolymph of two North American freshwater mussel species (*Quadrula quadrula* and *Amblema plicata*) following translocation. As with other studies, this was suggested to be due to a lack of tissue damage caused by the potential stressor (Steinagel et al., 2018). Because ALT and AST are generalised markers of stress in aquatic invertebrates (Fritts et al., 2015a; Liao et al., 2019), these studies are still useful in informing the design of the current field study, and in the interpretation of the results.

The lack of field studies that have specifically investigated heat stress in bivalves that have examined LDH, ALT, and AST in haemolymph is a gap in the literature. Given that bivalve species (including kākahi) must survive and reproduce in natural environments, not in a laboratory, it is important to expand our knowledge and understanding of their thermal physiology and vulnerability to anthropogenic climate change (ACC) in these settings, rather than solely in a laboratory setting. Contributing to filling this gap was one of the main goals behind this study, in the context of kākahi, a cultural and ecological keystone species in Aotearoa New Zealand. In combination with the laboratory study (Chapter 4), it contributes to our understanding of the thermal physiology of kākahi, one of the two aims of this thesis.

The research presented in this thesis includes field and lab studies and for this reason, some methodology was shared between field and lab. Both settings were necessary to achieve the research aims and provide context. This chapter describes the field study which involved two phases of field sampling from five locations in the Manawatū, Rangitīkei and Wairarapa regions of Aotearoa New Zealand. The first phase of sampling was conducted in January and February 2022, at summer water temperatures. The second phase was conducted between April and July 2022, at winter temperatures. Although some of the winter samples were technically collected in late autumn, they have been labelled as ‘winter’ for simplicity. In both phases, non-lethal haemolymph samples were collected from a small group of kākahi (n = ~10) at each site. The samples were analysed at Massey University to determine the LDH, ALT, and AST concentrations.

The purpose of the field study was to investigate the haemolymph concentrations of known heat stress biomarkers in kākahi, found in waterways that were surrounded by rural but modified habitat during 2022 summer and winter water temperatures. The main aim underpinning this investigation was to determine if the concentrations of heat stress biomarkers in haemolymph were different at the warmest time of the year compared to colder months. This study may allow the following question to be answered: are current summer water temperatures causing heat stress in kākahi populations across the Manawatū, Rangitīkei, and Wairarapa regions?

3.3 Materials and methods

3.3.1 Sampling sites

Field sampling was conducted at five sites across the Manawatū, Wairarapa, and Rangitīkei regions of the North Island, Aotearoa New Zealand (approximate locations shown below). Four sites were on private land and one was on public land. This section gives a description of each field sampling site, including the location, land use, and key physical characteristics.

Above: Approximate locations of the five study sites (1-5) in the North Island of New Zealand. Source of line map: Wikipedia.

Site 1: Private farm in Mauriceville, Wairarapa

The first site was on a privately-owned farm in Mauriceville, a small farming community located 20 km from Masterton (Figure 13). Most of the farm is hill country, with kākahi found in the Kōpuaranga River which runs through several of the flat areas of the farm. Being a river rather than a stream, this waterway is much wider, deeper, and faster flowing than the other sites. Parts of the river are surrounded by willow trees, some of which washed away in a recent flooding event (November 2021), leaving debris in the river (Figure 14). There is a wide range of sediment types in this river, with patches of fine silt, gravel and some larger rocks present. The distribution of kākahi was very patchy, with greater numbers in slower flowing areas, particularly close to the riverbanks. The surrounding vegetation is pasture for grazing farm animals. Summer samples were taken from this site in early January 2022, at a water temperature of 18.8˚C. Winter samples were taken from this site in late April 2022, at a water temperature of 13.2˚C.

Figure 13: Satellite image of the Mauriceville sampling site. The Kōpuaranga River flows through this farm. Kākahi were located and sampled at the position shown (blue marker) with an estimated latitude of -40.7611 and longitude of 175.7187. The length of the river sampled had a mixture of shade from a willow tree canopy as well as no tree cover. There was a mix of gravel and silty sediment, and a large amount of tree debris in the river, due to recent flooding. The flow rate was moderately fast, with the presence of deep pools in the lee of the river, where kākahi were found to accumulate. Source: Google Maps.

Figure 14: The location where kākahi were found and sampled from in the Kōpuaranga River in Mauriceville in April 2022.

Site 2: Private farm in Taueru, Wairarapa

The second site was on a privately-owned farm in Taueru (also called Tauweru on current topographic maps; Taueru is the spelling preferred by mana whenua), 10 km from Masterton, on the road to Castlepoint (Figure 15). This is a sheep and beef farm, located on hill country. The stream where the kākahi were found is a tributary of the Waipawa Stream, which flows into the Taueru River. The stream flows through a large stretch of regenerating bush in a small valley (Figures 16-19). The catchment of the stream originates in a large pine plantation. Sheep graze the area around the stream where kākahi are found but cattle are permanently excluded. The vegetation in the area surrounding the stream contained native grasses and sedges, overgrown pasture species, and regenerating kanuka, manuka, and totara trees. The trees and sedges provide some areas of the stream with shade, while other areas are exposed to the sun. When the summer samples were collected (early January 2022), the water level and flow rate were low. Stream levels were marginally higher in April, but this stream does not get particularly fast or high, except during flood events. The water temperature was 20.0˚C when haemolymph samples were taken in summer and 12.2˚C when samples were collected from this site in winter (late April 2022).

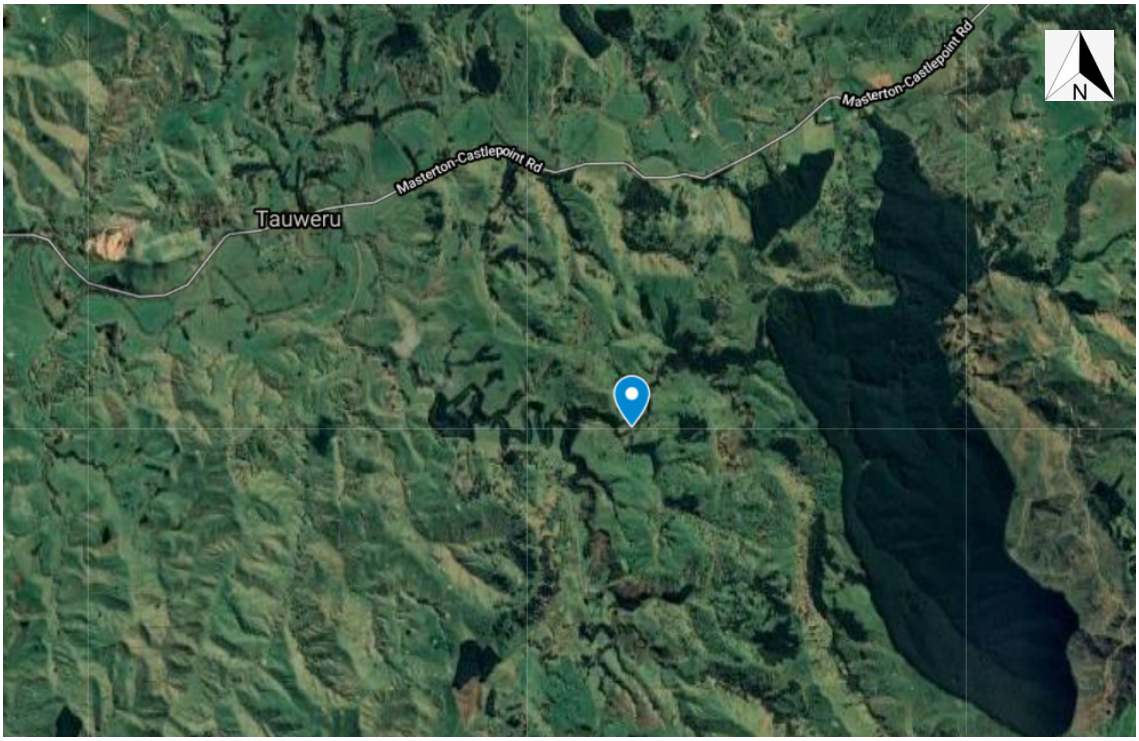

Figure 15: Location of the private farm in Taueru in relation to the Masterton-Castlepoint Road. The blue pin shows the approximate location where the sampled kākahi were located on the farm, with an estimated latitude of -40.9732 and longitude of 175.8415. On this map, a dark strip of regenerating native bush can be seen following the stream course. The large area of dark green to the right of the map is a large pine plantation. Source: Google Maps.

Figure 16: Regenerating native bush surrounding the stream.

Figure 17: The stream during winter sampling, in April 2022.

Figure 18: Large kākahi found in the stream at Taueru.

Figure 19: Section of the stream where kākahi were sampled in January 2022.

Site 3: Moonshine Valley, Aokautere, Palmerston North

This sampling site is in the Manga-o-tane Stream that runs through private property on Moonshine Valley Road, in Aokautere, a rural suburb of Palmerston North (Figure 20). The site is close to a regenerating and remnant reserve; Moonshine Valley Reserve and Tutukiwi Reserve, respectively. There are large pockets of planted native trees and regenerating bush surrounding the stream, including upstream and downstream of the sampling site. This length of the stream is largely shaded by the tree canopy year-round (Figures 21-23). Summer samples were taken at this site in mid-January 2022, at a water temperature of 18.4˚C. Winter samples were unable to be collected at the time of submission of this thesis due to the unseasonably high levels of rainfall between May and August 2022 making the kākahi habitat inaccessible. Should weather conditions allow, samples may be collected while winter temperatures remain.

Figure 20: Location of the Moonshine Valley sampling site, in Aokautere, Palmerston North. The blue pin shows the approximate location of where the kākahi were sampled, with an estimated latitude of -40.3800 and longitude of 175.6522. Source: Google Maps.

Figure 21: The Manga-o-tane Stream at Moonshine Valley. Kākahi were found partially under the bank of the stream in the silty areas to left of the centre in the photo.

Figure 22: Several kākahi siphoning in the stream at Moonshine Valley in summer (January 2022). The white triangles point to siphoning kākahi.

Figure 23: Kākahi found in the Manga-o-tane Stream, in Moonshine Valley.

Site 4: Simpson's Reserve, Hunterville

Simpson's Reserve is on public land five km north of Hunterville, in the Rangitīkei district. The Pourewa Stream, a tributary of the Rangitīkei River, flows south through this reserve (Figure 24). Pourewa is Ngāti Hauiti's (the Māori iwi) preferred spelling of the stream name (*pers. comm.* Hannah Rainforth, 2021). This section of the stream is surrounded by mature remnant podocarp forest and regenerating bush. However, both up- and downstream of Simpson's Reserve, the stream flows predominantly through exotic pine plantation and farmland. The stream is relatively narrow and has steep banks (Figures 25-27). In contrast to the sites at Mauriceville and Moonshine Valley, there is a more uniform flow rate at Simpson's Reserve. When summer samples were taken from this site in late January 2022, the water temperature was 20.0˚C. Due to the high levels of rainfall in the area between May and July of 2022, winter samples were unable to be collected at this site prior to submission of this thesis. Should weather conditions allow, samples may be collected while winter temperatures remain. Samples were collected under Department of Conservation Permit (97xxx-FAU).

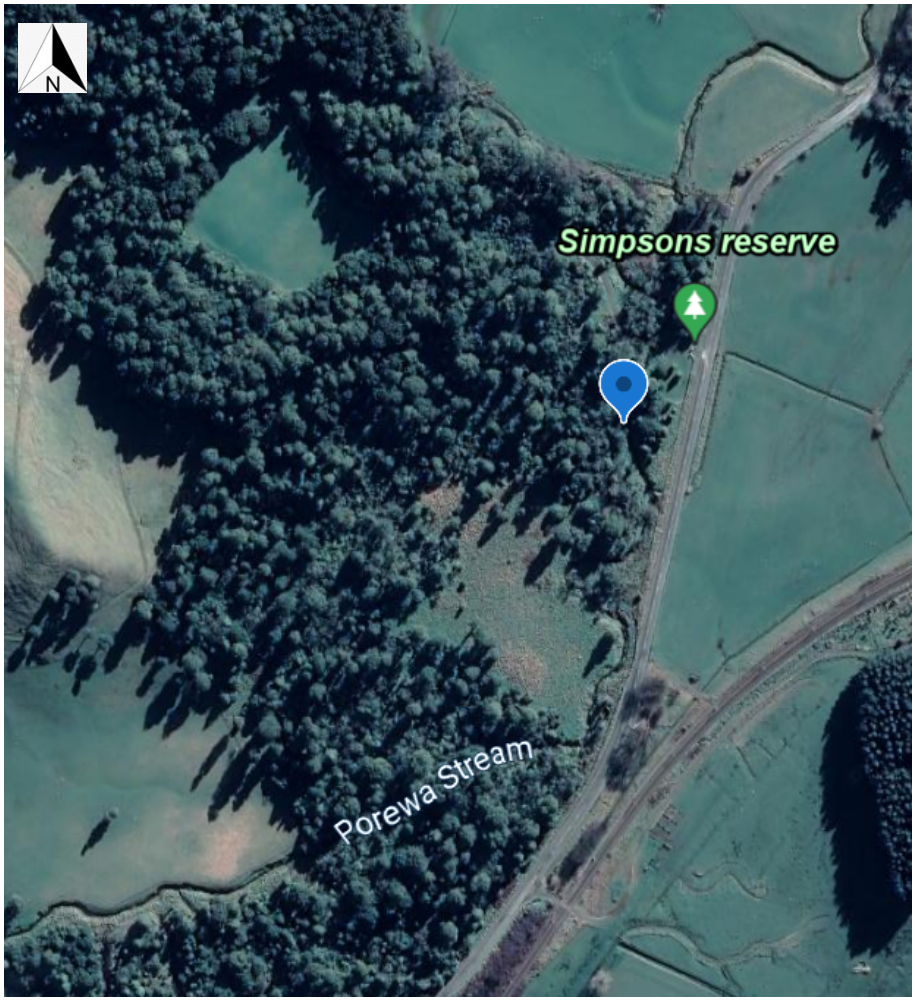

Figure 24: Satellite image of the Pourewa Stream at Simpson's Reserve, 5 km north of Hunterville. The stream name is spelled 'Porewa' on current topographic maps, 'Pourewa' is the preferred spelling of mana whenua. The blue pin indicates where kākahi were sampled, at an estimated latitude of -39.9033 and longitude of 175.6015. This section of the stream is surrounded by native bush, including mature podocarp specimens. The stream flows through farmland and exotic pine plantation forest upstream of the reserve, and predominantly farmland downstream of the reserve. Source: Google Maps.

Figure 25: Steep banks and a mixture of native and exotic trees, and pasture surround the Pourewa Stream.

Figure 26: Typical narrow section of the Pourewa Stream at Simpson's Reserve. There is a moderate flow rate, and some debris from the surrounding vegetation.

Figure 27: Entrance to Simpson's Reserve from Murimotu Road, Hunterville. The stream flows under the Warren steel truss bridge pictured (background) and is surrounded by the native bush which has a mixture of large native and exotic trees.

Site 5: Lake Horowhenua, Levin

The final field sampling site was Lake Horowhenua, which is located two km west of Levin, in the Horowhenua district (Figure 28). This shallow (<2m deep) coastal dune lake covers an area of 2.9 square kilometres. Its water supply comes from a series of streams, and the lake drains into the Hokio Stream (Tempero, 2013). Sixty-five percent of the lake's catchment runs through pasture, particularly dairy farms and market garden horticultural land (Figure 28). The water quality of Lake Horowhenua is very poor, and it is considered a hypertrophic lake (Gibbs et al., 2022). In summer, large blooms of cyanobacteria are common. Summer samples were taken from this site in late-February 2022, at a water temperature of 25.6˚C (Figure 29). Due to unseasonably high rainfall between May and August 2022, winter sampling was not possible at this site due to the lake level making kākahi inaccessible. Should weather conditions allow, samples may be collected while winter temperatures remain, and those data added to a future publication of the results of this thesis.

Figure 28: Satellite image of Lake Horowhenua including the surrounding farmland (west, north, and north-east). The edge of the township of Levin can be seen to the east of the lake (lower right). The blue pin indicates the approximate location where kākahi were sampled, at an estimated latitude of -40.6179 and longitude of 175.2455. The sampling site is close to Kohutoroa Marae (Māori iwi: Muaūpoko). Kākahi were found in the lake approximately 10-15 m away from the 2021-22 summer lake edge. Source: Google Maps.

Figure 29: Looking for kākahi in Lake Horowhenua with two members of the local iwi (Muaūpoko).

3.3.2 Haemolymph sampling

The equipment used for sampling kākahi haemolymph included 0.5 mL syringes and 29-gauge needles, 2 mL cryovials, a cryovial freezer box, a chilly bin filled with ice, wooden wedges for gently prying open the kākahi valves (shells), a headlamp, waders, a bathyscope, and a sharps container.

The following protocol was followed at every site. Upon arriving, a visual survey of the body of water was conducted to locate kākahi. At times, kākahi were easy to see as their siphons were visible. At other times, a bathyscope was used as a visual aid, while some locations required manual searches by hand. Kākahi were typically found in silty and sheltered areas of the stream, where the flow rate was low. The distribution of kākahi in any length of a stream or river was variable, with dense clusters (>10 specimens) of kākahi found in some parts of a stream/river, whereas the density could be much reduced (<2 specimens) in a similar area nearby. In Lake Horowhenua, kākahi were not typically found in clusters. Instead, there was often a metre or more between animals that were clearly visible.

Before sampling, several water temperature measurements were made, and a single mean sample recorded for each sampling site. Water temperature was typically measured mid-morning (10:00-11:00am) at each field site, with the temperature probe placed in the stream or lake bed near to the location and depth of sighted kākahi. A digital thermometer

and temperature probe (MASTECH MS6514, MGL APPA Corporation, Taipei, Taiwan) were used for all measurements. Haemolymph was only collected from kākahi that were greater than 40 mm in length. Where sufficient kākahi were located, a single haemolymph sample was collected from 10 individual kākahi (there was occasion for <10 samples at one site when locating kākahi became challenging). To collect haemolymph, each kākahi was taken out of the water and the tip of the wooden wedge gently inserted between the two shell valves, with care taken to avoid crushing the mantle and other tissues. The wedge was slowly and gently twisted to part the valves wide enough so that the anterior adductor muscle could be observed. It was often useful to wear a headlamp to illuminate the adductor muscle. A 29 g needle syringe was inserted into the anterior adductor muscle and 0.5 mL of haemolymph gently drawn out (Figure 30). It took 30 – 60 seconds to collect 0.5 mL of haemolymph but it could take slightly longer with smaller kākahi.

Figure 30: Collecting haemolymph from a kākahi in the field, using a wooden wedge to part the shell valves, and a 29 g needle and 0.5 mL syringe to collect a 0.5 mL sample from the anterior adductor muscle.

Haemolymph samples were transferred immediately to a pre-labelled (location, date, and sample number) cryovial. The cryovial was immediately placed into ice. Only one sample was collected from each kākahi. Ten haemolymph samples were taken at each site, where possible. The Mauriceville site in winter was the exception to this, where only five kākahi were located and sampled from. Once back at the lab, haemolymph samples were stored at -80˚C until assayed.

3.3.3 Haemolymph analyses

Kākahi haemolymph samples were assayed for lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) using commercially available kits.

Lactate dehydrogenase (LDH)

LDH activity in the haemolymph samples was measured using the Sigma-Aldrich Lactate Dehydrogenase Activity Assay Kit (MAK066, Merck, Sydney, Australia). Aliquots of each haemolymph sample were assayed as per the manufacturer's instructions. This colorimetric assay utilises the ability for LDH contained in a sample to reduce nicotinamide adenine dinucleotide (NAD) to NADH.

Briefly, each haemolymph sample was centrifuged at 10,000 g to separate any cellular debris. A 50 µL aliquot of supernatant was then collected and placed on flat-bottomed 96 wells. Concentrations (0, 1.25, 2.5, 5, 7.5, 10 and 12.5 nmole/well) of an NADH standard were also added to the plate. All unknowns, standard dilutions, and a control were assayed in duplicate. A volume of 50 µL of master reaction mix was added to each well. The plate was protected from light and agitated for 3 min on a horizontal shaker (150 rpm). Absorbance was then measured at 450 nm using a colorimetric plate reader (Multiskan FC, Thermo Fisher Scientific (Shanghai) Instruments Co. Ltd., Shanghai, China). This was repeated every 5 min up to a maximum of 15 min until the absorbance of the highest sample exceeded that of the 12.5 nmole/well standard. The plate was incubated in darkness at 37°C between each plate reader measurement. LDH activity measurements for each unknown sample were determined by interpolating their absorbances against the standard curve as per the manufacturer instructions. The limit of sensitivity of the assay was 0.12 milliunits/mL.

Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST)

Haemolymph samples were submitted to an accredited (International Accreditation New Zealand) commercial diagnostic laboratory (The Nutrition Lab, Massey University, Palmerston North, Aotearoa New Zealand) for quantifying ALT and AST concentrations. All samples were analysed in duplicate on a RX Daytona Plus clinical chemistry analyser (Randox Laboratories Limited, County Antrim, United Kingdom) using commercially available kits for ALT (Cat. No. AL8304) and AST (Cat. No. AL8306; Randox Laboratories Limited, County Antrim, United Kingdom). Molluscan ALT and AST have been previously quantified using standard clinical autoanalyzer kit chemistry.

3.3.4 Statistical analysis

The Welch's t-test (95% confidence interval, 0.05 significance level) was used to determine if there was a significant difference in LDH, ALT, and AST concentrations between summer and winter sampling groups at each field site. Possible correlations between the markers across the sites were evaluated using linear regression analysis. An analysis of variance (ANOVA) was used to determine if the mean concentrations of LDH, ALT, and AST were different between all sites. All tests were conducted using RStudio software (Rstudio® Desktop, Windows Version 2202.07.1-554, Boston, MA) using the

t.test(), lm(), and aov() functions. All data shown in graphs are displayed as means ± standard error of the mean (SEM).

3.4 Results

Mean LDH, ALT, and AST haemolymph concentrations were firstly compared between the summer and winter sampling groups at each site. The aim of this comparison was to investigate whether current summer water temperatures may be causing heat stress in local kākahi populations. Following this, field data were pooled to investigate any relationship between the physiological markers across the field sites. Note: Unfortunately, the Moonshine Valley, Simpson's Reserve, and Lake Horowhenua field sites experienced unseasonably high rainfall events which made sampling impossible during winter.

3.4.1 Mauriceville

Mean LDH, ALT, and AST haemolymph concentrations in kākahi sampled at the Mauriceville site in summer or winter were compared and shown in Figures 31- 33.

Mean LDH concentrations were similar in summer and winter (Figure 31). The mean LDH concentration in winter samples was 2.6 ± 0.4 milliunits/mL, which is slightly higher than the summer samples, which was 2.1 ± 0.7 milliunits/mL. There was no significant difference in between summer and winter samples at the Mauriceville site (Welch's t-test; $p = 0.51$).

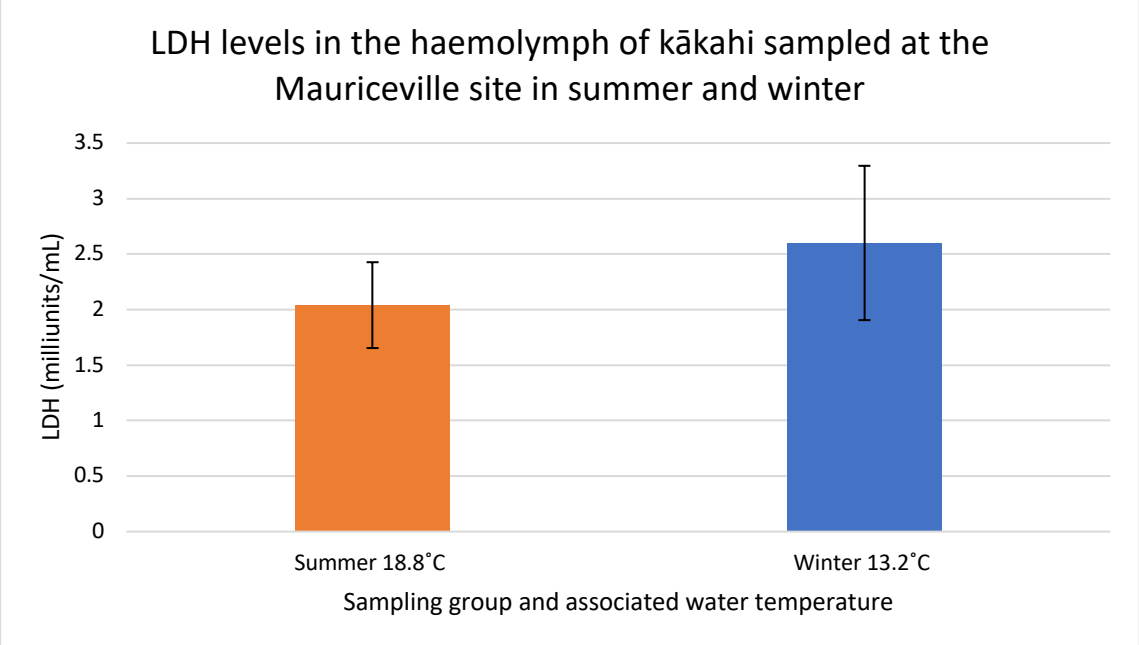

Figure 31: Mean LDH concentration (in milliunits/mL) in kākahi haemolymph in the summer (18.8˚C) and winter (13.2˚C) groups sampled at the Mauriceville (MV) field site. All data are mean ± SEM.

Mean ALT concentrations were similar in winter and summer (Figure 32). The summer group, which were sampled in January at a water temperature of 18.8˚C, had a mean ALT concentration of 9.2 ± 2.1 U/L, compared to 12.8 ± 2.2 U/L when kākahi were sampled at a temperature of 13.2 ˚C. There was no significant difference in mean ALT concentration between the summer and winter groups (Welch's t-test; $p = 0.26$).

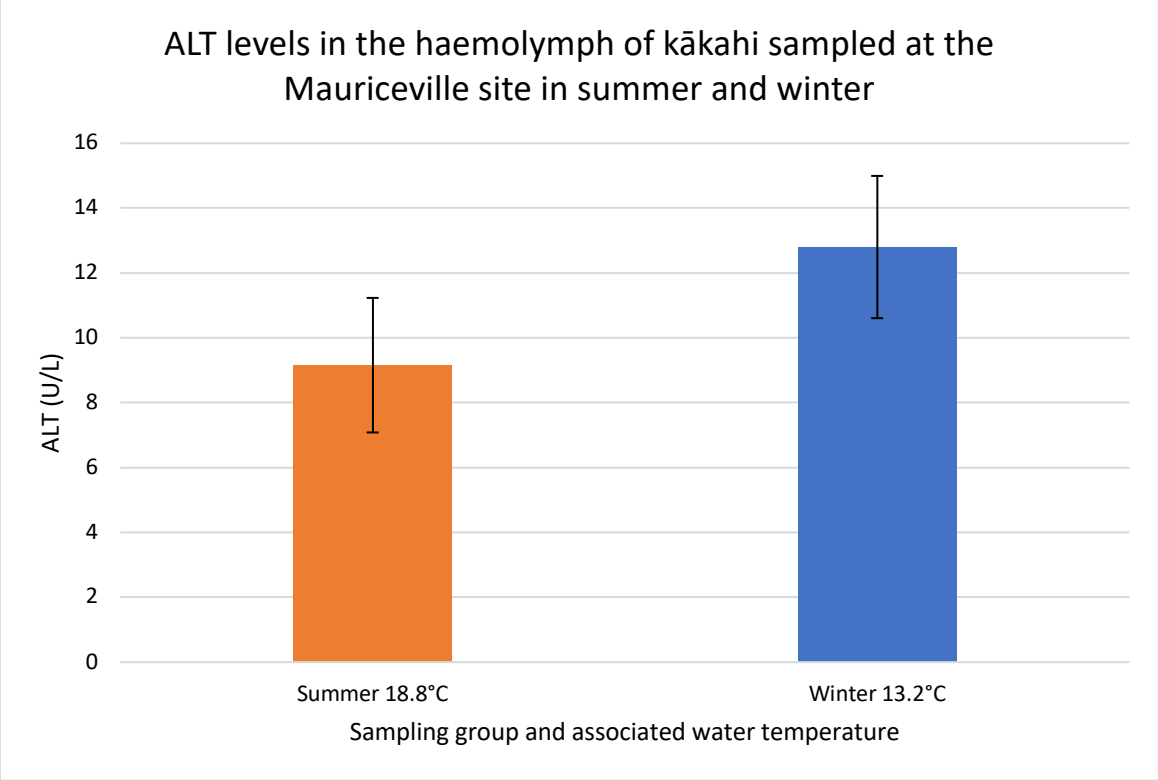

Figure 32: Mean ALT concentration (in units per litre) in kākahi haemolymph in the summer (18.8˚C) and winter (13.2˚C) groups sampled at the Mauriceville (MV) field site. All data are mean ± SEM.

Mean AST concentrations were higher in summer, when the water temperature was 18.8˚C, than in winter when the water temperature was 13.2 ˚C (Figure 33). The mean AST haemolymph concentration was 36.9 ± 7.7 U/L in the summer samples which was higher than in the winter samples that had a mean concentration of 25.3 ± 6.4 U/L. However, the difference in mean AST concentration between summer and winter samples at this site was not significant (Welch's t-test; $p = 0.27$).

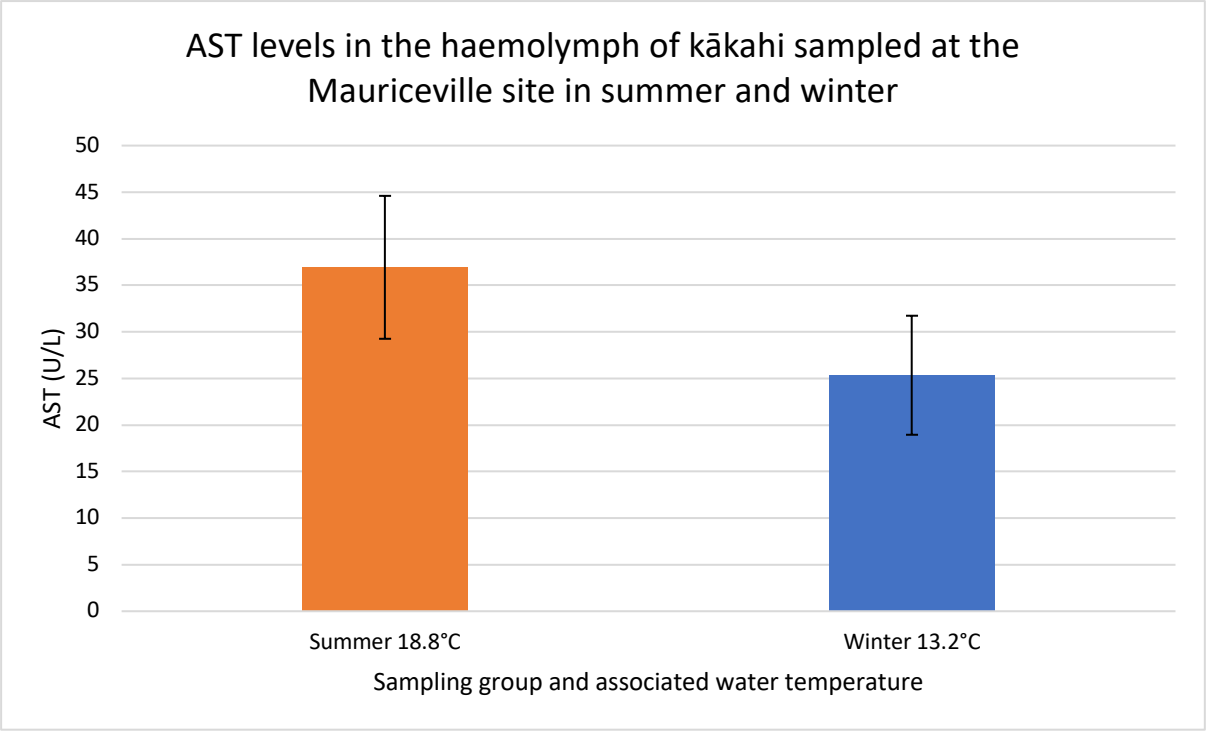

Figure 33: Mean AST concentration (in units per litre) in kākahi haemolymph in the summer (18.8˚C) and winter (13.2˚C) groups sampled at the Mauriceville (MV) field site. All data are mean ± SEM.

3.4.2 Taueru, Castlepoint

Mean LDH, ALT, and AST haemolymph concentrations in kākahi sampled in summer or winter at the Taueru site are presented below (Figures 34-36).

Mean LDH concentrations were somewhat similar in winter and summer, with the winter group appearing to have slightly higher concentrations than the summer group (Figure 34). Kākahi sampled in summer at a water temperature of 20.0˚C, had a mean LDH concentration of 1.8 ± 0.4 milliunits/mL, compared to 3.1 ± 0.9 milliunits/mL when the winter water temperature was 12.2˚C. The difference between summer and winter samples at the Taueru site was not significant (Welch's t-test; $p = 0.21$).

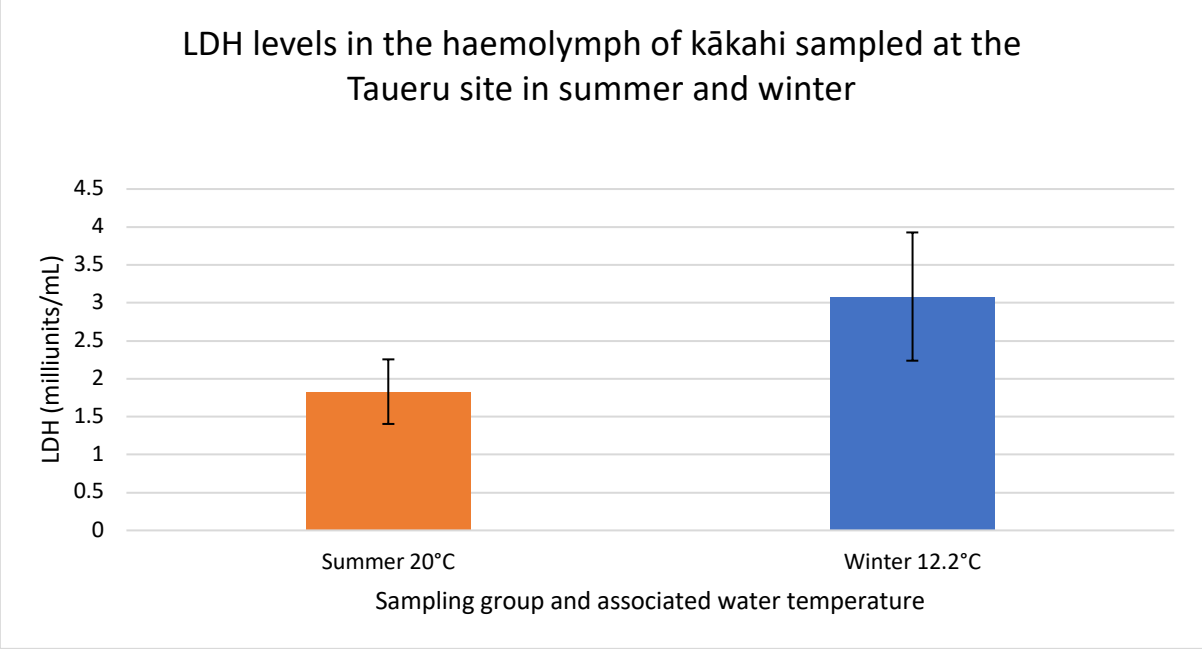

Figure 34: Mean LDH concentration (milliunits/mL) in kākahi haemolymph in the summer (20.0˚C) and winter (12.2˚C) groups sampled at the Taueru field site. All data are mean ± SEM.

There was no difference in mean ALT concentration in haemolymph sampled from kākahi in summer and winter at the Taueru site (Figure 35). The mean ALT concentration in the summer group was 8.1 ± 1.1 U/L, compared to 8.0 ± 0.7 U/L in the winter group. There was no significant difference between summer and winter samples at the Taueru site (Welch's t-test; $p = 0.93$).

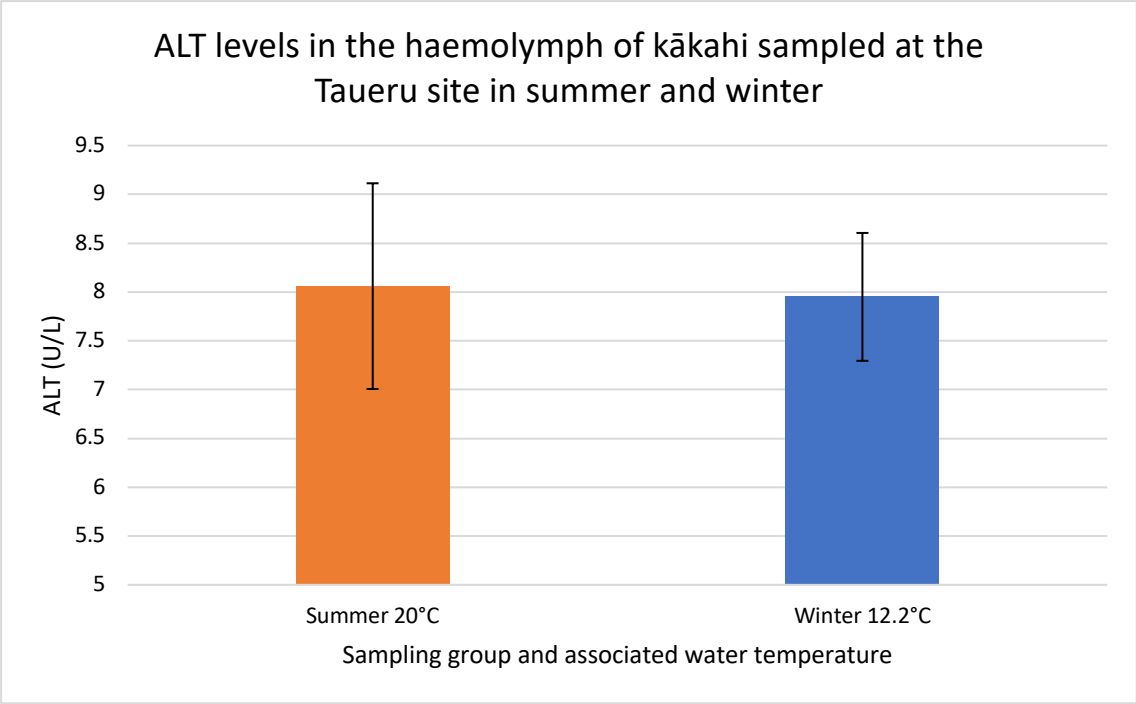

Figure 35: Mean ALT concentration (in units per litre) in kākahi haemolymph in the summer (20.0˚C) and winter (12.2˚C) groups sampled at the Taueru field site. All data are mean ± SEM.

Mean AST concentrations in kākahi haemolymph at the Taueru site were similar in winter and summer (Welch's t-test, p = 0.43; Figure 36). The summer group, which were sampled at a water temperature of 20.0˚C, had a mean AST concentration of 20.3 ± 2.5 U/L, compared to 23.2 ± 2.6 U/L when kākahi were sampled at a temperature of 12.2˚C in winter.

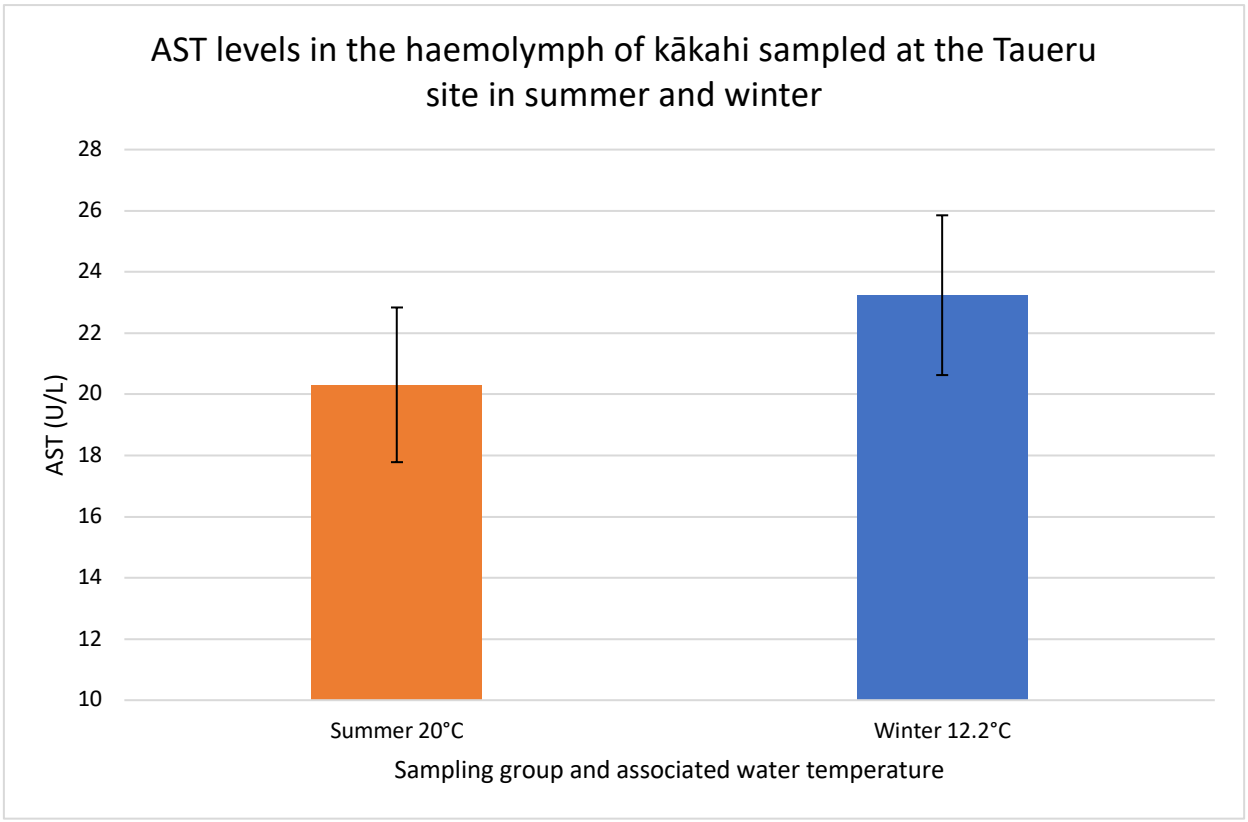

Figure 36: Mean AST concentration (in units per litre) in kākahi haemolymph in the summer (20.0˚C) and winter (12.2˚C) groups sampled at the Taueru field site. All data are mean ± SEM.

3.4.3 Simpson's Reserve

Due to high levels of rainfall at Simpson's Reserve that caused sampling difficulties, only the mean LDH, ALT, and AST haemolymph concentrations in summer are provided for this site (Figures 37-39). Therefore, a comparison between the summer and winter groups is not given for the following three figures.

Mean LDH concentration in the haemolymph of kākahi at Simpson's Reserve in summer, at a water temperature of 20.0˚C was 1.3 ± 0.1 milliunits/mL (Figure 37).

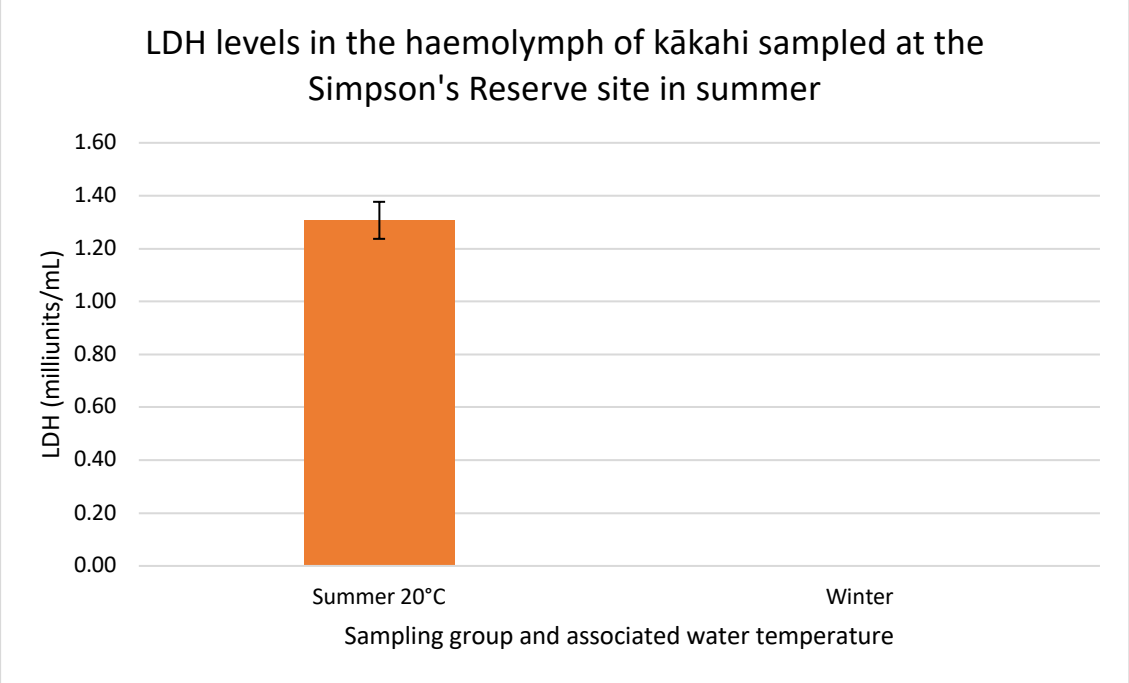

Figure 37: Mean LDH concentration (milliunits/mL) in haemolymph in kākahi sampled at the Simpson's Reserve field site during summer water temperatures of 20.0˚C. All data are mean ± SEM. There is a blank space for the winter group as these samples have not yet been collected and analysed.

The mean ALT concentration in haemolymph was 7.1 ± 0.5 U/L (Figure 38) in kākahi sampled during summer at Simpson's Reserve when the water temperature was 20.0˚C.

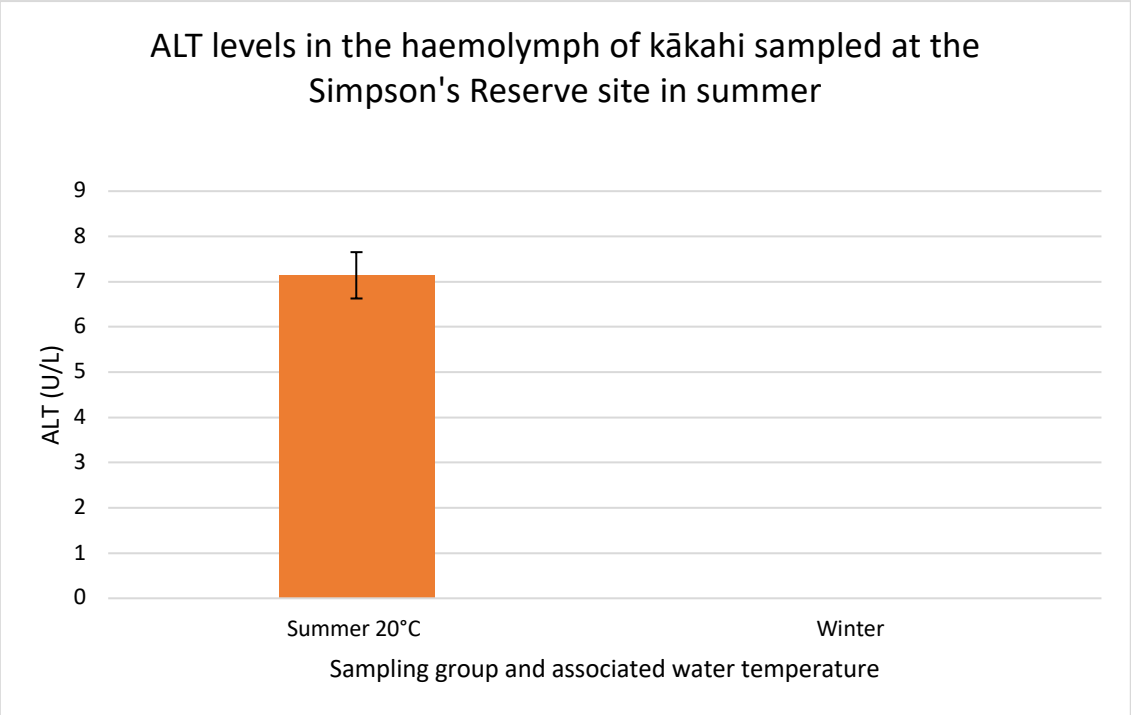

Figure 38: Mean ALT concentration (Units/Litre) in kākahi haemolymph in the summer (20.0˚C) group sampled at the Simpson's Reserve field site. All data are mean ± SEM. There is a blank space for the winter group as these samples have not yet been collected and analysed.

The mean AST concentration in haemolymph was 12.3 ± 1.5 U/L (Figure 39) in kākahi sampled during summer at Simpson's Reserve when the water temperature was 20.0˚C.

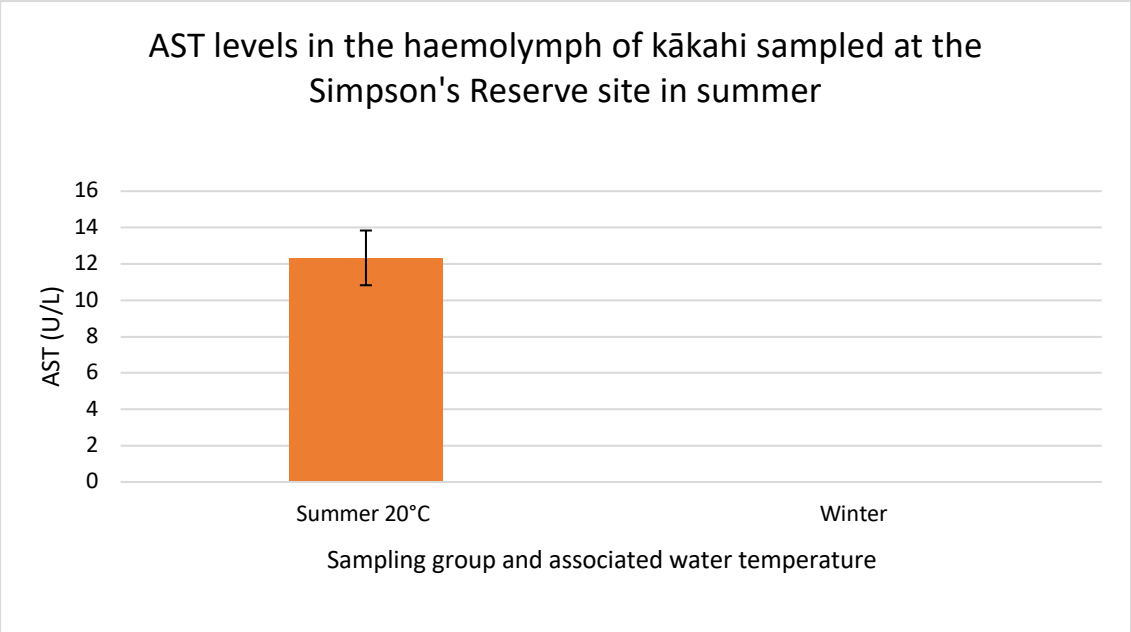

Figure 39: Mean AST concentration (Units/Litre) in kākahi haemolymph in the summer (20.0˚C) group sampled at the Simpson's Reserve field site. All data are mean ± SEM. There is a blank space for the winter group as these samples have not yet been collected and analysed.

3.4.4 Moonshine Valley

As with the Simpson's Reserve site, only the mean concentrations of the markers in summer could be provided for Moonshine Valley. The mean LDH concentration in summer and the mean concentrations of all three markers in winter are awaiting analysis. Therefore, only the mean ALT and AST haemolymph concentrations in summer have been provided for Moonshine Valley (Figures 40-41).

The mean ALT concentration in haemolymph was 12.2 ± 2.9 U/L (Figure 40) in kākahi sampled during summer at the Moonshine Valley site when the water temperature was 18.4˚C.

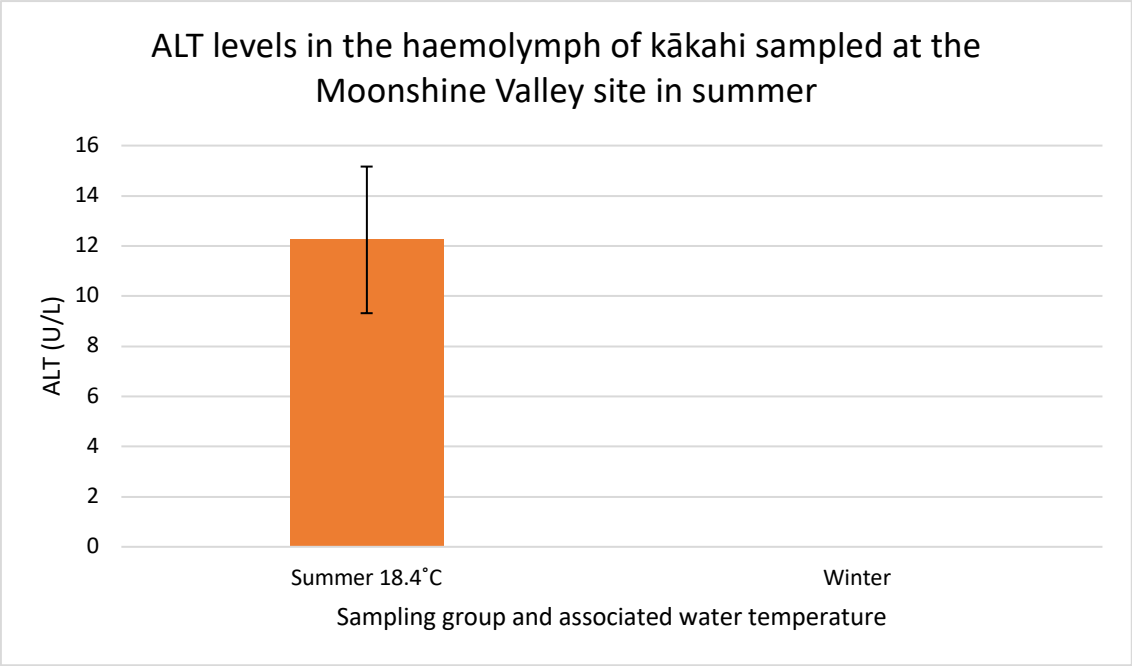

Figure 40: Mean ALT concentration (Units/Litre) in kākahi haemolymph in the summer (18.4˚C) group sampled at the Moonshine Valley field site. All data are mean ± SEM. There is a blank space for the winter group as these samples have not yet been collected due to adverse stream conditions.

The mean AST concentration in haemolymph was 45.8 ± 10.7 U/L (Figure 41) in kākahi sampled during summer at the Moonshine Valley site when the water temperature was 18.4˚C.

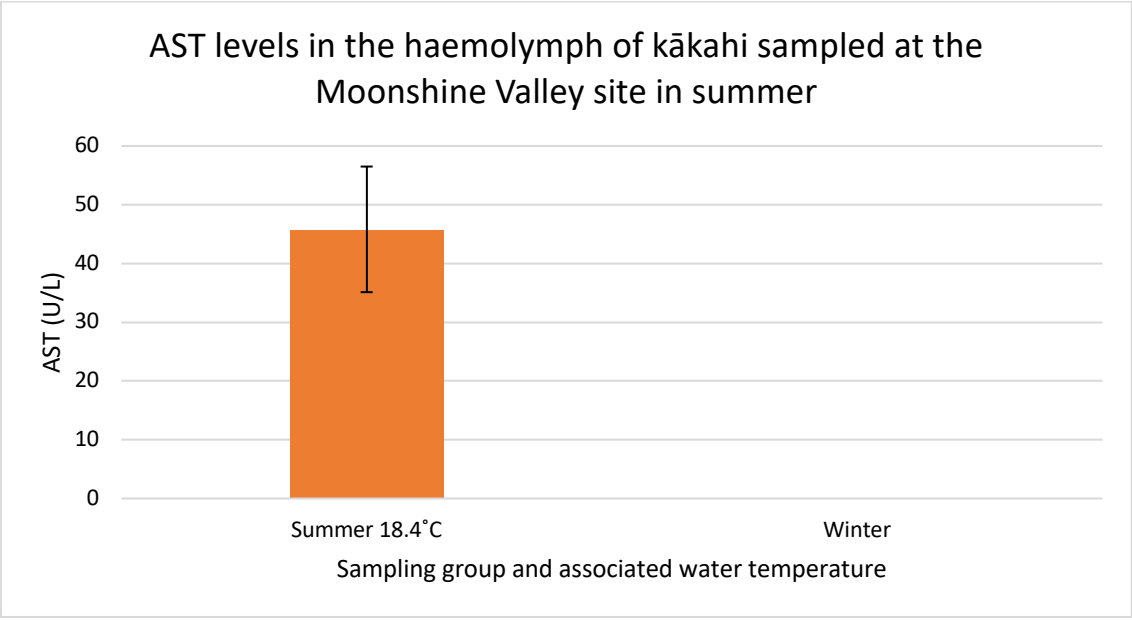

Figure 41: Mean AST concentration (Units/Litre) in kākahi haemolymph in the summer (18.4˚C) group sampled at the Moonshine Valley field site. All data are mean ± SEM. There is a blank space for the winter group as these samples have not yet been collected due to adverse stream conditions.

3.4.5 Lake Horowhenua

The final field site was Lake Horowhenua. As with the Moonshine Valley site, only the mean ALT and AST haemolymph concentrations in summer were able to be provided. These results are presented below (Figures 42 and 43).

The mean ALT concentration in haemolymph was 10.1 ± 1.0 U/L (Figure 42) in kākahi sampled during summer at the Lake Horowhenua site when the water temperature was 25.6˚C.

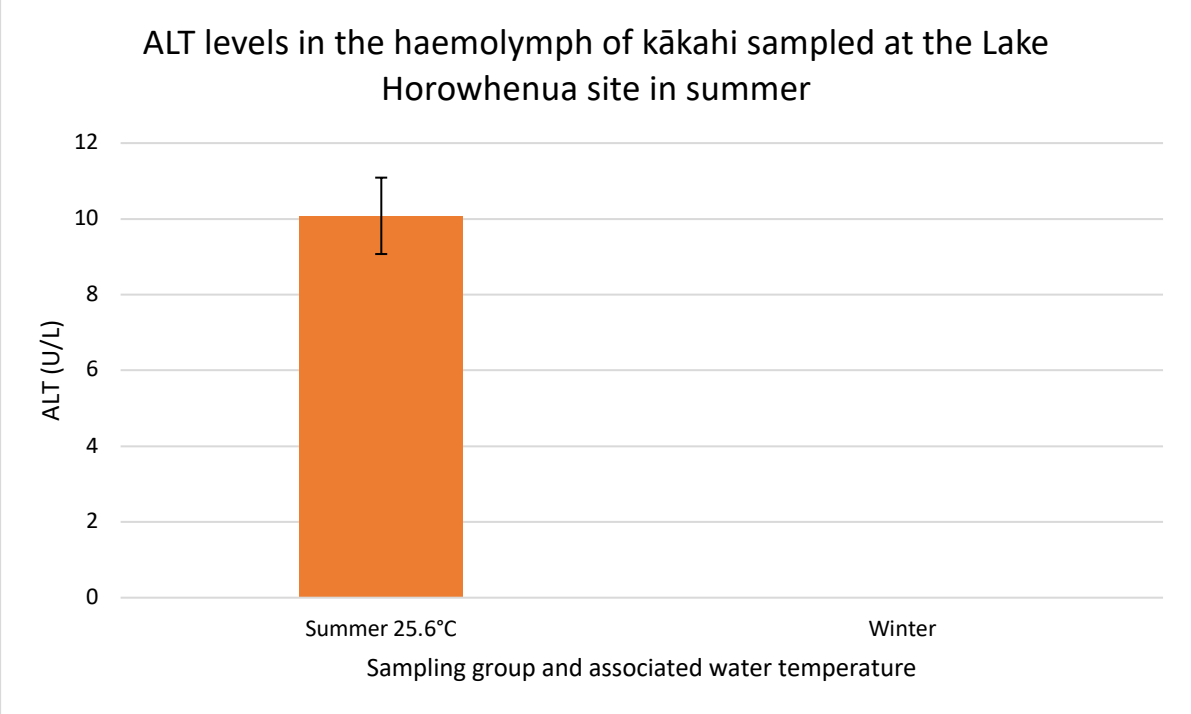

Figure 42: Mean ALT concentration (Units/Litre) in kākahi haemolymph in the summer (25.6˚C) group sampled at the Lake Horowhenua field site. All data are mean ± SEM. There is a blank space for the winter group as these samples have not yet been collected due to adverse lake conditions.

The mean AST concentration in haemolymph was 23.1 ± 3.0 U/L (Figure 43) in kākahi sampled during summer at the Lake Horowhenua site when the water temperature was 25.6˚C.

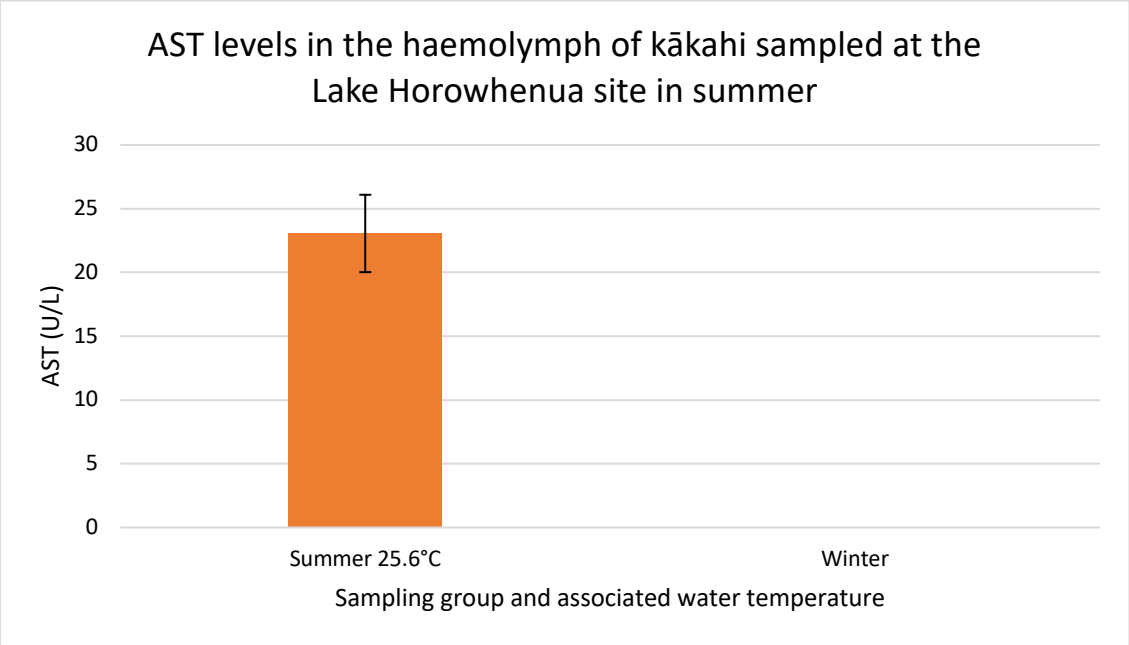

Figure 43: Mean AST concentration (Units/Litre) in kākahi haemolymph in the summer (25.6˚C) group sampled at the Lake Horowhenua field site. All data are mean ± SEM. There is a blank space for the winter group as these samples have not yet been collected due to adverse lake conditions.

Table 1 provides a summary of the mean LDH, ALT, and AST haemolymph concentrations in kākahi sampled from all five field sites. Mean LDH and ALT concentrations were similar at all the sites where these markers were measured, in both summer and winter sampling groups. Mean LDH concentrations ranged from 1.3 ± 0.1 milliunits/mL to 3.1 ± 0.9 millunits/mL across all sites and sampling groups. Mean ALT concentrations ranged from 7.1 ± 0.5 U/L to 12.8 ± 2.2 U/L. The mean AST concentration was more variable across the sites, ranging from the lowest value of 12.3 ± 1.5 U/L in the summer samples at Simpson's Reserve, to the highest value of 45.8 ± 10.7 U/L in the summer samples at Moonshine Valley.

An analysis of variance (ANOVA) was conducted to determine if the mean concentrations of LDH, ALT, and AST in summer were significantly different at all sites. There was no significant difference in mean LDH in summer samples taken from Mauriceville, Taueru, and Simpson Reserve (ANOVA; $p = 0.296$). There was no significant difference in mean ALT between summer samples taken from all sites (ANOVA; $p = 0.251$). There was a significant difference in mean AST between summer samples taken from all sites (ANOVA; $p = 0.002$).

Table 1: Mean LDH, ALT, and AST haemolymph concentrations in kākahi sampled at the five different field sites at summer and winter water temperatures. All data is given as mean ± SEM. NS = no sampling possible.

	LDH (milliunits/mL)		**ALT** (U/L)		**AST** (U/L)	
Site (Summer, Winter ˚C)	Summer	Winter	Summer	Winter	Summer	Winter
Mauriceville (18.8, 13.2)	2.1 ± 0.7	2.6 ± 0.4	9.2 ± 2.1	12.8 ± 2.2	36.9 ± 7.7	25.3 ± 6.4
Taueru (20, 12.2)	1.8 ± 0.4	3.1 ± 0.9	8.1 ± 1.1	8.0 ± 0.7	20.3 ± 2.5	23.3 ± 2.6
Simpson's Reserve (20, NA)	1.3 ± 0.1	NS	7.1 ± 0.5	NS	12.3 ± 1.5	NS
Moonshine Valley (18.4, NA)	NS	NS	12.2 ± 2.9	NS	45.8 ± 10.7	NS
Lake Horowhenua (25.6, NA)	NS	NS	10.1 ± 1.0	NS	23.1 ± 3.0	NS

3.4.6 Investigating the relationship between different biomarkers at all the field sites

After assessing the concentrations of the heat stress markers at each site individually, the data were pooled to determine if there was any correlation between the physiological markers across the sites. Firstly, AST was plotted against LDH at each site (Figure 44).

There is no clear correlation between the summer and winter concentrations of AST and LDH across sites (Figure 44), although data are missing from Simpson's Reserve (SR), Moonshine valley (MSV), and Lake Horowhenua (LH) due to delayed analysis of samples, so it is difficult to make definitive conclusions. The r^2 for the available data shown on this graph was 0.006 ($p = 0.29$), giving no evidence of a relationship between AST and LDH concentrations in summer and winter samples at the Mauriceville (MV) and Taueru (CP) field sites, the only sites with both summer and winter data.

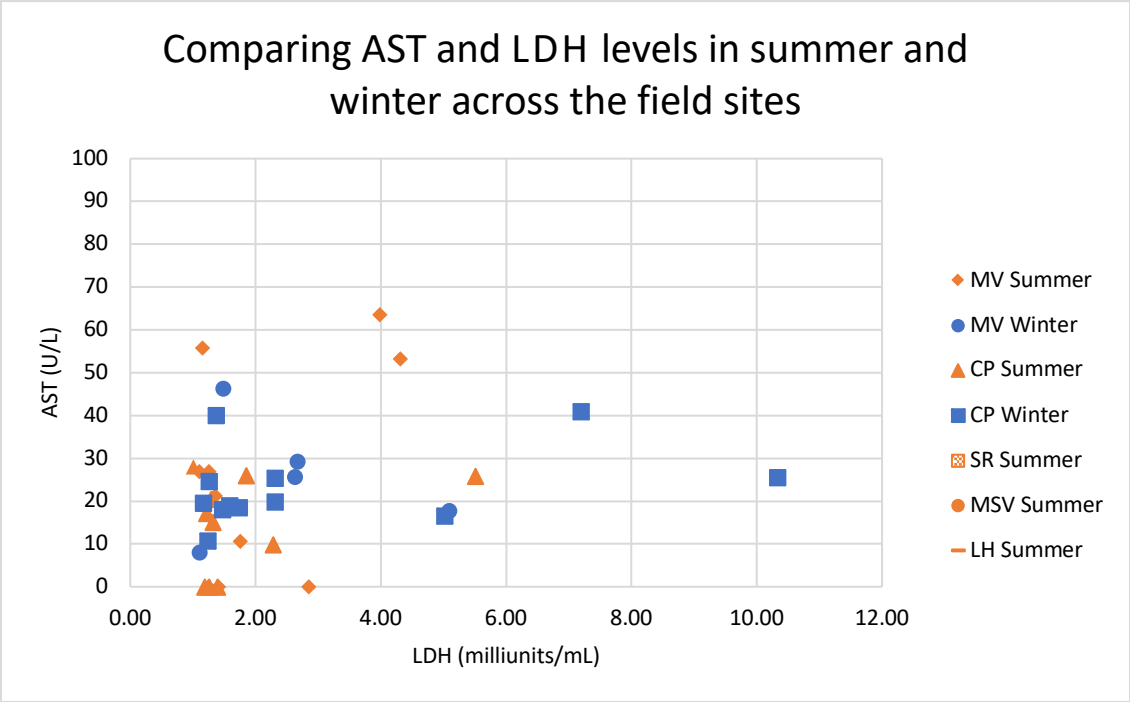

Figure 44: AST versus LDH concentrations in summer and winter samples at all the field sites. There are missing data from the SR, MSV, and LH sites due to these data not being collected and analysed yet. The summer data are missing due to the LDH concentrations from these groups not being available. The winter AST and LDH results from these sites were also not available at this time. Therefore, this graph shows the correlation between AST and LDH haemolymph concentrations in summer and winter at only the Mauriceville (MV) and Taueru (CP) sites.

Secondly, AST and ALT concentrations in summer and winter samples were compared across the sites (Figure 45).

There appears to be a weak, positive relationship between AST and ALT across the field sites (Figure 45), particularly so at the MSV site. However, the data are highly variable, with outliers skewing the general trend. There are also several data points where ALT is high while AST remains low, and vice versa. The r^2 value for this graph was 0.14 (p = 0.002), indicating no significant relationship between AST and ALT haemolymph concentrations in summer and winter across the field sites.

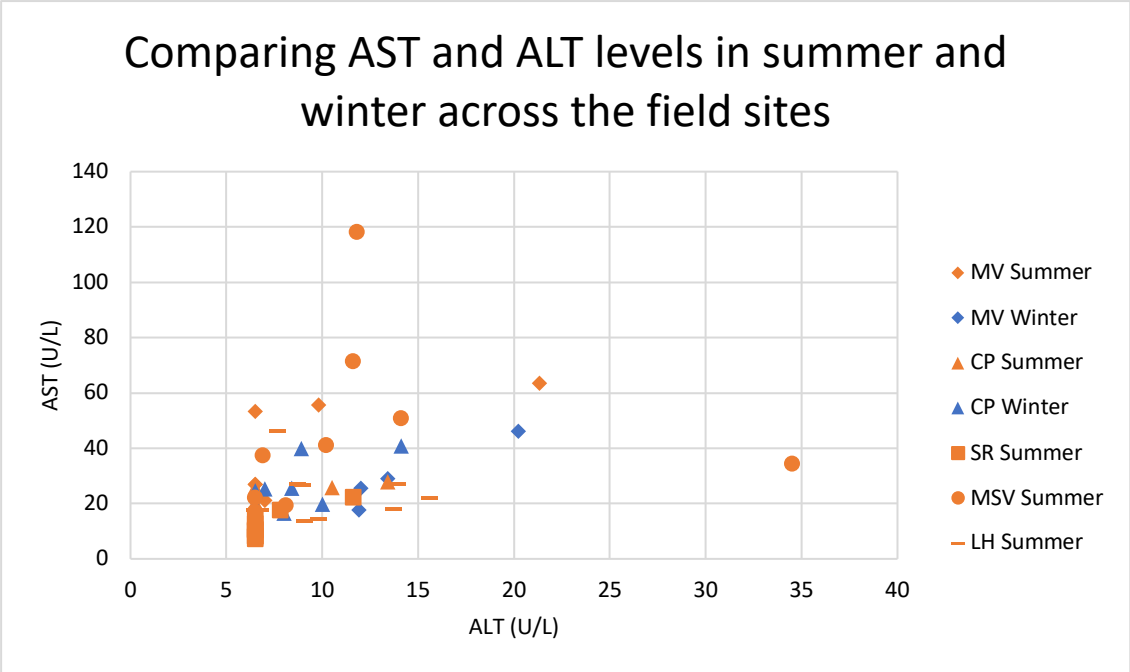

Figure 45: Correlation between AST and ALT concentrations sampled from kākahi in summer and winter at all the field sites. The winter concentrations for the SR, MSV, and LH sites are missing due to delayed collection and analysis of these samples.

ALT and LDH haemolymph concentrations in summer and winter samples were compared across the sites (Figure 46).

Figure 46 shows the relationship between ALT and LDH in summer and winter at the Mauriceville (MV) and Taueru (CP) sites only. There was no obvious correlation, with both winter and summer data points at low and high concentrations of ALT and LDH. The lack of a relationship between these two markers at these sites is supported by an r^2 value of 0.037 ($p = 0.15$).

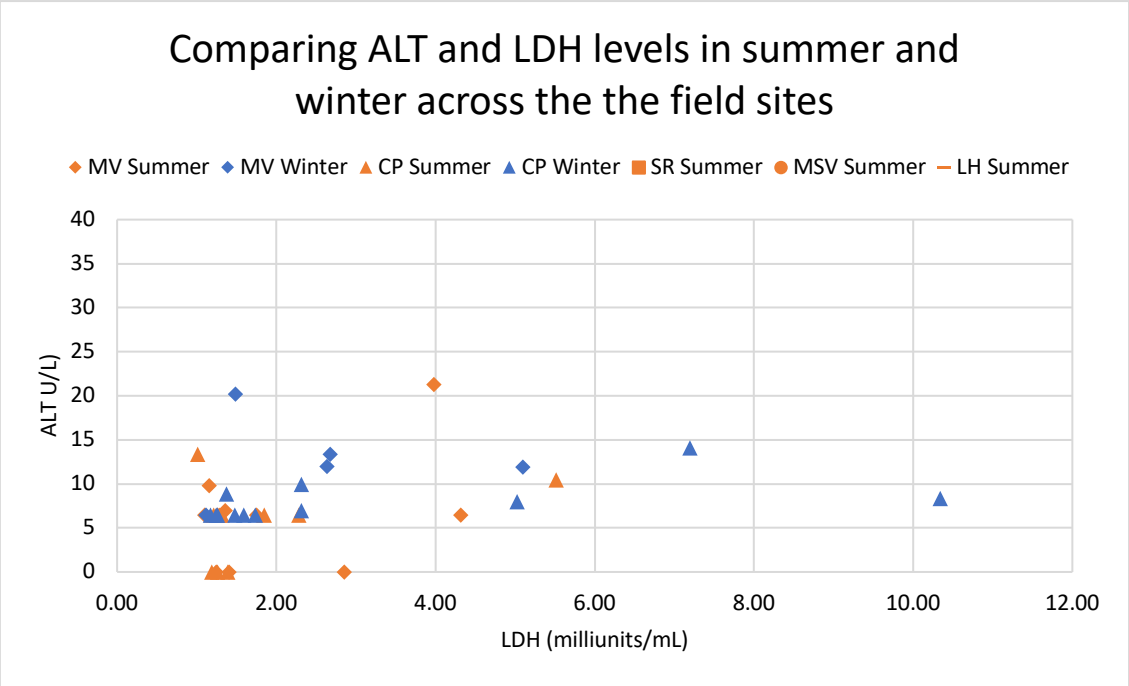

Figure 46: Correlation between haemolymph ALT and LDH concentrations in kākahi sampled in summer and winter at all the field sites. There are missing data from the SR, MSV, and LH sites due to these data not being collected and analysed yet. The summer data are missing due to LDH results from these groups being unavailable. The winter LDH and ALT results from these sites were also not available at this time. Therefore, this graph shows the correlation between LDH and ALT concentrations in summer and winter samples at the Mauriceville (MV) and Taueru (CP) sites.

3.5 Discussion

The field study targeted the first aim of this thesis (see Chapter 2, section 2.8), which was to increase our knowledge of the thermal physiology of kākahi. This was achieved by determining if there were any differences in known markers of heat stress at current (2022) summer and winter water temperatures in wild kākahi. The outcomes of this study may also have wider implications for kākahi conservation and future research because it describes, to my knowledge, the first successful application of non-lethal haemolymph sampling in kākahi as a method of measuring the concentration of heat stress biomarkers at current environmental conditions. The non-lethal nature of a 0.5 mL haemolymph sample was confirmed through a short pilot trial that was conducted using kākahi from Lake Horowhenua which is described in Chapter 4. A similar methodology has been used in other freshwater mussel species (Gustafson et al., 2005; Karlsson et al., 2013; Fritts et al., 2015a). It is a relatively simple technique that does not require specialised equipment, making it ideal for use in the field. Given its non-lethal nature, it may be a useful tool for monitoring stress in kākahi populations and for other research using these animals, without negatively impacting population sizes.

In addition to validating the usefulness of non-lethal haemolymph sampling of kākahi in the field, this chapter describes haemolymph concentrations of several heat stress biomarkers in five local populations at this point in time. This allowed a snapshot to be captured of how current environmental temperatures in summer and winter may be affecting kākahi physiology in 2022.

Typical average summer air temperatures in the main centres closest to the field sites (Palmerston North and Masterton) have ranged between 17˚C and 19.5˚C between 2018 and 2022 (Figure 47). The water temperatures measured at most of the field sites ranged between 18.4˚C and 20˚C, which are similar to the average summer air temperatures in the two regions over the last five years. Lake Horowhenua is an exception, but summer water temperatures of 25˚C and above are typical for this lake (LAWA, 2022). Typical average winter air temperatures (May-July) in Palmerston North and Masterton have ranged between 10.0 and 12.3˚C between 2018 and 2022 (Figure 47). The winter water temperatures were 12.2˚C at Taueru and 13.2˚C at Mauriceville. Therefore, the 2022 winter water temperature at Taueru was within the range of winter air temperatures recently recorded in the region, but was slightly outside this range at the Mauriceville site.

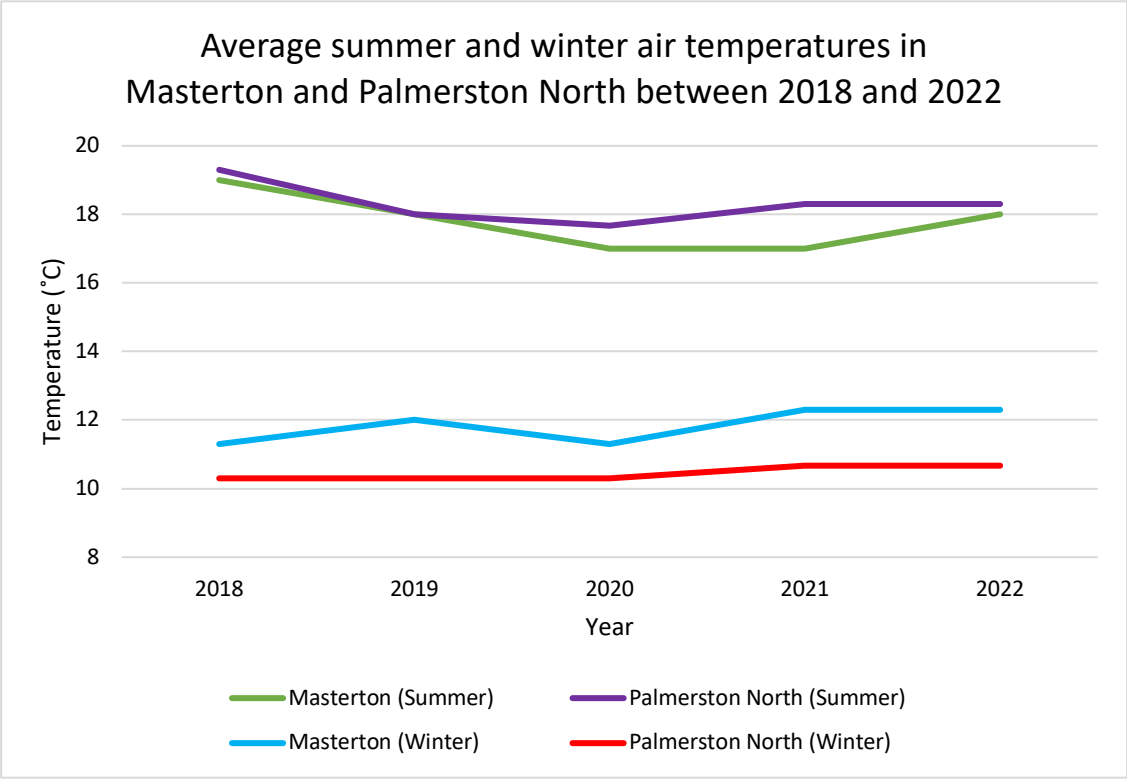

Figure 47: Average air temperatures in summer and winter in Masterton and Palmerston North between 2018 and 2022. The overall average for the summer period of each year was calculated using the average temperatures in December, January, and February of each summer, respectively. The overall average for the winter period of each year was calculated using the average temperatures in May, June, and July of each winter. Temperature data were retrieved from the Palmerston North and Masterton pages of the Time and Date webpage (Time and Date AS, 2022).

At the Mauriceville site, there was minimal difference in mean LDH concentration in the haemolymph between the summer and winter samples. This suggests that neither current summer (18.8˚C) nor winter (13.2˚C) water temperatures cause an increase in anaerobic energy production in this kākahi population. This result may be partially explained by the larger volume of water in this river compared to the other sites. The larger water volume may buffer the temperature rise that occurs in summer, reducing the impact on the physiology of the kākahi population.

The mean ALT and AST haemolymph concentrations at this site were somewhat contradictory, as ALT appeared to be slightly higher in winter (though this was not statistically significant) but AST was almost 10 U/L higher in summer (also not statistically significant). Heat stress is not the only potential cause of elevated AST concentrations in the circulation, as this is a generalised marker of stress in aquatic invertebrates (An & Choi, 2010; Fritts et al., 2015a). AST haemolymph concentrations have been found to increase in response to salinity stress in ark shells (*Scapharca broughtonii*) (An & Choi, 2010) and in oysters (*Crassostrea virginica*) with a parasite infection (Douglass & Haskin, 1976). It could be that some of the kākahi at this site were experiencing other forms of stress, such as disease, that caused an (non-significant) elevation of AST in the haemolymph, rather than the 18.8˚C temperature causing tissue damage via increased oxidative stress.

At the Taueru site, mean LDH was slightly higher in winter at a temperature of 12.2˚C than in summer at a temperature of 20.0˚C. However, this difference was not statistically significant. This indicates that current summer water temperatures do not challenge the energy system of kākahi, as I would expect an increase in LDH in summer if this was the case. There was essentially no difference in mean ALT concentration between the summer and winter groups at this site. Mean AST concentration was slightly elevated in winter, though this was not a significant difference. Therefore, it is unlikely that current summer temperatures cause tissue damage from increased oxidative stress in the kākahi at this site. A larger sample size would strengthen these conclusions.

Unfortunately, it was not possible to compare the mean haemolymph concentrations of LDH, ALT, and AST in summer and winter at Simpson's Reserve, Moonshine Valley, or Lake Horowhenua. The weather conditions between May and August of 2022 made it unsafe to collect the winter samples from these locations. These data will instead be presented at a later date, in the published manuscript of this thesis.

Given the many variables that likely contribute to physiological stress in a wild population of freshwater bivalves, such as food availability, temperature, oxygen concentration, and contaminants to name a few, I expected the haemolymph concentrations of LDH, ALT, and AST to vary considerably between the field sites (Table 1). In addition to the biotic factors above, the physical geography varied significantly between the stream, river, and lake habitats that kākahi were sampled. Given the connections between the physical environment and the ecology and physiology of animals (Huey, 1991), one would expect physiological stress to be different between the field sites. Interestingly, there was no significant difference in LDH or ALT haemolymph concentrations between the sites. This suggests that none of the individual kākahi populations were experiencing higher rates of metabolic disruption compared to the others. There was, however, a significant difference in AST haemolymph concentrations between the different sites. The summer

temperatures were within 2˚C of each other at all sites except Lake Horowhenua, which suggests that the cause of this variation in AST cannot be attributed solely to temperature.

In future studies, it would be useful to fully characterize potential changes in the environment at the field sites between sampling events. Kākahi live in highly dynamic environments, where the stressors present and the interactions between stressors changes over time. Acknowledging the impacts that changes such as high rainfall or drought events may have on the stream/lake/river environment would enhance our understanding of how kākahi respond to environmental stress. It would also be helpful to measure other potential sources of stress in the field environment such as dissolved oxygen concentration, common pollutant levels (such as nitrogen and phosphorous), and pH. This would offer a clearer understanding of why some local kākahi populations may be more stressed than others, and if summer temperatures are a contributing factor.

Overall, the results from this chapter indicate that there was little correlation between the haemolymph concentrations of LDH, ALT, and AST at the field sites (Figures 44-46). It is difficult to say whether this was an expected result or not, due to the lack of previous studies that investigated the concentrations of all three of these markers in a wild population of bivalves. It would also be useful to repeat this study across a greater number of field sites, and to increase the sample size at each site. Several studies have measured the haemolymph concentrations of both ALT and AST in bivalve species at different temperatures (Park et al., 2009; An & Choi, 2010). However, these studies were all conducted in a controlled environment in response to a specific, elevated temperature. Therefore, it does not make sense to situate the findings from the field study within this section of the literature. Instead, the findings of the laboratory study (Chapter 4) will be compared with the findings of these field studies.

This study aimed to determine whether kākahi are experiencing physiological heat stress at current summer water temperatures in streams, rivers and lakes across the Manawatū, Rangitīkei, and Wairarapa regions. The results suggest that current summer water temperatures in 2022 occurring in these areas are not causing heat stress in the kākahi populations that reside there, at least with the markers chosen here.

Chapter 4: Laboratory experiment to investigate potential heat stress in kākahi under projected water temperatures

4.1 Abstract

Exposing aquatic animals to an elevated water temperature in a controlled environment is a common method for studying an organism's physiological response to the increase in temperature predicted to occur under ACC. In this laboratory study, LDH, ALT, and AST were measured in the haemolymph of kākahi exposed to a range of temperatures predicted to occur under ACC. Two groups of kākahi were kept at 20˚C and served as control groups. This temperature was equivalent to that of the environment they were collected from. Haemolymph was non-lethally collected from one group on Day 0 of the experiment and the other group on Day 7. Two other groups of kākahi were exposed to an increase of water temperature of either 26˚C or 32˚C and maintained at that temperature for 7 days from Day 0. This temperature increase was achieved gradually, following a recommended acclimation protocol for freshwater bivalves. Haemolymph samples were taken from each kākahi on Day 7, the last day of the temperature experiment.

LDH, ALT, or AST did not increase in kākahi exposed to 26˚C or 32˚C, in comparison to those kept at 20˚C. The results from this chapter suggest that kākahi do not experience lethal or sub-lethal heat stress following a 7-day exposure to 26˚C or 32˚C. Therefore, they are likely to be resilient to short periods of warming between 26 and 32˚C under ACC, but their response to longer periods of elevated temperatures needs to be investigated in the future.

4.2 Introduction

Collecting aquatic molluscs from their natural habitats and exposing them to predicted future water temperatures in a laboratory is a common method of investigating the physiological response and vulnerability to ACC. This has been conducted using a wide range of bivalve groups, including oysters (Park et al., 2009; Zhang et al., 2022), clams (Zhou et al., 2019), marine mussels (Coppola et al., 2018) and freshwater mussels (Falfushynska et al., 2014; Payton et al., 2016). The biomarkers, sampling technique, and tissue used to assess heat stress differs markedly between each of these studies.

A significant increase in lactate dehydrogenase (LDH) activity in the gill tissue of the duck mussel (*Anodonta anatina*) was measured in response to moderate (25˚C) and extreme (30˚C) warming (Falfushynska et al., 2014). This increase was attributed to an increase in anaerobic glycolysis, due to suppression of the aerobic energy system during heat stress. LDH is usually measured in tissues such as the gills and digestive gland, rather than in the haemolymph (Zhang et al., 2022). However, these sampling methods often have a lethal outcome for the study species. Increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) haemolymph concentrations have been found in

two freshwater mussel species (*Villosa vibex* and *Villosa lienosa*) at 30˚C (Fritts et al, 2015a). In pacific oysters, a temperature challenge (elevation) of 30˚C caused a significant increase in AST haemolymph concentration, but not in ALT (Park et al., 2009). In both studies, an increase in ALT or AST is attributed to an increase in tissue damage caused by heat-induced oxidative stress. In this study, we chose to measure LDH, ALT, and AST in kākahi haemolymph to maintain a non-lethal sampling approach. These three heat stress biomarkers have been shown to change in response to temperature challenges in several other bivalve species. A temperature challenge in this chapter refers to an elevation in water temperature. Given that previous studies have commonly measured LDH in other tissues, this study will seek to determine if there is any appreciable change in LDH levels in the haemolymph.

The laboratory study presented in this chapter was a major component of this MSc research. A pilot experiment was conducted prior to both the field study presented in Chapter 3 and lab experiment but is presented alongside the lab experiment in this chapter. This pilot study was necessary to validate our laboratory housing, feeding choices, and non-lethal haemolymph and tissue sampling protocols. Following the completion of the pilot experiment, the lab fish tanks were prepared for housing 50 kākahi for the temperature experiment. This involved collecting stream water, sediment of the type kākahi had been located in, conditioning the external cannister filters to the stream water and sediment, and establishing an algal culture to feed to the kākahi for the duration of the experiment.

Kākahi were collected from one of the previously used field sites and translocated to the lab fish tanks at Massey University, Palmerston North. The kākahi were allowed a period of acclimation, before being exposed to the treatment temperatures for the main lab experiment. The aim of the temperature experiment was to simulate potential future summer water temperatures that kākahi may experience due to ACC. In comparison to the field study, the laboratory experiment allowed us to examine the same haemolymph markers but in kākahi kept in a controlled environment. Therefore, any changes in the concentrations of the measured markers can be more confidently attributed to the change in temperature.

By measuring the concentrations of heat stress biomarkers at different water temperatures, we may be able to determine the heat stress response that kākahi may face under different emission scenarios. This is a commonly used method to predict the potential vulnerability or resilience of various bivalve species to increasing water temperatures under ACC (Park et al., 2009; Fritts et al., 2015a; Payton et al., 2016). Adapting this existing methodology for the laboratory study allowed the following research question to be answered: Do kākahi haemolymph LDH, ALT, and AST concentrations under projected water temperatures indicate resilience or vulnerability to ACC?

4.3 Materials and methods

4.3.1 Aquariums and filtration

Both the pilot study and temperature experiment were conducted in the Freshwater Laboratory in the School of Natural Sciences, Massey University, Palmerston North. This is a temperature- and light-controlled room. Kākahi were housed in four 60 L aquariums located side by-side on a bench (Figure 48). Each tank was filtered by an individual external cannister filter set to a flow rate of 1,100 L/h (Aqua One® Aquis 1200 filter). Each tank was also fitted with an adjustable Aqua One (100W, Item Code 11304; 150W, Item code 11305) or Eheim glass heater (200W, EHEIM Thermocontrol 200, Item Code 3617010) to allow the water temperature to be set. Two of the tanks were designated as control tanks that were kept at 20˚C for the duration of the experiment. The remaining tanks were assigned to one of two temperature treatment groups, one where the water temperature would be raised to 26˚C, and the other where the temperature would be raised to 32˚C. These temperatures were chosen based on different emission scenarios provided by the IPCC. A detailed explanation of these scenarios is provided in section 4.3.4.4.

The first 20˚C tank had a 100W heater installed, while the second 20˚C tank and the 26˚C tank had 150W heaters installed. The 32˚C tank had a 200W heater installed. Each tank had a digital thermometer attached to the front glass plane at the same height in the tank as the kākahi would be located (Figure 49). The temperature displayed on the in-tank thermometer was checked with a separate digital thermometer and temperature probe (MASTECH MS6514, MGL APPA Corporation, Taipei, Taiwan) to ensure the correct temperature was achieved. The room temperature was set at 18˚C and the room remained on a 16:8 light:dark cycle for the duration of the laboratory study. The tanks were initially filled with filtered, non-chlorinated reverse-osmosis (RO) water and the filters were left running for one week. The tanks were later dosed with stream water from one of the study sites, to mitigate the effects to kākahi of the low ion content of RO water, and the lack of microbial content necessary to colonise the filters. The tanks were allowed to “run in” and conditions to stabilise prior to introduction of any kākahi. Once the kākahi were translocated into the tanks, glass or polystyrene tank lids were placed on top of the tank to minimise heat loss and evaporative water loss. Aeration of the tank water was provided by placing the outlet water pipe from the filter just below the waters surface in the tank, thus introducing some degree of turbulent flow.

Figure 48: Aquarium and filtration set up in the Freshwater Laboratory. The four aquariums were set up next to each other on a lab bench, with an external cannister filter located behind each.

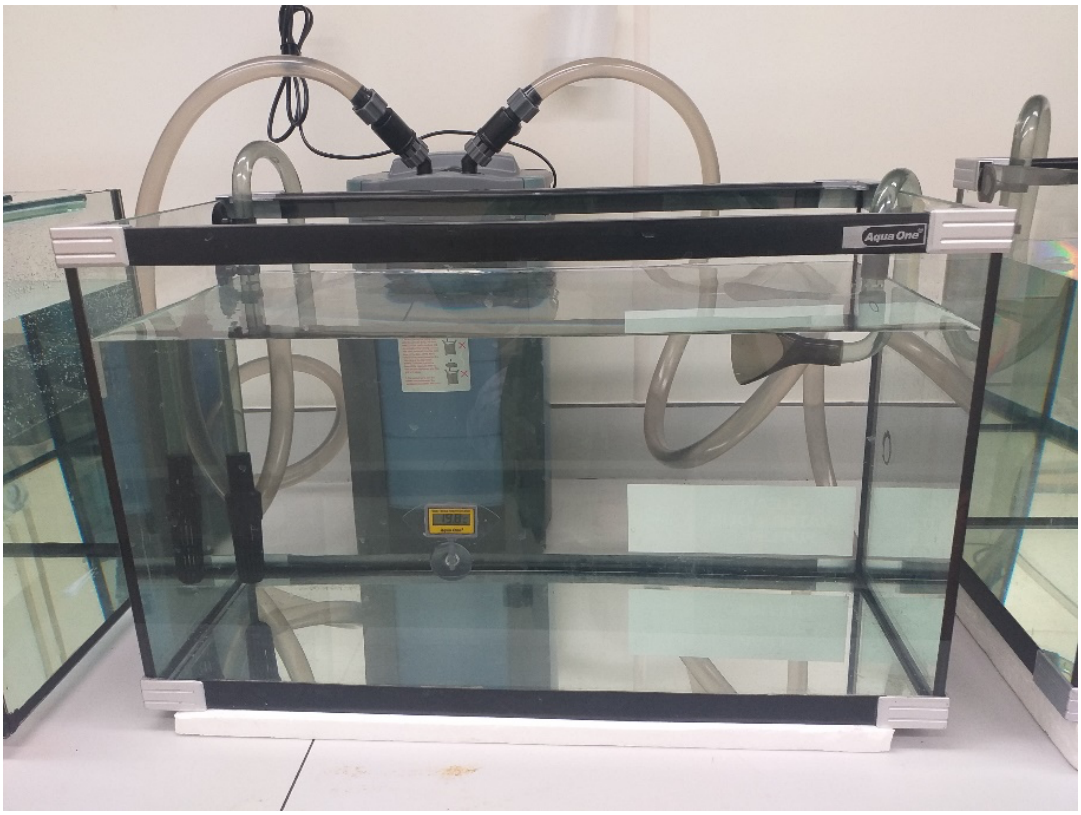

Figure 49: Individual aquarium and filter setup, with thermometer (small yellow device) shown attached to the internal surface of the front glass panel. The heater is yet to be installed in this tank.

4.3.2 The pilot experiment

At the end of November 2021, eight mature kākahi were collected from Lake Horowhenua and transported back to Massey University in a 20 L insulated bin filled with lake water. During the collection, water from the lake was also collected in a separate 20 L container. In the lab, 10 L of the lake water was used to dose one aquarium. This was done to bring the tank water closer to normal conditions for the kākahi, and to encourage the filter to establish its own microbiota. Plastic cups were placed on the bottom of the tank, each one was weighed down by a clean rock (Figure 50). Each kākahi was then placed into a cup in the tank, sitting siphon end up (Figure 51).

These kākahi remained in captivity for a 16-day period. This pilot experiment allowed us to trial the suitability of the tank system intended for the main temperature experiment and to determine that being housed and fed with yeast or algae for that period of time was not detrimental to the health and behaviour of the kākahi. We also trialled haemolymph and tissue sample biopsy techniques to validate the non-lethal nature of a 0.5 mL haemolymph sample (Fritts et al., 2015a) and small (20-30 mg) tissue biopsy (Naimo et al., 1998).

Figure 50: Plastic cups weighed down by rocks in one of the tanks, ready for kākahi translocation.

Figure 51: A kākahi from Lake Horowhenua, after being placed into one of the plastic cups in the tank. In this photo, the kākahi has extended its foot, to position itself in the cup.

Each day, kākahi were fed a suspension of ½ teaspoon of baker's yeast mixed in 250 mL of filtered RO water. Ammonia levels were monitored closely using the API® Freshwater Master Test Kit (RM002073-04-1020, API® brand, Mars Fishcare), with water changes being completed if the levels were deemed too high (≥4.0 ppm). On day 6 of captivity, a single kākahi was placed in cooled water (4˚C) and placed in a lab fridge for 15 minutes after which a 2 mL lethal haemolymph sample was taken from the anterior adductor muscle. A small drop of haemolymph was placed on a haemocytometer and observed under a microscope (Olympus IX71, Olympus, Tokyo, Japan) to check for the presence of haemocytes. Haemocytes were abundant, validating this sampling method for collecting haemolymph from the anterior adductor muscle of kākahi. This kākahi was then humanely euthanised by first chilling at 4°C for 15 min followed by −20°C for 15 min, before being dissected for our own familiarisation with their anatomy. Three individual samples of the mantle, posterior adductor muscle, gill, anterior adductor muscle, and two different parts of the foot (distal margin/tip of foot; base of foot) were taken and snap frozen in liquid nitrogen before being stored at -80˚C.

On day 8 of captivity, 0.5 mL haemolymph samples were taken from the remaining seven kākahi, following the protocol outlined in Chapter 3 (section 3.3.2). These kākahi were not chilled prior to haemolymph sampling. All kākahi survived this process and began siphoning (feeding) shortly after being returned to the fish tank, validating our sampling method as non-lethal in the short term. To validate our tissue biopsy technique, a small (24 mg) foot snip was taken from one of the kākahi, and a small (15 mg) mantle snip from

another kākahi, both undertaken on day 12 of captivity. Briefly, a wooden wedge was used to gently part the valves and a small pair of forceps and curved iris scissors used to gently grip and snip a small amount of foot or mantle tissue. Both kākahi survived for the remainder of the pilot study and demonstrated siphoning behaviour when observed shortly after being returned to the fish tank. This validated the short-term non-lethality of our tissue sampling method.

Unfortunately, after 14 days of captivity, two kākahi died. Neither had been subject to foot or mantle tissue sampling. A post-mortem showed that the deceased kākahi had poor body condition and their gills exhibited an abundance of filtered yeast. Based on this, we believe that these deaths were caused by a nutritional deficiency or imbalance associated with the yeast diet. The yeast suspension was deemed unsuitable as a diet for kākahi. From this point on it was decided (following consultation with a scientist familiar with long-term maintenance of kākahi in captivity) we would only feed kākahi cultured single-celled algae for the temperature experiment. The remaining kākahi were humanely euthanised after 16 days of captivity.

4.3.3 Culturing and feeding algae

Kākahi were fed single-celled algae for the temperature experiment. Algal cultures were grown in the lab under the guidance of algal scientist Dr Maxence Plouviez. It was decided that a single species of single-celled freshwater algae (*Chlorella vulgaris*) would be grown in culture to feed the kākahi during the temperature experiment. This species was selected due to it being ubiquitous in clean Aotearoa New Zealand waterways and due to its hardiness and reliability of being less prone to culture crashes that would limit the food supply for the kākahi therefore interrupting the experiment (*pers. comm.* Maxence Plouviez, 2022). *C. vulgaris* cultures were established six weeks prior to kākahi being translocated to the lab.

Blue-Green medium (BG-11) for *C. vulgaris* culture:

C. vulgaris were cultured on BG-11 media (Andersen et al., 2005). BG-11 media was created from five stock solutions as needed. The chemicals required for each stock solution, their concentration, and the required volume of each stock solution are provided in Table 2.

Table 2: BG-11 media stock solutions A-D and trace element. The chemicals needed in each solution, the required concentration of each in the stock, the required volume of each stock solution, and the final concentration of each chemical in the BG-11 media.

Stock solution	Chemicals needed	Concentration in stock (g/L)	Volume of stock (mL) required for 1L of BG-11	Final concentration in BG-11 (g/L)
Stock A	$NaNO_3$	30	50	1.5
	K_2HPO_4	62		3.1
	KH_2PO_4	30.4		1.52
Stock B	$MgSO_4$, $7H_2O$	7.5	10	0.075
Stock C	$CaCl_2$, $2H_2O$	3.6	10	0.036
Stock D	Citric acid	6	1	0.006
	Ferric ammonium citrate	6		0.006
	Na_2EDTA, $2H_2O$	0.98		0.00098
Trace element	H_3BO_3	2.86	1	0.00286
	$MnCl_2$, $4H_2O$	1.81		0.00181
	$ZnSO_4$, $7H_2O$	0.222		0.000222
	$CuSO_4$, $5H_2O$	0.079		0.000079
	Na_2MoO_4, $2H_2O$	0.39		0.00039
	$CoCl_2$, $6H_2O$	0.0404		0.0000404

Before preparing the stock solutions, all glassware was sprayed with 70% ethanol and rinsed three times with distilled water before being wiped dry with paper towels. To prepare stock solution A, three beakers were labelled with the name of each chemical and the amount that needed to be weighed. Then, 30 g of $NaNO_3$, 62 g of K_2HPO_4, and 30.4 g of KH_2PO_4 were weighed out separately into their own beakers (Table 2).

A magnetic stirrer was then placed in each beaker along with some distilled water. Each chemical was dissolved in the water using a stirring plate. Once dissolved, the magnetic stirrers were removed using a magnetic rod. The first chemical solution was then poured into a 1 L volumetric flask, followed by some distilled water. This was repeated for the other two chemicals. The flask was then filled to the 1 L line with distilled water. A seal was placed on the top and the solution was gently mixed by inversion. Then, the finished stock solution was transferred into a 1 L Duran bottle and sealed with the bottle cap. The

bottle was labelled with the solution name, the individual chemicals present in solution, name, and date.

The same process was repeated for stock solutions B, C, D, and trace element, using the chemical quantities stated in Table 2. Stock solutions A, B, C, and D (not trace element) were then autoclaved before being used to make up BG-11. Once the solutions had been autoclaved and cooled, 2 L of BG-11 media was made up. Table 2 states the volumes of each solution required to make 1 L of BG-11. A 2 L culture was required for efficient feeding of the kākahi, so these volumes were doubled to make 2 L of BG-11.

To make up 2 L of BG-11, 100 mL of Stock A was added to a 2 L volumetric flask, using a measuring cylinder. Then, 20 mL of Stock B was added, using a 10 mL pipette twice. The pipette tip was rinsed with distilled water, before adding 20 mL of Stock C. The tip was rinsed again, before adding 2 mL of Stock D. The tip was rinsed once more, before adding 2 mL of trace element solution. The flask was then filled to the 2 L line by adding distilled water. The finished media was autoclaved and allowed to cool overnight.

Inoculating the algae culture into the prepared media

Once the 2 L bottle of media had been autoclaved and cooled, it was ready to be distributed into smaller flasks that had been cleaned with ethanol and rinsed with distilled water. The algae were kept growing in several flasks at once, so that a backup was available should a culture crash. The steps below were followed to inoculate and grow the required culture of *Chlorella vulgaris*. A pre-existing culture of *C. vulgaris* was used for the initial inoculation.

Firstly, 50 mL of BG-11 was measured using a measuring cylinder and poured into a 250 mL conical flask. The top of the flask was covered with a cotton top and the neck of the flask was labelled with BG-11, date, and name. The cotton top was then covered with tin foil and autoclave tape. The flask was then ready for autoclaving before use. Following this, 150 mL of media was measured and poured into a 500 mL flask. The steps above were repeated to cover and autoclave the flask. The flasks were then ready for inoculation.

10-20% of an existing culture, which worked out as approximately 5.6 mL, was inoculated into the 250 mL flask using a 10 mL pipette. This was done next to a Bunsen burner, so that the neck of the flask could be waved over the burner before putting the cotton top back on, limiting the risk of contamination. This process was repeated to inoculate 16.7 mL into the 500 mL flask. Both flasks were then placed in the shaking incubator (at 25 ± 1˚C under continuous agitation (165 rpm), constant illumination (40 µmol·photons·m-2·s-1 at the culture surface, and in an atmosphere of 2% (vol) CO_2 in air)) and left to grow.

After approximately seven days, 200 mL of culture from the flasks was inoculated into the 2 L Duran bottle of media and attached to a bubbler (air), to accelerate algal growth (Figure 52). At the same time, another 250 mL flask was inoculated as a backup. After 7-14 days (based on a dry cell weight and colorimetric for chlorophyll measurement), the 2 L bottle of algae culture was ready for centrifuging and resuspension prior to feeding.

Figure 52: 2 L Duran bottle of *Chlorella vulgaris* culture, attached to the bubbler, growing in the microbiology lab.

Centrifuging and resuspending the algae culture

Before centrifuging the 2 L culture, a 10 mL pipette was used to remove 6 mL of culture to measure dry cell weight and absorbance. These were necessary for approximating the quantity of algae in the culture and, therefore, for calculating the quantities for feeding. To centrifuge and resuspend a 2 L culture, the culture was divided between four 500 mL centrifuge bottles.

The bottles were then placed into a high-speed refrigerated centrifuge (Hitachi himac, CR 22GII) and centrifuged at 230 g for 15 minutes. Following centrifugation, the supernatant was discarded leaving the algae pellet in each bottle. 100 mL of distilled water was then added to each bottle and vigorously shaken to resuspend the algae in the water. All resuspended algal solutions were then poured into a clean 500 mL Duran bottle and labelled. The algae solution was stored at 4˚C.

Calculating rates of feeding for kākahi

Each tank containing kākahi was fed approximately 3.1×10^9 cells of algae per day. This quantity was calculated based on an optimum range of algal density for feeding of between 20-100×10^3 cells/mL (*pers. comm.* Karen Thompson, National Institute of Water and Atmospheric Research, Hamilton, New Zealand) and based on the volume of the tanks.

To calculate the volume of algae needed based on cell count, a measure of optical density (absorbance) was made of the resuspended algal culture following the centrifugation to remove BG-11. Briefly, 1 mL of algae culture was placed in a spectrophotometer tube. 2 mL of distilled water was added, to give a 1 in 3 dilution. The spectrophotometer was set at 683 nm, to give an absorbance reading for chlorophyll. Based on previously validated findings from Plouviez (2017), cell number per mL can be approximately inferred from optical density value. Once cell number per mL was known, this was then multiplied by 500 mL (the total volume of the resuspended culture) to give total cell number in 500 mL. Once this was known, the volume needed for each tank, per day was calculated.

Feeding volumes of subsequent batches of algae were calculated based on ongoing dry cell weight (DCW) rather than cell number. This was because DCW is a more accurate measurement of biomass than estimating cell number based on optical density.

Calculating the dry cell weight of the algal culture

Briefly, 0.45 µm filter papers were marked with a pencil and then placed on a piece of aluminium foil, with the edges slightly lifted to stop the papers falling off. Small forceps were used to handle the filter papers at all times to avoid contamination. The foil with the papers was carried in a desiccator to a heating oven. The filter papers (on aluminium foil) were placed in the oven for 24 hours at 105˚C. After 24 hours, the papers and foil were placed back in the desiccator for transport to an analytical balance. Each filter paper was weighed, and the weight was recorded. The paper was kept in the desiccator until use.

For measuring the DCW, a dry filter paper was inserted into a Büchner funnel, which was then placed on a Büchner flask and vacuum was applied. An aliquot of 2.5-5 mL of resuspended algae was then pipetted onto the filter paper and left until all liquid was drawn into the flask under vacuum. The filter was then rinsed with distilled water. The paper was then placed back in the desiccator and carried back to the oven, to be left in the oven at 105˚C for 1 hour. The paper was then weighed (using the same balance) and the initial paper weight was subtracted from the paper + algae weight to give the dry cell weight for a known volume of medium.

As an example of the workflow, our first batch of *C. vulgaris* culture used to feed the kākahi, had a DCW of 0.609 g in a 500 mL bottle. Based on cell count calculations, we fed each tank of kākahi 19 mL of culture per day. This was calculated to be 0.02387 g

(24 mg) of algae per tank, per day. The volume fed from subsequent batches of *C. vulgaris* culture were adjusted as needed based on DCW measurements, to ensure that approximately 24 mg of algae was fed to each tank of kākahi every day of the experiment.

4.3.4 Temperature challenge experiment: Climate change simulation

4.3.4.1 Preparation, collection, and translocation

The Freshwater Lab was prepared for the translocation of 50 kākahi for the temperature experiment. Sediment was collected from the Manga-o-tane Stream in Moonshine Valley, Aokautere. This included a mix of fine silt and gravel. These two sediment types were combined and placed on the base of all four empty tanks and levelled to an even depth of 5 cm (deep enough for kākahi, H. Rainforth *pers. comm.*). The tanks were then filled with 25-30 L of chlorine-free filtered RO water. An additional 20-25 L of stream water, from the waterway where the kākahi were collected from, was added to each tank and allowed to circulate with filtration for 2 weeks, allowing the filter and tank microbiota to develop and stabilise well before introducing the kākahi (Figure 53). The filters were not activated for the 24 hours after the sediment and water was added, this allowed the sediment to settle, therefore minimising clogging of the filters.

The heaters in each aquarium were tested to make sure the water temperature could be maintained at the experimental temperatures (26˚C and 32˚C). Once this was confirmed, the heaters were switched off, and the water allowed to stay at an ambient 20˚C (heat from the filters kept each tank at this temperature despite the room temperature being set to 18˚C).

Two weeks after sediment was added to the tanks, 50 kākahi were collected from the field. An initial manual survey of the field site indicated >100 kākahi for a 50 m stretch of the stream, similar densities were found 100 m down- and upstream of the 50 m stretch where kākahi were collected from. Only kākahi that were >4 cm in length were collected. During collection, kākahi that met the size requirement were put in a catch bag, which remained in the stream until 50 suitable kākahi were found (Figure 54). Fifty kākahi were required because there were four groups of kākahi required for the temperature experiment, two control (n=12 each) and two treatment (n=13 each) groups. The experimental treatments are explained in greater depth in section 4.3.4.4.

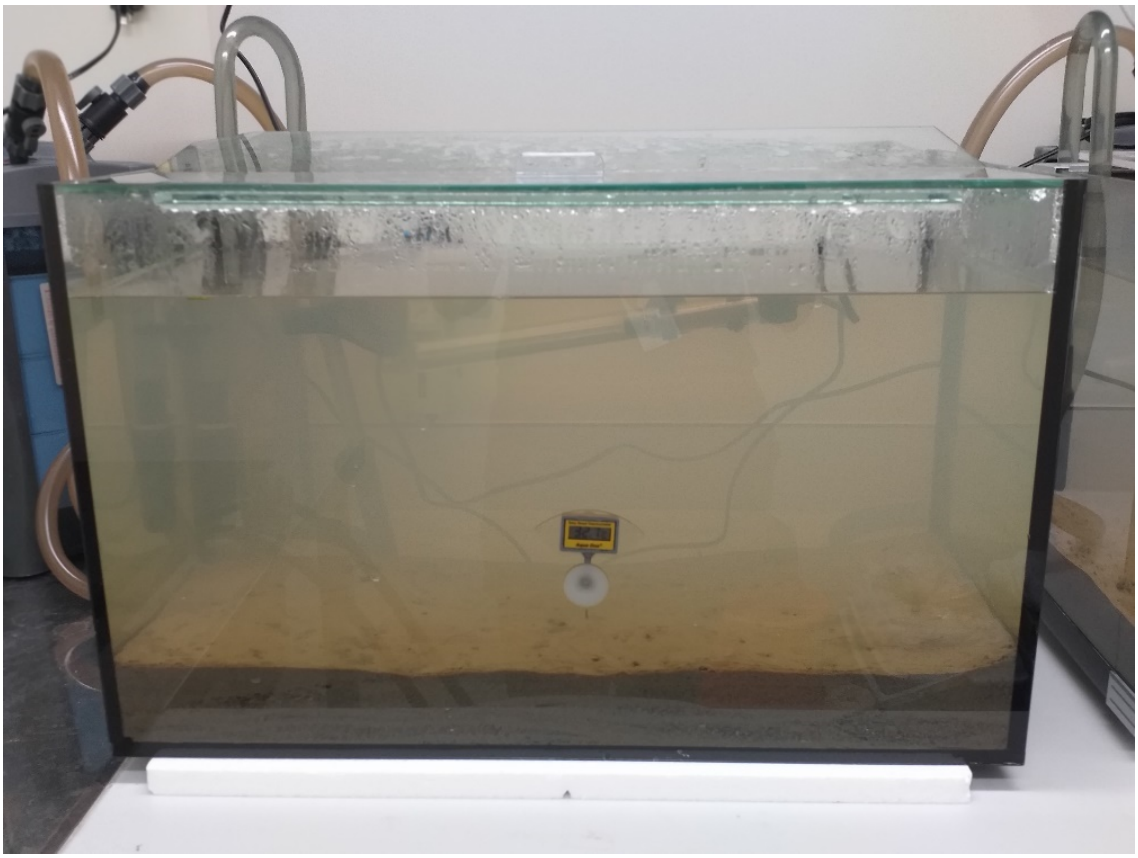

Figure 53: What the tanks looked like after adding sediment and being filled with a mixture of chlorine-free filtered RO water and stream water. The water began to look much clearer 24 hours after this photo was taken, as the fine sediment suspended in the water column settled. Following this period, the filters were switched on and left on for the microbiota to develop and mature.

Figure 54: Representative photo of the collection site from which the kākahi used in the lab experiment were taken. All kākahi were collected along a 10 m length of the stream, each kākahi was placed in the catch bag (blue bag) until all 50 animals were found.

Once 50 kākahi were found and placed in the catch bag, they were transferred into two 20 L insulated bins (25 in each) filled with stream water, for transportation back to the lab. Once back in the Freshwater Lab, the kākahi were placed siphon side up in the sediment of the tanks (Figure 55). Twelve kākahi were placed in each of the two 20˚C tanks, and 13 in the 26 and 32˚C aquariums. Kākahi were randomly assigned to tanks but care was taken to ensure an even distribution of sizes were present in each treatment.

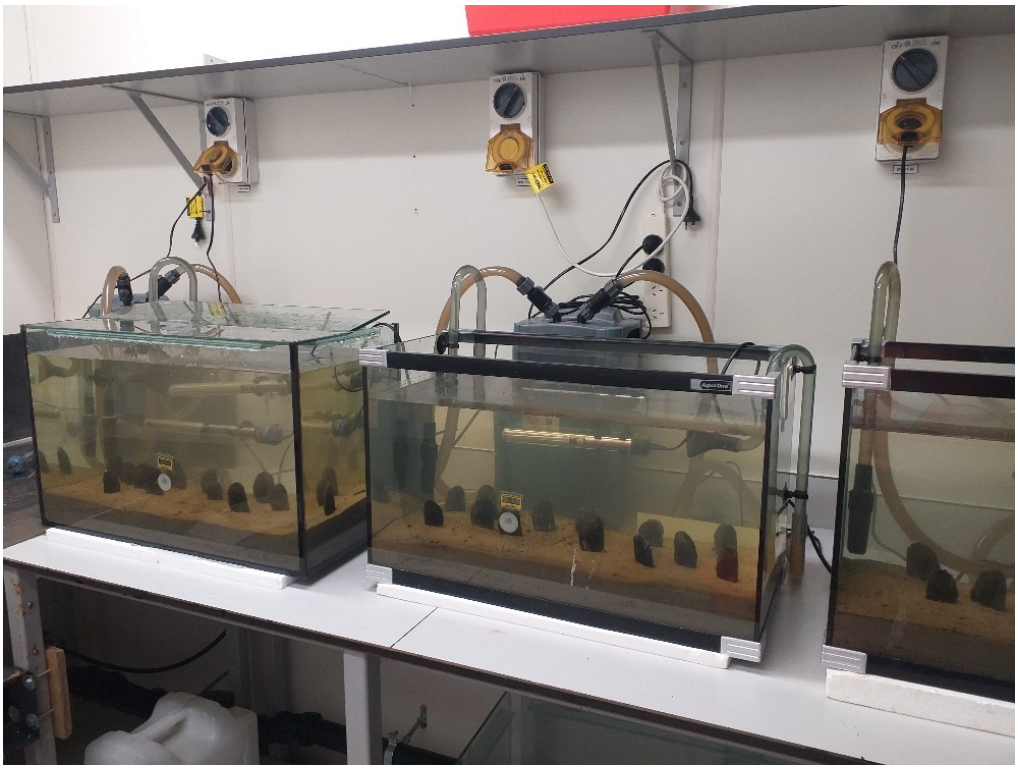

Figure 55: Kākahi immediately after being placed in the sediment of the tanks.

Within 20 minutes of being placed in the tanks, many kākahi had repositioned themselves, with many partially or fully submerging themselves in the sediment and demonstrating siphoning (Figure 56).

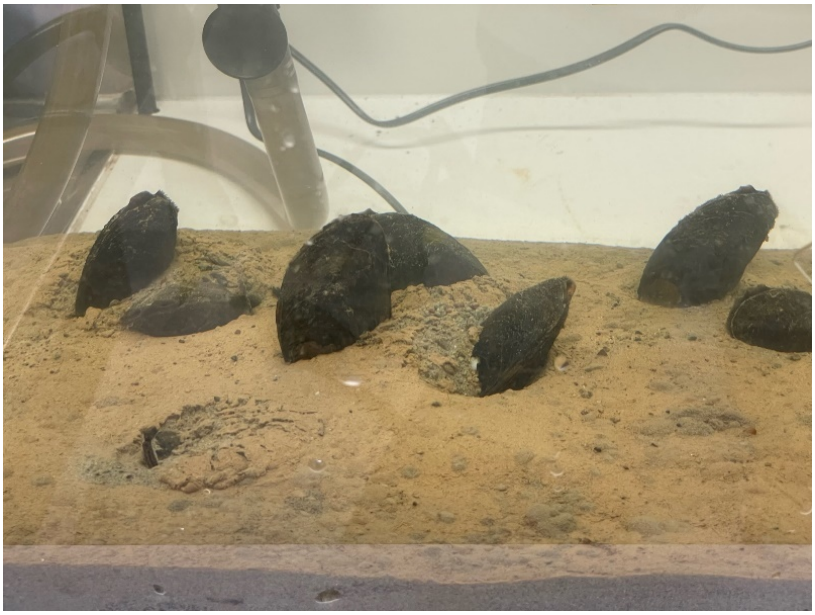

Figure 56: Shortly after translocation, the kākahi began to move around and reposition themselves in the sediment. Some changed which edge of the body was settled on the sediment, while others burrowed down so that some or most of the animal was submerged.

4.3.4.2 Acclimation

The kākahi were allowed to acclimatise for 9 days prior to any change in tank temperature. Each tank was kept at 20˚C. Each tank was dosed daily with a precise amount single-celled algae suspended in chlorine-free filtered RO water (see section 4.3.3). The external filters were switched off for a 3–4 hour period during feeding to minimise the loss of algae to the filter and allow the algae to remain in the water column for kākahi to filter feed.

4.3.4.3 Environmental monitoring

The ammonia, pH, and nitrate levels in each tank were monitored on alternate days to ensure these variables were not adding a source of stress for the kākahi. This was achieved using the API® Freshwater Master Test Kit (RM002073-04-1020, API® brand, Mars Fishcare). The colonising microbiota in the external filters kept ammonia levels low (≤0.25 ppm) in all tanks throughout the temperature experiment. Nitrate levels also remained low (5.0 ppm) in all tanks over this period. The pH of all tanks remained between 7.2 and 7.6 for the length of the experiment.

4.3.4.4 Increasing the temperature of the tanks

After nine days of acclimation to the tank conditions, the temperature treatments began. The two experimental tanks were raised to their target temperatures over a period of 12 hours, at a rate of 1˚C of increase per hour. This rate was chosen based on the recommendations given in the ASTM International Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels. These guidelines state that temperature should be increased by no more than 3˚C per hour (Ingersoll et al., 2006). To avoid mortality due to acute heat shock, a rate on the lower end of this recommendation was selected. The tanks remained set at the experimental temperatures for seven days. This period was selected so that the physiological response to a chronic temperature challenge could be investigated. Acute temperature exposures are considered to be up to 48 hours, while a chronic exposure could be any period >48 hours (Payton et al., 2016). Some studies investigated biomarker concentrations after 14 days (Falfushynska et al., 2014) and others after three or seven days (Fritts et al., 2015a). For this study, seven days was selected as a mid-range period of chronic exposure compared to previous studies. Tanks 1 & 2 remained at 20˚C for this period, tank 3 was raised to 26˚C, and tank 4 to 32˚C (Table 3). Following the seven-day temperature challenge, tanks 3 and 4 were gradually returned to 20˚C over 12 hours to allow the kākahi to reacclimatise to the pre-challenge temperature.

Table 3: Thermal regime of laboratory temperature experiment. Tank 1 was the baseline control, used to measure haemolymph markers prior to the seven-day temperature challenge. Tank 2 was the second control, used to distinguish between the temperature-challenge and any other potential stress caused by being in captivity for an additional seven days. Kākahi in tanks 3 and 4 were exposed to the experimental temperatures. These aimed to simulate a potential future scenario that kākahi could experience under ACC, based on different emission situations.

Tank number	Tank 1	Tank 2	Tank 3	Tank 4
Treatment	Control 1 – baseline concentrations before 7-day experiment began.	Control 2 – captivity related stress following 7-day experiment.	Experimental temperature: ACC scenario 1	Experimental temperature: ACC scenario 2
Temperature	20˚C	20˚C	26˚C	32˚C

Tank 1 remained at 20˚C as the baseline control group. The kākahi in this group were sampled at the end of the nine days of acclimation, to give a baseline level of the measured heat stress markers at the acclimation temperature. Tank 2 also remained at 20˚C as a second control group. The kākahi in this tank were sampled at the end of the seven-day experimental period. This allowed any additional stress that the kākahi may have been experiencing due to an additional week in captivity to be controlled for. Concentrations of stress biomarkers have been found to increase in wild mussels after a period of captivity, even if they were not exposed to additional stressors such as elevated temperature (Boutet et al., 2022). Having this second control group allowed greater confidence that any changes in the heat stress markers between this group and the two experimental temperature groups could be attributed to the increase in temperature.

The temperatures that tanks 3 and 4 were raised to were chosen carefully, based on current local temperature data and predictions for future water temperatures, based on different IPCC emission scenarios (Figure 57).

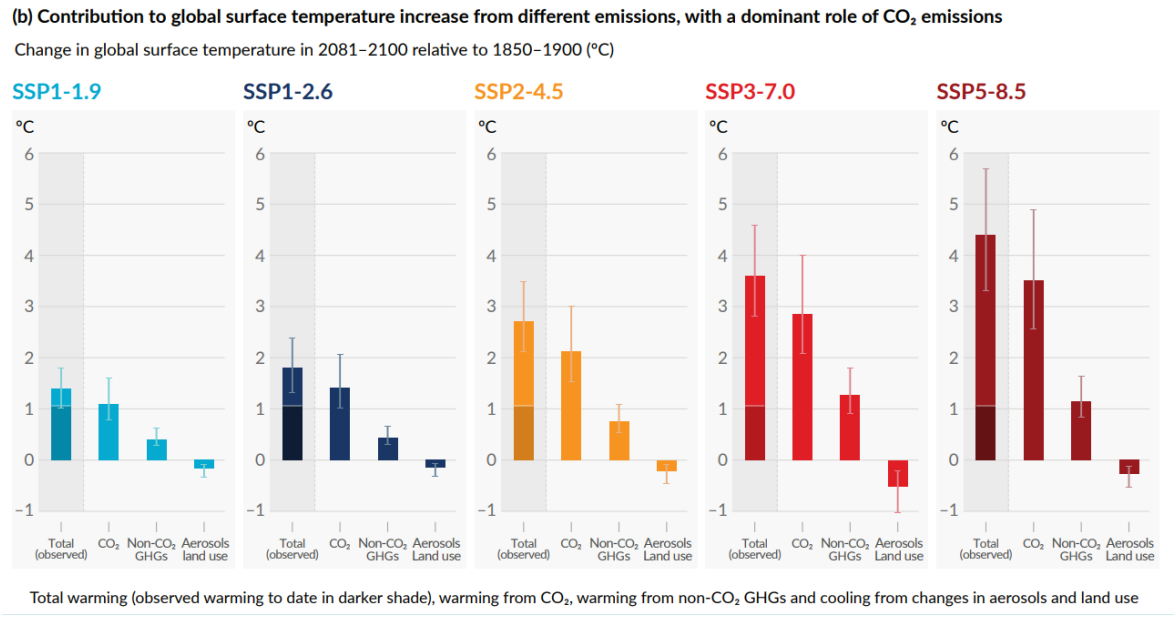

Figure 57: Possible changes in global surface temperature in 2081-2100 given by the IPCC, based on different emission scenarios. The four scenarios are given as SSPx-y, which refers to the Shared Socio-economic Pathway. This model is based on the understanding that the level of emissions over the next 80 years depends on the global socio-economic situation. The 'x-y' refers to "the approximate level of radiative forcing (in watts per square metre, or $W.m^{-2}$) resulting from the scenario in the year 2100" (IPCC, 2021).

The temperatures chosen were based on the total observed warming from IPCC's SSP scenarios (Figure 57). These values take into account all contributors to global warming but focus on CO_2 emissions as the dominant factor. These scenarios were used rather than the previously described IPCC RCP pathways (Chapter 2, Figure 2), because they consider the likelihood of emission reductions occurring, based on which climate policies are or are not implemented. Under SSP1-1.9, the 'best case scenario', emissions are expected to peak between 2040 and 2060, then plateau and reduce as previously implemented climate policies start to take effect. Under this SSP, average global temperature is expected to be 3˚C above pre-industrial temperatures.

Tank 3, which was raised to 26˚C, represents a moderate warming scenario, based on SSP2-4.5 (Figure 57). This temperature represents two scenarios for local kākahi. Firstly, kākahi in Lake Horowhenua are already regularly experiencing temperatures of 26˚C in the summer months (LAWA, 2022). Secondly, this temperature represents a potential water temperature that could be experienced by kākahi in the field stream, under SSP2-4.5. The river, of which the field stream is a tributary, experiences maximum summer temperatures of 24.5˚C (LAWA, 2022). Under SSP2-4.5, this could increase to between 25.6 and 27˚C by 2100. The experimental temperature of 26˚C was chosen as a middle point between these values. The concentrations of heat stress markers detected in kākahi housed in tank 3 after the seven-day experiment, may be indicative of what we would expect wild kākahi in the field stream to be experiencing under SSP2-4.5. The results, therefore, give insight into how moderate warming may affect kākahi physiology.

Tank 4, which was raised to 32˚C, simulates the temperature that some local kākahi may experience by 2100 under SSP5-8.5, which is the highest emission scenario that the IPCC has modelled (Figure 57). At present, Lake Horowhenua, and the kākahi that live in it, experiences temperatures of up to 28˚C in summer (LAWA, 2022). Under SSP5-8.5, this ecosystem is expected to continue warming to temperatures between 30.2 and 32.6˚C by 2100. Therefore, the experimental temperature of 32˚C was chosen as the highest likely temperature that local kākahi could experience by 2100. The results would therefore give insights into the question of whether kākahi could survive the 'worst case scenario' if sufficient climate policies are not implemented in the coming years. The concentrations of measured heat stress markers help determine how severely their physiological function may be affected, and whether this may have long term consequences for population survival and ecosystem health.

4.3.4.5 Sampling and analysis

Haemolymph samples were taken from all 50 kākahi following the protocol that was described in Chapter 3 (3.3.2). Samples were collected from tank 1 (baseline control) kākahi on day 0, before the temperature treatments began, to measure baseline concentrations of the heat stress markers. Samples were collected from kākahi in tanks 2, 3, and 4 after the seven-day experimental period. All samples were collected within the same two-hour period. The cryovials of haemolymph were kept at -80˚C until sample analysis could take place. Haemolymph samples were assayed for LDH, ALT, and AST as previously described in section 3.3.3.

Small tissue biopsies were also taken from four kākahi in each tank, at the time of haemolymph sampling. Once the haemolymph sample had been collected, the wooden wedge was left inside the shell valves to allow access to the foot of the kākahi. Using small dissection scissors, a 20-30 mg sample of the foot tissue was taken. These tissue samples were placed directly into RNA*later*® solution (Ambion, AM7021), before being placed in the -80˚C freezer for later analysis.

4.3.4.6 Statistical analysis

A one-way ANOVA was used to test the differences in mean LDH, ALT, and AST concentrations between the four groups of kākahi in the lab experiment. A post-hoc Tukey HSD test was also used to compare the second control group (Day 7, 20˚C) with the 26˚C and 32˚C treatment groups. A simple linear regression was used to test the correlation between the markers. All tests were conducted in RStudio (RStudio® Desktop, Windows Version 2202.07.1-554, Boston, MA) using the aov(), TukeyHSD(), and lm() functions. All data shown in graphs are displayed as means ± standard error of the mean (SEM).

4.4 Results

All 50 kākahi survived transport, acclimation, and the seven-day temperature experiment. No mortalities occurred at either of the treatment temperatures. The mean haemolymph concentrations of LDH, ALT, and AST were compared between each group of kākahi in the lab experiment (Figures 58-60).

The mean LDH haemolymph concentrations were similar in both control and treatments groups of kākahi (Figure 58). The 32˚C group appears to have a slightly higher LDH concentration, but the standard error of the mean is large, as the error bars overlap with the concentrations of the other three groups. The second control group (Day 7; D7 20˚C) had a LDH concentration that was higher than the baseline control. The 26˚C treatment group showed a slightly lower mean LDH concentration than the second control and was very similar to the baseline control group. The one-way ANOVA showed that the difference in mean LDH between the lab groups was not significant at 5% ($p = 0.82$). The post-hoc Tukey HSD test showed there was also no significant difference between mean LDH in the second control group (D7 20˚C) and the 26˚C group ($p = 0.98$) or between the second control group and the 32˚C group ($p = 0.97$).

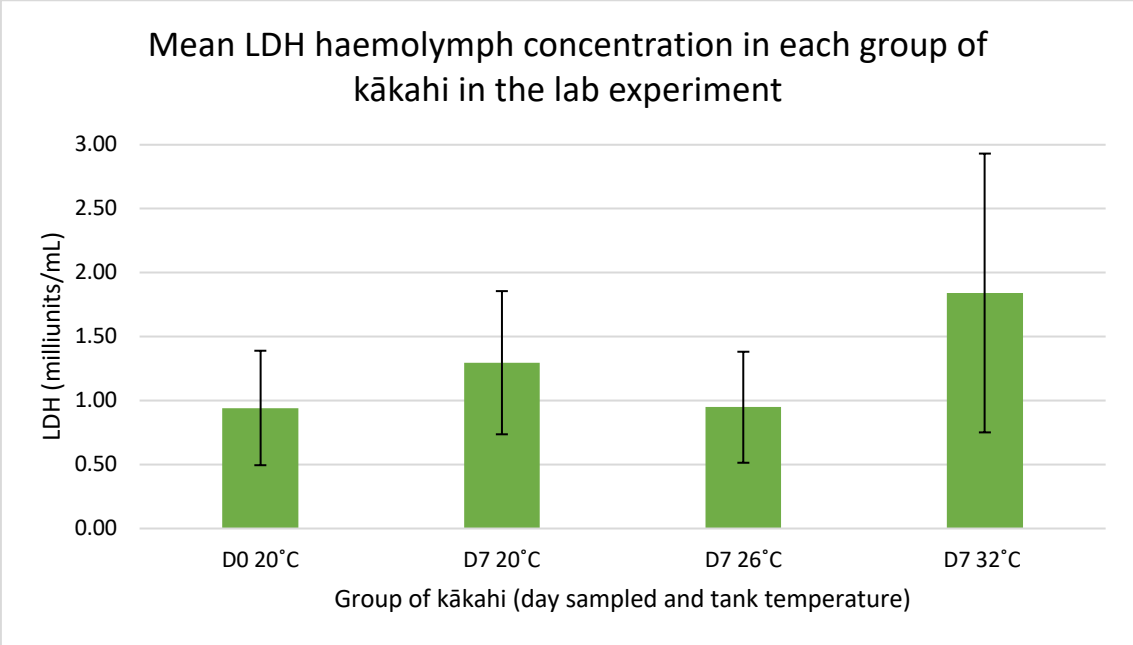

Figure 58: The mean haemolymph LDH concentration (in milliunits per mL) in samples taken from each group of kākahi in the main temperature experiment in the laboratory. The group name under each bar includes which day of the experiment the samples were taken (Day 0 or Day 7) and the water temperature they were exposed to. All data are mean ± standard error of the mean (SEM).

Mean ALT haemolymph concentrations were very similar between the lab groups (Figure 59). The one-way ANOVA showed that there was no significant difference in mean ALT concentration between the four groups ($p = 0.21$). The post-hoc Tukey HSD test showed

there was also no significant difference between the mean ALT in the second control group (D7 20˚C) and the 26˚C group ($p = 0.95$) or between the second control group and the 32˚C group ($p = 0.68$).

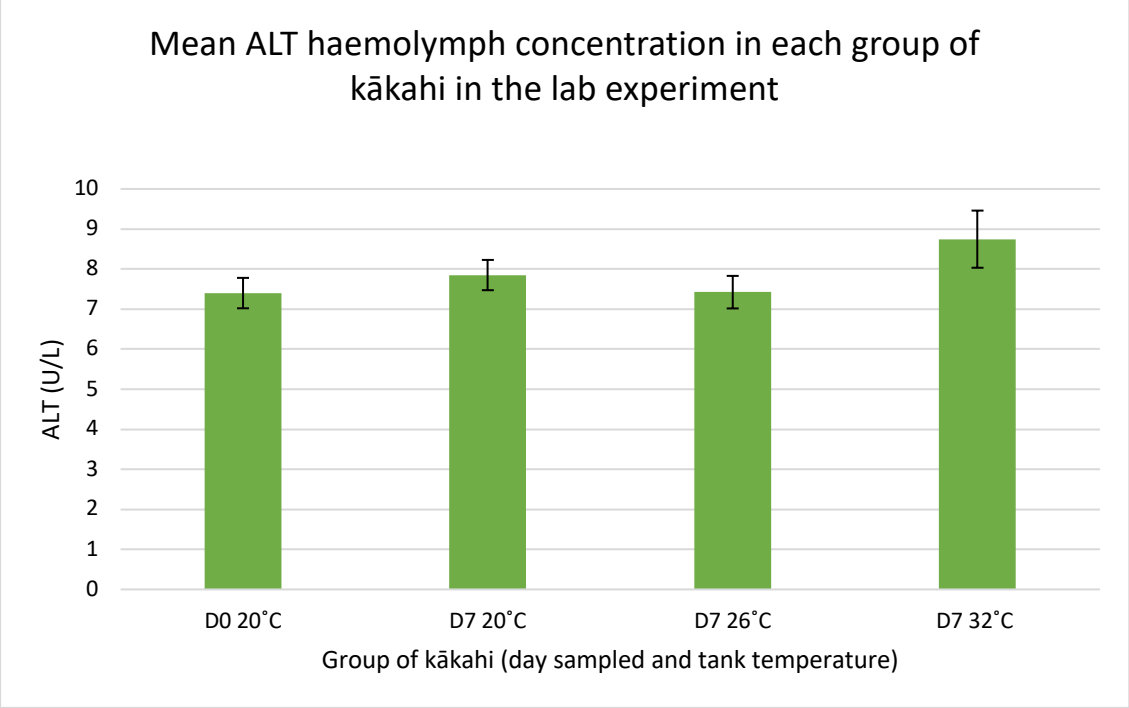

Figure 59: The mean haemolymph ALT concentration (in units per litre) in samples taken from each group of kākahi in the main temperature experiment in the laboratory. The group name under each bar includes which day of the experiment the samples were taken (Day 0 or Day 7) and the water temperature they were exposed to. All data are mean ± SEM.

Mean AST haemolymph concentrations were similar amongst the treatment groups (Figure 60). The 32˚C group had a higher AST concentration compared to the first control group (D0 20˚C) but not the second control group or the 26˚C group. The one-way ANOVA showed that there was not a significant difference in mean AST concentrations between the four groups ($p = 0.20$). The post-hoc Tukey HSD test showed there was also no significant difference between the mean AST in the second control group (D7 20˚C) and the 26˚C group ($p = 0.89$) or between the second control group and the 32˚C group ($p = 0.93$).

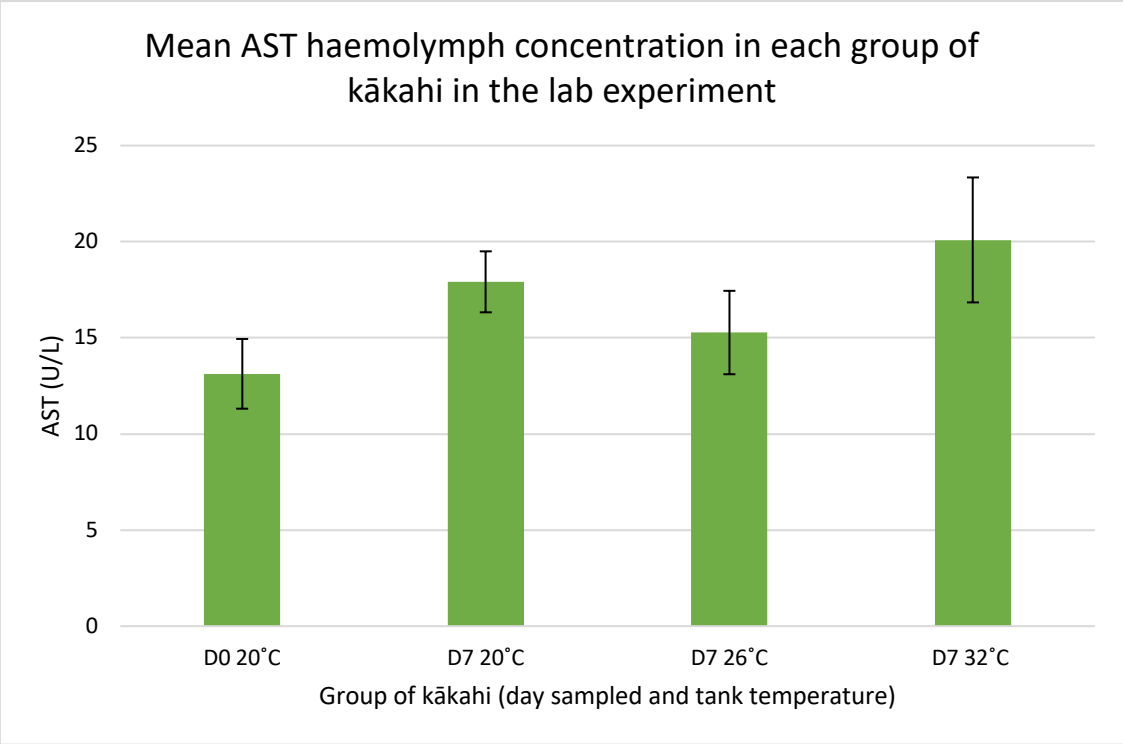

Figure 60: The mean haemolymph AST concentration (in units per litre) in samples taken from each group of kākahi in the main temperature experiment in the laboratory. The group name under each bar includes which day of the experiment the samples were taken (Day 0 or Day 7) and the water temperature they were exposed to. All data are mean ± SEM.

After looking at the mean concentrations of each marker in the four lab groups, the concentrations of LDH, ALT, and AST in all 50 individual kākahi in the lab experiment were grouped by temperature treatment. These results are presented below in Figures 61-63.

The spread of LDH concentrations is similar among the two control and two treatment groups (Figure 61). The 32˚C treatment group had one kākahi with a much higher LDH concentration than the rest in that group and those in other groups. Excluding that one outlier, all groups had a range of LDH concentration between <1 and 7 milliunits/mL.

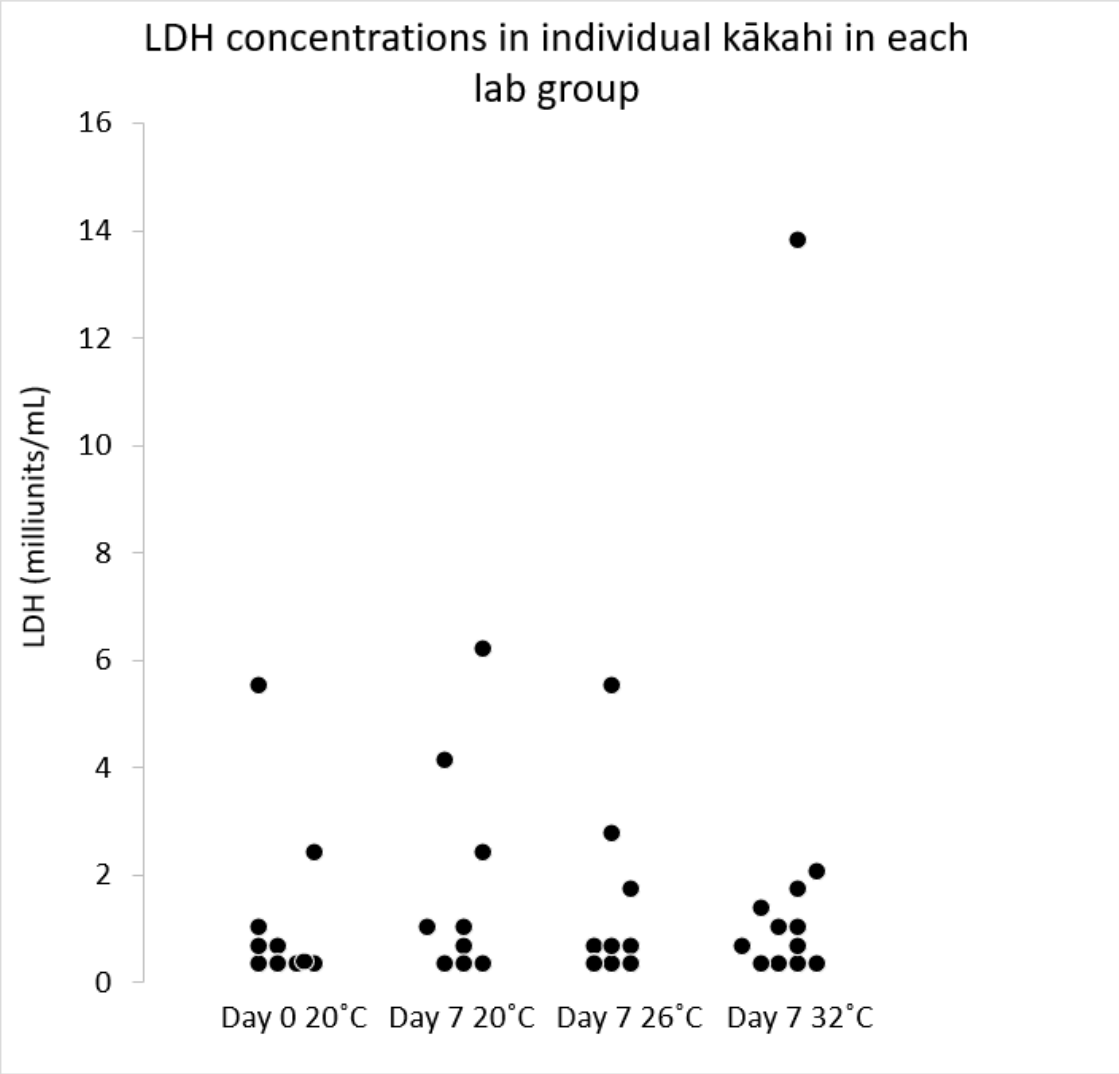

Figure 61: LDH concentrations (in milliunits per mL) measured in the haemolymph of each individual kākahi in the four lab groups.

The 32˚C lab group had several kākahi with ALT concentrations that were elevated compared to the two control groups (D0 and D7 20˚C) (Figure 62). ALT concentrations were very similar among kākahi in the two control groups and the 26˚C treatment group, with all kākahi having concentrations of 6-11 U/L. The exception was one kākahi in the 26˚C group having a slightly higher ALT concentration that was comparable to the three kākahi in the 32˚C group with elevated ALT concentrations.

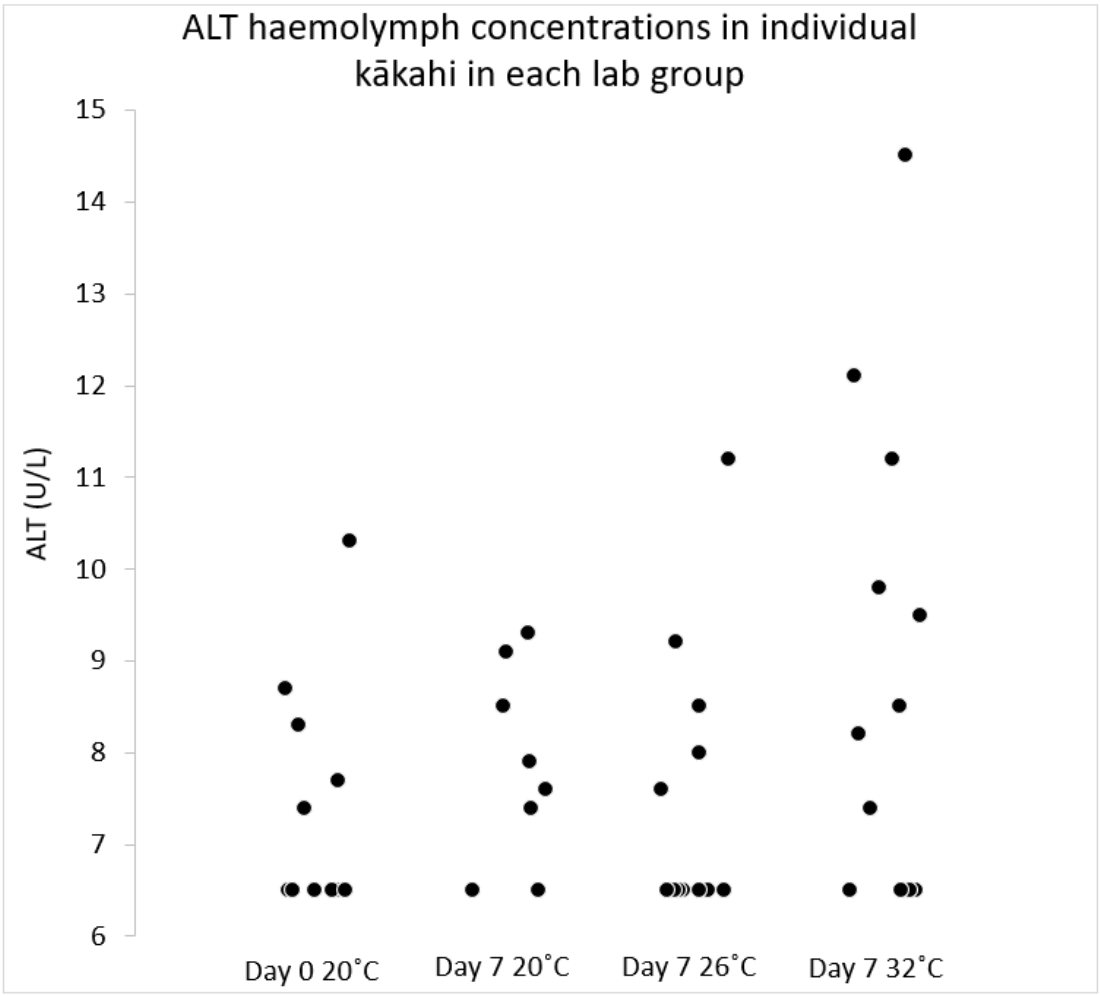

Figure 62: ALT concentrations (in units per litre) measured in the haemolymph of each individual kākahi in the four lab groups.

Similar to the individual concentrations of ALT (Figure 62), there were several kākahi in the 32˚C group that had higher AST concentrations than kākahi in any of the other groups (Figure 63). One kākahi in the 26˚C group also had a higher AST concentration than any of the kākahi in either of the control groups. For the majority of kākahi however, all groups had a variety of individual AST concentrations between 6 and 27 U/L.

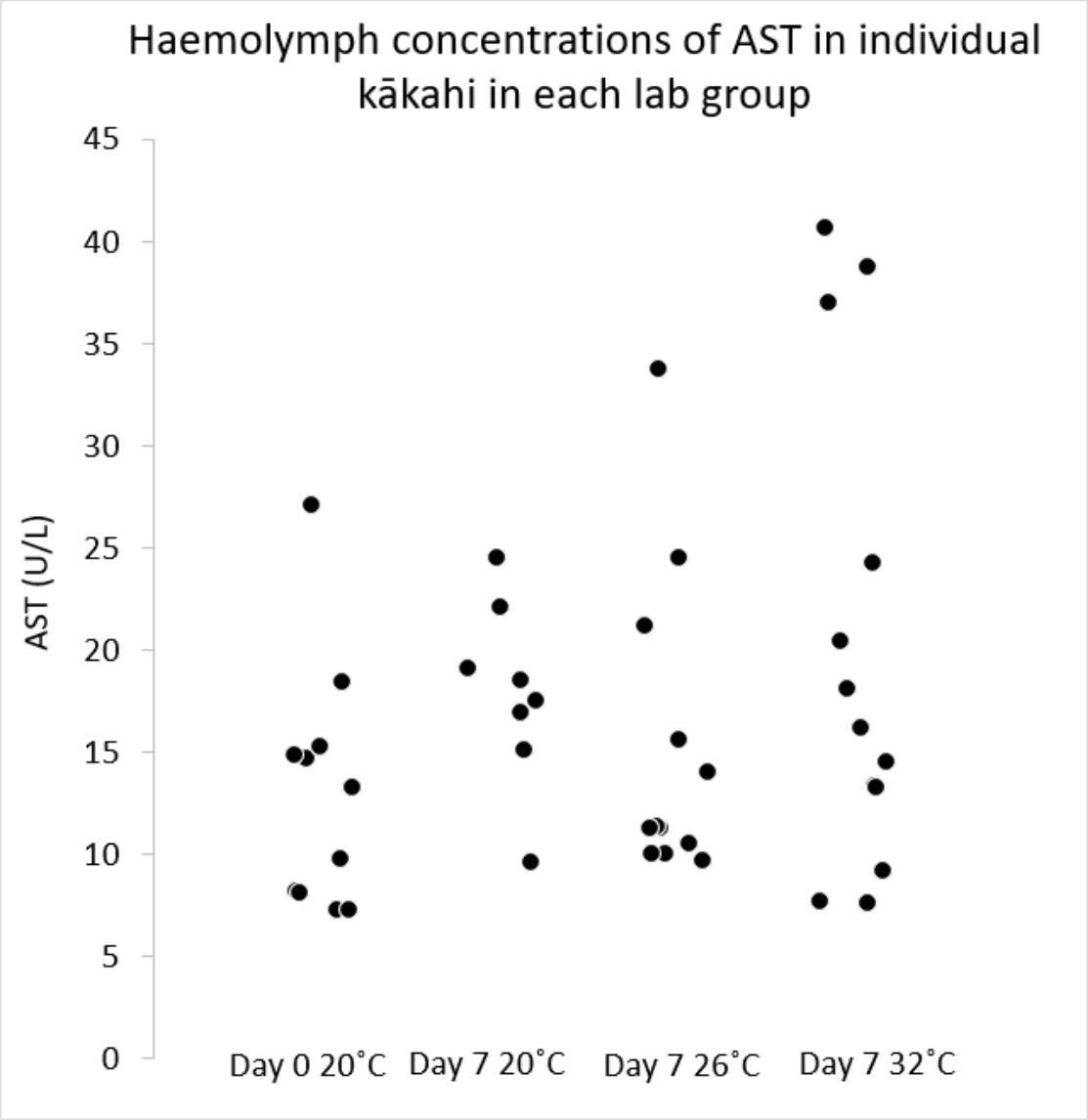

Figure 63: AST concentrations (in units per litre) measured in the haemolymph of each individual kākahi in the four lab groups.

After looking at the mean concentrations of each marker and the concentrations in the individual animals of each group, focus was given to correlations between the markers. Firstly, ALT was plotted against LDH (Figure 64).

There does not seem to be a clear relationship between LDH and ALT concentrations in any of the lab groups (Figure 64). The data is very variable, with some of the control groups having low ALT but high LDH concentrations, and vice versa. There is one data point from the 32˚C treatment group that shows a strong positive correlation between ALT and LDH, but this is an outlier from the rest of the samples. The adjusted R-squared value (r^2) for this data was 0.33 ($p = 3.052 \times 10^{-5}$) which suggests there is no relationship between ALT and LDH haemolymph concentrations in the lab samples.

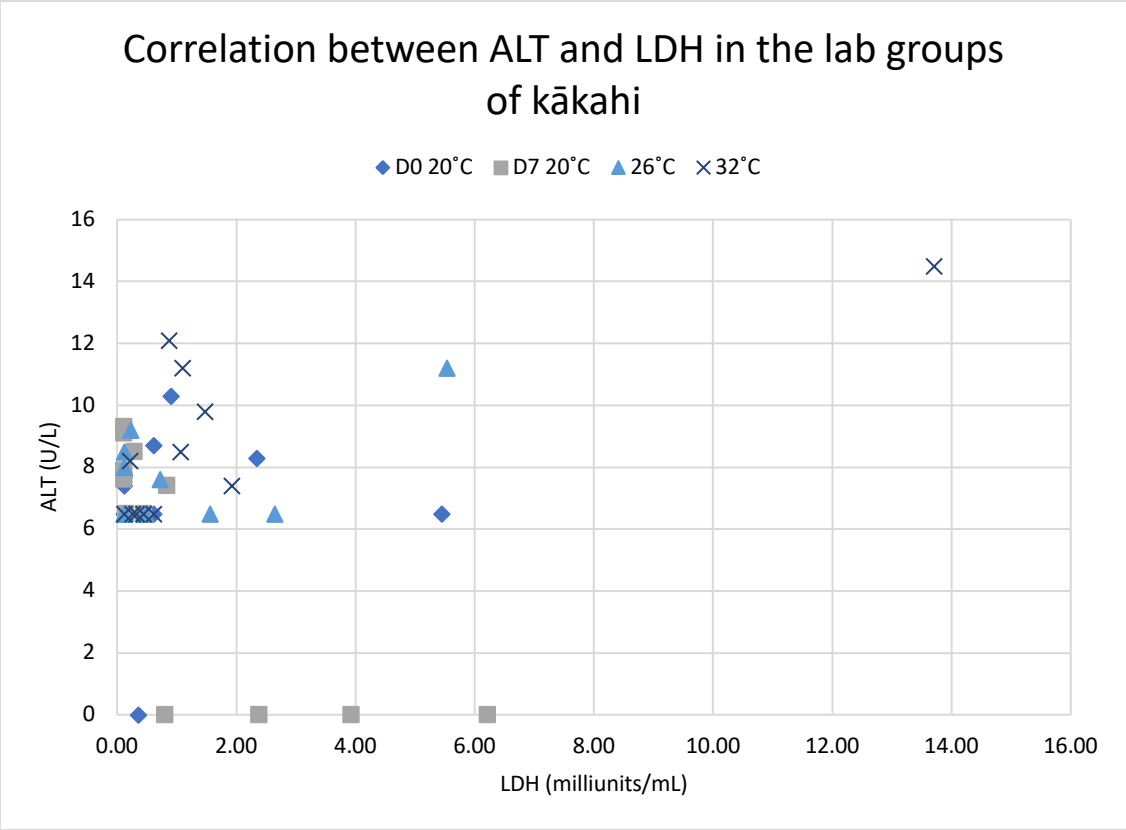

Figure 64: ALT plotted against LDH for all the lab groups of kākahi. Each symbol represents one of the four lab groups (two control groups and two treatment groups).

Secondly, AST was plotted against LDH, to investigate any correlations between these two markers (Figure 65).

There was no correlation between AST and LDH concentrations in the lab groups of kākahi (Figure 65). This was confirmed by an r^2 value of -0.018 ($p = 0.61$), which suggests there is no relationship between these two markers in the lab groups of kākahi.

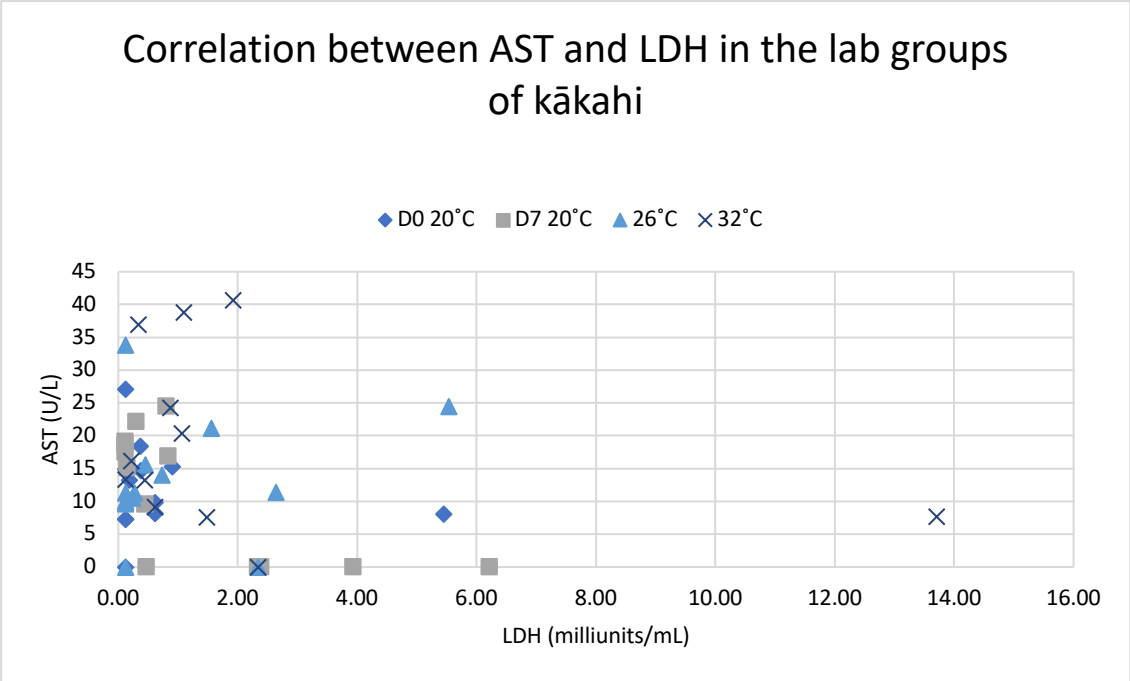

Figure 65: AST plotted against LDH for the four lab groups of kākahi to investigate the correlation between the two markers.

Lastly, AST was plotted against ALT (Figure 66).

There appears to be a weak, positive correlation between AST and ALT in the lab groups of kākahi (Figure 66). Therefore, as AST increases, ALT tends to increase as well. However, the adjusted r^2 value for this graph was 0.26 ($p = 0.003$), providing no evidence of a positive, linear relationship between ALT and AST in the haemolymph of the lab kākahi.

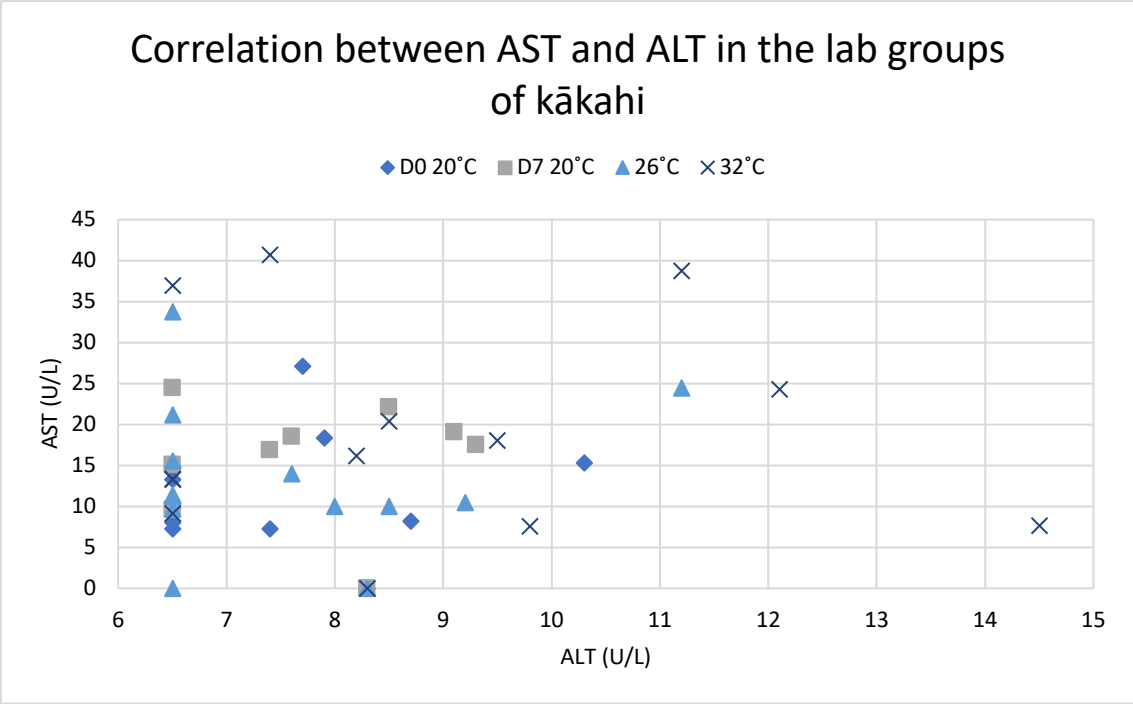

Figure 66: AST plotted against ALT in all four lab groups of kākahi to investigate the correlation between these two markers in the lab experiment.

4.5 Discussion

The lab experiment addresses the second aim of this thesis (Chapter 2.8) which was to provide insight into the vulnerability of kākahi to possible future water temperatures. The experiment outlined in this chapter is, to my knowledge, the first to investigate the impacts of a chronic temperature challenge on kākahi physiology in a controlled environment. The first key finding from this study was that there were no mortalities at 26˚C or 32˚C. This suggests that the upper thermal limit of kākahi is above 32˚C. The summer water temperatures of streams, rivers, and lakes in the Manawatū, Wairarapa, and Rangitīkei regions are not predicted to increase beyond 32˚C in any of the SSP emission scenarios (Figure 57) (IPCC, 2021). Therefore, based on the results in this chapter, it is unlikely that ACC will cause widespread mortality from heat shock (7 days duration or shorter) in kākahi populations in these regions. In addition to the physiological measures taken, normal siphoning behaviour was observed consistently for all four treatment groups throughout the experiment. While this was not quantitatively recorded, it is indicative that kākahi are able to maintain normal physiological function during a seven-day exposure to 26 and 32˚C.

The mean haemolymph concentrations of LDH, ALT, and AST indicate that the single stressor of a water temperature of 26˚C or 32˚C does not cause significant heat stress in kākahi. However, when considering the individual concentrations of these markers, it is possible that some of the kākahi in the 32˚C group were experiencing sub-lethal heat stress. It is unlikely that any of the changes observed in LDH, ALT, and AST

concentrations in the two treatment groups was caused by non-heat, captivity-related stress, because there were no significant differences in any of the markers between the Day 0 20˚C and Day 7 20˚C control groups. In addition, there were no outliers in the individual concentrations of any markers in either of these groups (Figures 61-63). Therefore, variation in marker concentrations of the treatment groups can be confidently attributed to the temperature challenge.

The haemolymph concentration of LDH was only high in one kākahi at 32˚C, which would have driven the average for the group upwards. Therefore, I cannot confidently conclude that LDH was elevated in the 32˚C group. It may be that the level of heat stress experienced by the kākahi in this group was not high enough to promote an increase in energy production via anaerobic glycolysis or that an increase in LDH activity is not reflected in haemolymph. A larger sample size would be helpful in future research to strengthen this conclusion. It may also be useful to measure LDH in tissue samples from the foot, gill or mantle, to determine if LDH remains low in these tissues during exposure to 32˚C. It would also have been useful to have a positive control group of kākahi that were exposed to a treatment that was known to elicit a significant increase in haemolymph concentrations of LDH, ALT, or AST. Unfortunately, this was not possible due to the absence of a known stressor that causes this response in kākahi. This would also have required a greater number of kākahi to be housed in captivity, which we wanted to avoid.

In contrast to the LDH results, there were several kākahi (3 out of 13) in the 32˚C group that showed an elevation in ALT and AST above concentrations seen in the control groups. While the mean ALT and AST concentrations in the control groups and the 32˚C group were not significantly different, this result may still be important. The largest difference was in AST, with some individual kākahi having an AST concentration that was 15-20 U/L higher than the highest value measured in either of the control groups (Figure 63). The kākahi in the 32˚C group had a less marked elevation in ALT, with several animals showing concentrations 2-3 U/L above the highest control value. This result is similar to what was observed in Park (2009), where AST concentrations were significantly elevated in Pacific oysters (*Crassostrea gigas*) exposed to 30˚C, while there was no significant change in ALT concentrations at this temperature.

In two studies, AST and ALT concentrations in the haemolymph have been shown to peak at water temperatures near the upper thermal limit of bivalve species (Park et al., 2009; Fritts et al., 2015a). In both studies, these increases are attributed to tissue damage induced by oxidative stress. Due to the lack of statistical significance, I cannot confidently conclude that exposure to a water temperature of 32˚C causes an increase in oxidative stress and, therefore, tissue damage in kākahi. It is possible that 32˚C did cause an increase in oxidative stress and tissue damage in some of the kākahi in the 32˚C group, which would be indicative of sub-lethal heat stress occurring at this temperature. Several factors have been found to contribute to individual variability in the physiological response to a temperature challenge. For example, smaller shell size was related to higher AST concentrations in *V. lienosa* and *V. vibex* during a temperature challenge (Fritts et al., 2015b). We did not measure and include shell size in the analysis of the results in this chapter. It is possible that the individual kākahi that had elevated ALT and AST concentrations in the 32˚C group were smaller than the others. The possible interaction between shell size and heat stress response in kākahi should be investigated in a future

experiment. Some individuals may also have higher copy numbers of genes involved in the heat-stress response, such as HSP70, making them more tolerant to a temperature challenge (Payton et al., 2016). In future, a greater sample size would also enable stronger conclusions to be made about the impact of a 32˚C temperature challenge on ALT and AST concentrations in kākahi haemolymph.

The lack of a statistically significant increase in AST and ALT in the haemolymph of kākahi after a seven-day exposure to 32˚C may be attributed to the time-dependent manner that these markers are known to increase in. In Fritts (2015a), both AST and ALT were significantly elevated in two freshwater mussel species (*Elliptio crassidens* and *Villosa vibex*) after a three-day exposure at 25˚C, 30˚C, and 35˚C. However, after seven days, ALT declined while AST remained elevated. In An & Choi (2010), both AST and ALT peaked after 48 hours of exposure at 30˚C in arks shells (*Scapharca broughtonii*) then began to decline. In future, it would be useful to measure AST and ALT in kākahi haemolymph at several time points over a seven-day exposure to 32˚C, to determine if the concentrations of these markers peak at 48 or 72 hours, like they have been demonstrated to do in other species.

In the 26˚C treatment group, there was very little change in the haemolymph concentrations of LDH, ALT, and AST in comparison to the control groups. This can be seen when looking at the mean concentrations of each marker at this temperature (Figures 58-60) and the individual concentrations in each kākahi (Figures 61-63). The absence of an increase in LDH indicates that being exposed to 26˚C does not cause a switch from primarily aerobic metabolism to primarily anaerobic metabolism in kākahi, or that this is not detectable in haemolymph in this species. Further, it is unlikely that metabolic depression was occurring in kākahi at 26˚C, as this response has been previously observed in other bivalves as a sharp increase in LDH concentrations in the tissue or haemolymph (Falfushynska et al., 2014; Matozzo et al., 2018; Zhang et al., 2022).

As with LDH, mean haemolymph concentrations of neither AST nor ALT in the 26˚C treatment group were elevated above those of the control groups. This indicates that this group of kākahi did not experience an increase in oxidative stress and subsequent tissue damage due to a seven-day exposure to 26˚C. The results from this chapter suggest that seven days at 26˚C does not induce heat stress in kākahi. It is possible that the kākahi in a field stream, may regularly experience summer temperatures of 26˚C under SSP2-4.5 by 2100 (IPCC, 2021). Given that a seven-day exposure to this temperature does not appear to have a significant impact on kākahi physiology, these animals may be thermally resilient to this degree of warming. At this stage, I cannot conclude on whether kākahi may be vulnerable to a warming scenario where summer temperatures reach 32˚C based on the results from this chapter. It may be that a longer exposure (>7 days) to 32˚C causes an increase in haemolymph concentrations of the chosen markers in more individual kākahi, or that this is dependent on other factors such as shell size. Therefore, further research is required before the vulnerability of kākahi to the SSP5-8.5 warming scenario can become clearer.

Chapter 5: Identification of heat shock protein 70 in kākahi tissue

5.1 Abstract

Increased expression of heat shock protein 70 (HSP70) is a useful biomarker of heat stress in aquatic animals, including freshwater mussels. However, before changes in expression can be measured, the genetic sequence for the HSP70 gene must be identified. This had not been attempted in kākahi before. The aim of the research presented in this chapter was to successfully design primers that would amplify the HSP70 gene in DNA extracted from kākahi tissue. This would enable future studies to measure changes in HSP70 expression in response to elevated water temperature, providing another biomarker of heat stress and increase our knowledge of how ACC may affect kākahi physiology.

This chapter describes the process of primer design, DNA extraction, endpoint PCR, gel electrophoresis and sequencing. Primers were designed based on the consensus HSP70 sequences of other molluscs. DNA was extracted from kākahi gill tissue and multiple cycles of endpoint PCR and gel electrophoresis were run with different primer combinations. This process was optimised by adjusting the primer concentrations and annealing temperature, as necessary. The primers were successful at amplifying regions of the kākahi DNA. However, the sequencing results showed that the amplified DNA was not aligned with the HSP70 sequence of other molluscs.

Following this outcome, the same primers were used to attempt to amplify HSP70 in the New Zealand green-lipped mussel (*Perna canaliculus*). Unfortunately, the desired HSP70 gene sequence was not amplified, suggesting the primers did not target that section of DNA. Nonetheless, this process provided key baseline information for future studies to continue from, with necessary improvements having been identified.

5.2 Introduction

Heat shock proteins (HSP) are a crucial part of the physiological response to heat stress in almost all organisms (Riezman, 2004). HSP are nuclear and cytosolic chaperones that are conserved in prokaryotic and eukaryotic organisms (Ahmed et al., 2020). During heat stress (and other cellular disruptions) HSP are recruited to target misfolded proteins that accumulate in the cytosol. HSP bind to the exposed hydrophobic regions of misfolded or unfolded proteins, facilitating the refolding of that protein into its correct formation (Matambo et al., 2004). HSP also increase the activity of protein degradation pathways, which is important for maintaining proteostasis during heat stress (Somero, 2020). HSP are expressed at a basal during physiological conditions (Manzon et al., 2022). However, when heat stress is detected in a cell, heat-inducible HSP are rapidly upregulated to prevent cellular damage (Tabuchi & Kondo, 2013).

There are many different specific HSP, and different animals possess a variable number of HSP genes. The individual HSP are grouped into families based on molecular weight (Christians et al., 2002). One of the most highly conserved HSP families is HSP70, which has a molecular mass of 70 kDa. Within eukaryotic cells, HSP70 sequences share a base similarity of 60-78% (Kiang & Tsokos, 1998). All proteins that belong to the HSP70

family bind to misfolded proteins using allosteric mechanisms, which involve ATP binding and hydrolysis (Mayer, 2013).

HSP70 has been identified and sequenced in many mollusc species, including marine mussels (Bultelle et al., 2021) and several freshwater bivalves (Payton et al., 2016; Xia et al., 2017). The HSP70 gene has not been identified in kākahi. To my knowledge, no molecular or genomic investigations have been performed in kākahi to date. The investigation of the presence of HSP70 in kākahi tissue was an additional aspect of this research project. The aim of the research presented in this chapter was to provide baseline information that can be used in future studies of kākahi thermal physiology. Measuring the levels of HSP70 expression under different temperature regimes is a common method of assessing vulnerability to specific environmental temperatures due to HSP70 proteins being heavily involved in thermotolerance, and they are considered to be cellular thermometers (Hassan et al., 2019).

Thermally resilient species may show higher basal expression of HSP70 and a less marked upregulation of the gene during heat stress, compared to thermally sensitive species (Payton et al., 2016). This means that the thermally resilient species expends less energy during heat stress, because a lower level of HSP upregulation is required to prevent cellular damage. To apply this methodology to future studies of climate change vulnerability in kākahi, identification of HSP70 must be achieved first. This chapter describes the process of designing primers, extracting DNA from kākahi tissue, endpoint PCR, gel electrophoresis, and DNA sequencing in an attempt to identify HSP70.

5.3 Materials and methods

5.3.1 Primer preparation

The primers were designed based on the consensus sequences of HSP70 sequences from other mollusc species that were available on the National Center for Biotechnology Information (NCBI) database (see appendix). Two different forward primers and three different reverse primers were designed using the primer design function of Geneious 10.2.6 (http://www.geneious.com/) using standard conditions (Table 4). All primers were ordered from Integrated DNA Technologies (IDT, IA, USA).

Table 4: Names and sequences of the five primers (two forward and three reverse) that were designed for use with kākahi DNA, based on the consensus HSP70 sequences of other mollusc species.

Primer name	Primer sequence
HSP70 K 28F	5’-CAAGTTGCCATGAATCCACA-3’
HSP70 K 321F	5’-ACTTGCTACAAAAGATGCCG-3’
HSP70 K 465R	3’-ACCACCTCCCAGATCAAAAA-5’
HSP70 K 536R	3’-GTATCCCCAGCAGTTGATCT-5’
HSP70 K 667R	3’-CTCTTTCACAAGCCGTTCTC-5’

Once the primers arrived, each one was rehydrated to a final concentration of 100 µM with nuclease free water. They were then placed in the fridge for storage for two days until they were needed for the PCR.

5.3.2 DNA extraction

DNA extraction from kākahi tissue samples was completed using the Qiagen DNeasy Blood and Tissue Kit (Düsseldorf, Germany). Firstly, two 25 mg samples of kākahi gill tissue were finely chopped using a scalpel blade. Each sample of chopped tissue was then transferred into a 1.5 mL microtube, labelled 1 and 2. To each tube, 180 µL of ATL buffer was added, followed by 20 µL of 20 mg/mL proteinase K. Each tube was lightly vortexed for 5 seconds to mix the tissue with the buffer and enzyme. The tubes were then incubated for 1.5 hours at 56˚C, until the tissue was completely lysed and completely clear.

Following incubation, the tubes were vortexed for 15 seconds, then 200 µL of Buffer AL was added to each sample and mixed by vortexing. To enhance binding of the DNA to the column 200 µL of 100% ethanol was added to each tube, and vortexed again to mix. The samples were then pipetted into DNeasy Mini spin columns placed inside 2 mL collection tubes. These tubes were centrifuged at 6000 g for 1 minute. The flow-through solution and collection tube were then discarded. The spin columns were placed in new 2 mL collection tubes. To wash the bound DNA, 500 µL of Buffer AW1 was then added to each column, followed by another 1-minute centrifugation at 6000 g. The flow-through and collection tubes were once again discarded, and the spin columns were inserted into new collection tubes. A second wash with 500 µL of Buffer AW2 was added to each spin column, followed by centrifugation at 20,000 g for 3 minutes to dry the DNeasy membrane.

The DNeasy spin columns were then transferred to 1.5 mL microcentrifuge tubes and 100 µL of Buffer AE was then pipetted directly onto the DNeasy membrane. This was incubated at room temperature for 1 minute, then centrifuged at 6000 g for 1 minute to elute. Following elution, DNA concentration and purity were assessed using a Nanodrop 2000 (ThermoFisher, MA, USA). To do this, 2 µL of DNA solution was pipetted onto the Nanodrop measurement platform. Once this measurement was recorded, the DNA samples were kept in the freezer until the PCR was initiated.

5.3.3 Endpoint PCR and gel electrophoresis

Several cycles of endpoint polymerase chain reaction (PCR) were performed to test and optimise for the amplification of the desired region of the kākahi genome. The aim of the first cycle of PCR was to determine if it was possible to obtain a successful product using the designed primers. Subsequent PCRs were performed to minimise the number of mis-primed products. Six combinations of the designed primers (Table 4) were prepared to investigate the HSP70 gene in kākahi.

A total reaction volume of 20 µL was prepared to consist of 1 µL of template DNA, 1x Solis Biodyne HotFirePol (Solis Biodyne, Estonia), 1 µM of each primer (Integrated DNA Technologies (IDT), IA, USA), all made to a final concentration with nuclease free

water. For each primer combination, two 20 µL PCR solutions contained 1 µL of kākahi template DNA, and in one solution, the DNA template was replaced with 1 µL of nuclease free water to provide a negative control.

The PCR was performed in an Eppendorf Mastercycler (Hamburg, Germany) using the following cycling conditions; 95˚C for 15 minutes to denature the hot start *Taq* followed by 40 cycles of denaturation at 95˚C for 30 seconds, annealing at 50˚C for 45 seconds and extension at 72˚C for 45 seconds. A seven-minute final extension at 72˚C was performed after the cycles.

To optimise the size of the PCR products, several optimisation PCRs were performed after the initial cycle. These subsequent PCR solutions had a reduced primer concentration of 0.2 µM. The PCRs were performed with the same cycling conditions as before, but with annealing temperatures of 55˚C, 56˚C, and 60˚C. For all optimisation PCRs, only the HSP70 K 28F primer was used in combination with all three reverse primers (Table 4).

A gel was prepared prior to commencing the electrophoresis of the PCR products. Firstly, two 0.5 g agarose tablets (Bioline, London, United Kingdom) were dissolved in 100 mL of 1x TAE buffer (Tris-acetate-EDTA) (40 mM Tris, 20 mM Acetic acid and 1 mM EDTA). The solution was microwaved for 1 minute at 50% power. This was repeated twice, until the solution was clear. To visualise the DNA, 4 µL of RedSafe Nucleic Acid Staining Solution (iNtRON, Seongnam, South Korea) was then added to the solution and gently swirled to mix. The casting tray was set up with two 15-well combs inserted. The gel solution was then poured into the casting tray. The gel was checked for the presence of any air bubbles, before being left to set for 1-2 hours. In the subsequent optimisation PCRs, only one 15-well comb was required for each gel.

Once the gel was set, the combs were removed and 20 µL of the PCR products were pipetted into the wells. The size of the PCR products was determined in comparison with a 100bp ladder (Hyperladder, 100bp, Bioline, London, United Kingdom). In the first cycle of PCR, 5 µL of this ladder was pipetted into wells 1 and 11 (Figure 67). In the subsequent optimisation PCRs, the ladder was only pipetted into well 1. The electrophoresis tank was set to run for 30 minutes at 70Volts. Once this was completed, the gel was placed under the UV transilluminator, to visualise the amplicons. Two subsequent PCRs were run on a gel for 2 hours, at 75V. Another two subsequent PCRs were run for 1 hour, at 100V.

5.3.4 Sequencing of the PCR products

The selected amplicons were cut out of the gel and sent for bi-directional Sanger sequencing to the Massey Genome Service (Massey University, New Zealand). Each amplicon was covered in 50 µL of DNA elution buffer and placed in the fridge overnight. The next day, the DNA was prepared for sequencing as per the Massey Genome Service's requirements: for a total of 20 ng of DNA, 4 pmol of primer were used for each sequencing reaction. The resulting sequence was visually assessed for quality in Geneious 10.2.6 (http://www.geneious.com/) and compared to previously published sequences using the BLAST tool in NCBI (Altschul et al., 1990).

5.3.5 PCR using DNA from the New Zealand green-lipped mussel

Once the sequencing results had been analysed, the PCR and gel electrophoresis processes were repeated using DNA extracted from the New Zealand green-lipped mussel (*Perna canaliculus*). The primers used were HSP70 K 28F and all three reverse primers (Table 4). The PCRs were performed using the same cycling conditions as the previous cycles, with annealing temperatures of 55˚C and 60˚C. The products from both PCRs were loaded onto separate gels and run for two hours at 50V. One amplicon was cut out of the second gel and sent away for bi-directional Sanger sequencing following elution and addition of the correct primers, as per the requirements of the Massey Genome Service.

5.4 Results

5.4.1 The concentration and purity of the extracted kākahi DNA

The reading taken using the Nanodrop 2000 (ThermoFisher, MA, USA) revealed that both samples of DNA extracted from kākahi gill tissue contained high quality DNA with minimal contamination. The concentrations and purity measurements of each sample are presented in Table 5.

Table 5: Final concentration and purity of the two DNA samples following extraction from kākahi gill tissue.

Sample	Concentration (µg/µL)	Purity (260:280)
1	427.1 µg/µL	1.90
2	199.5 µg/µL	1.88

5.4.2 Results from the gel electrophoresis of the PCR products using kākahi DNA

The first cycle of PCR and gel electrophoresis was successful in amplifying the DNA from the kākahi gill tissue. All the primer combinations were successful in producing products in the gel (Figure 67).

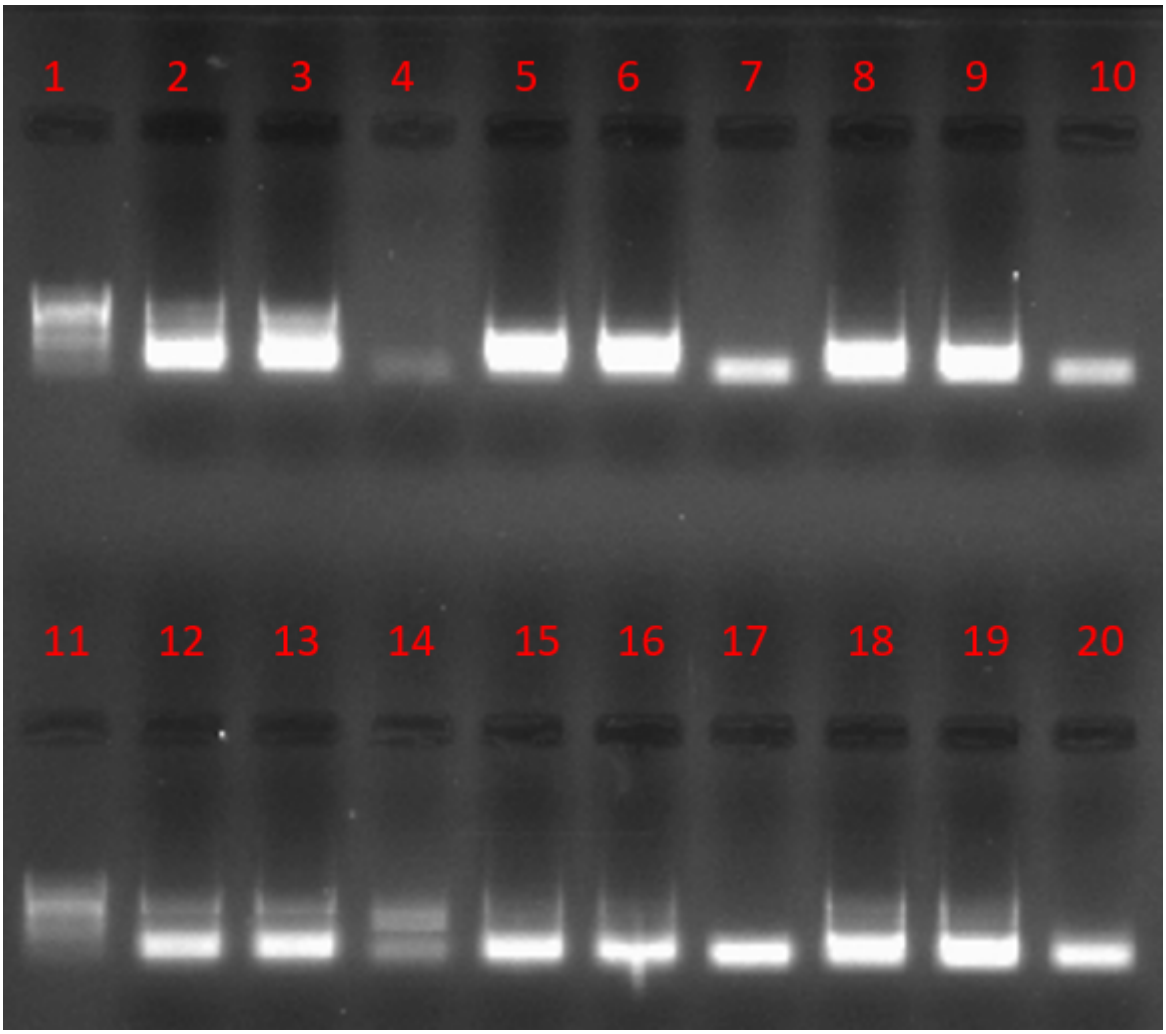

Figure 67: Results of PCR with an annealing temperature of 50˚C of kākahi DNA, using all primers (Table 4). Wells 1 and 11 contain the 100bp ladder. Wells 2-4 used primers HSP70K 28F and HSP70 K 465R. Wells 5-7 used primers HSP70 K 28F and HSP70 K 536R. Wells 8-10 used primers HSP70 K 28F and HSP70 K 667R. Wells 12-14 used primers HSP70 K 321F and HSP70 K 465R. Wells 15-17 used primers HSP70 K 321F and HSP70 K 536R. Wells 18-20 used primers HSP70K 321F and HSP70 K 667R.

While the first round of PCR successfully amplified the DNA (Figure 67), the amplicons were very thick, with several of the wells producing multiple amplicons. The HSP70 K 28F primer in combination with all three reverse primers (Table 4) produced the strongest amplicons in this first gel, so in every subsequent cycle only these three combinations were used. The second round of PCR, which had a lower primer concentration (0.2 µM), also successfully amplified the DNA (Figure 68).

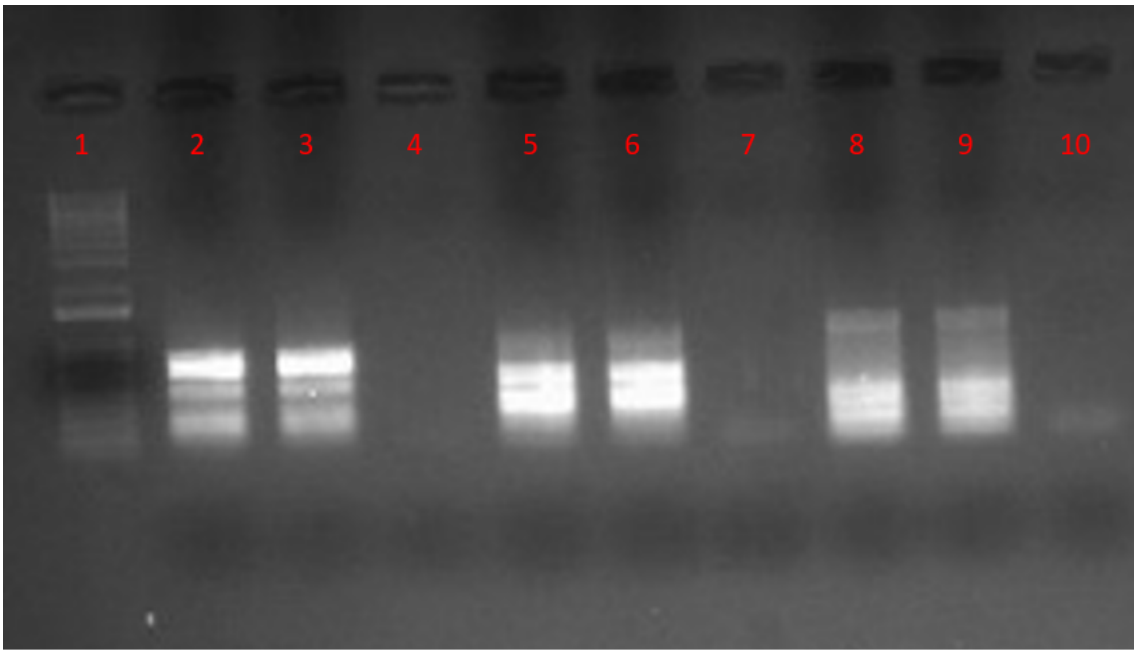

Figure 68: Results of PCR with an annealing temperature of 50˚C of kākahi DNA, using primers HSP70 K 28F, HSP70 K 465R, HSP70 K 536R, and HSP70 K 667R (Table 4). The ladder is in well 1. Wells 2-4 used primers HSP70K 28F and HSP70 K 465R. Wells 5-7 used primers HSP70 K 28F and HSP70 K 536R. Wells 8-10 used primers HSP70 K 28F and HSP70 K 667R.

Lowering the primer concentration was not enough to reduce the number of amplicons to an acceptable level (Figure 68). Following this outcome, the annealing temperature was increased to 60˚C and as a result, the third round of PCR successfully reduced the number of the amplicons on the gel (Figure 69).

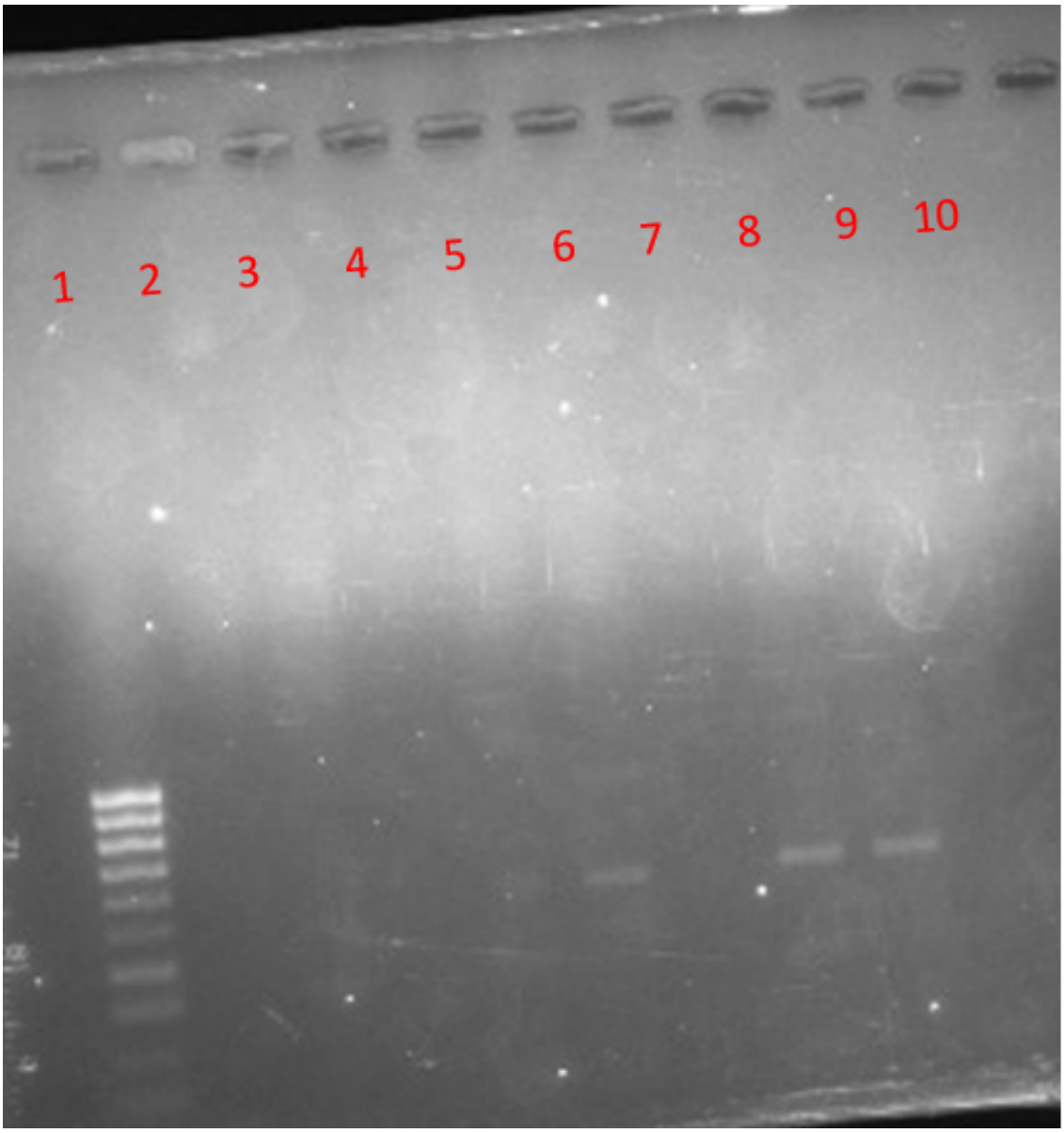

Figure 69: Results of PCR with an annealing temperature of 60˚C of kākahi DNA, using primers HSP70 K 28F, HSP70 K 465R, HSP70 K 536R, and HSP70 K 667R (Table 4). The ladder is in well 1. Wells 2-4 used primers HSP70K 28F and HSP70 K 465R. Wells 5-7 used primers HSP70 K 28F and HSP70 K 536R. Wells 8-10 used primers HSP70 K 28F and HSP70 K 667R.

The third round of PCR achieved the desired aim of reducing the number of amplicons produced in each well (Figure 69). However, the number of amplicons was reduced slightly more than was ideal, so a fourth PCR and gel were run with an annealing temperature of 56˚C (Figure 70).

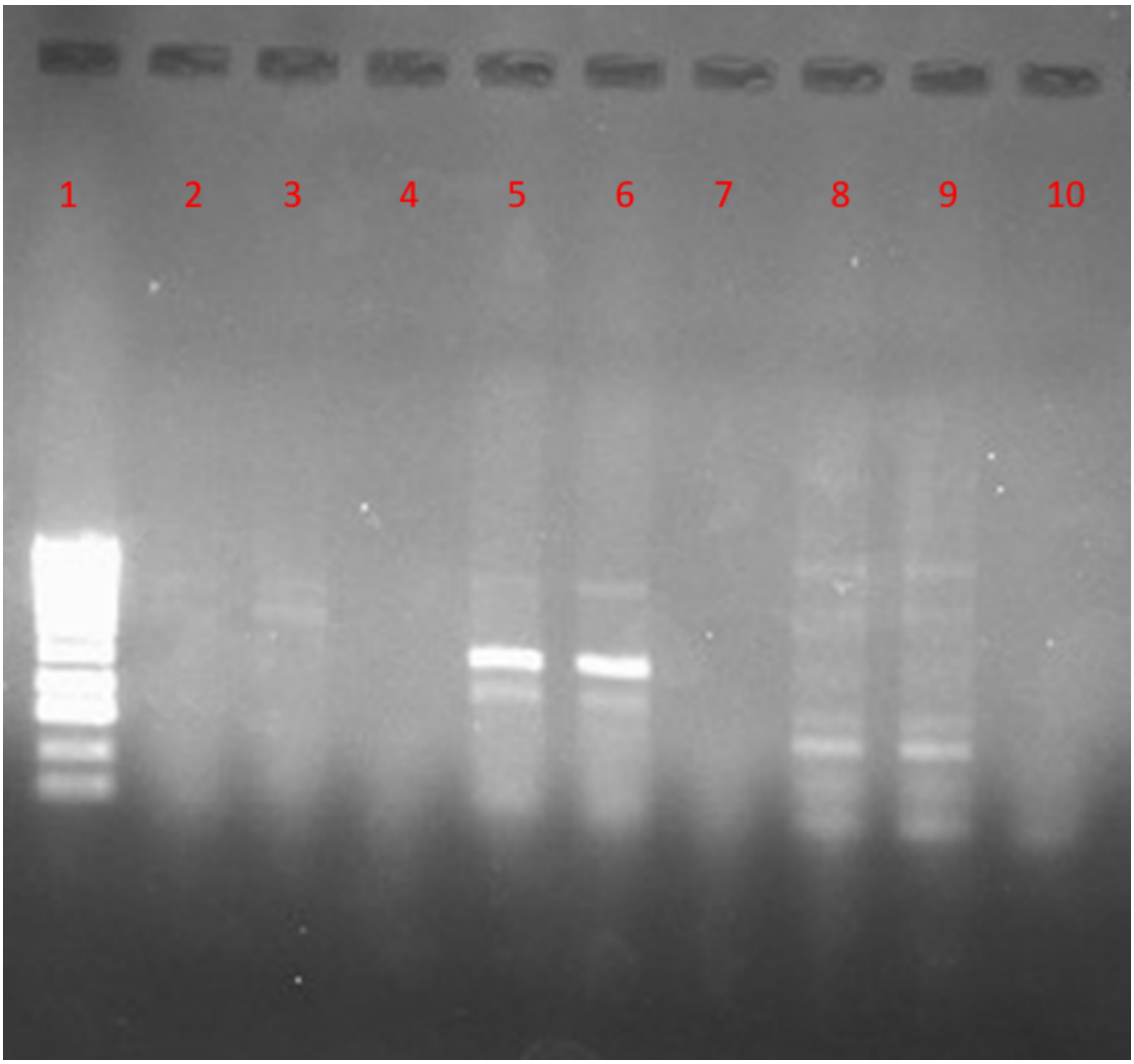

Figure 70: Results of PCR with an annealing temperature of 56˚C of kākahi DNA, using primers HSP70 K 28F, HSP70 K 465R, HSP70 K 536R, and HSP70 K 667R (Table 4). The ladder is in well 1. Wells 2-4 used primers HSP70K 28F and HSP70 K 465R. Wells 5-7 used primers HSP70 K 28F and HSP70 K 536R. Wells 8-10 used primers HSP70 K 28F and HSP70 K 667R.

The amplicons in this gel would have given enough product to cut out and send away for sequencing (Figure 70). However, a couple of the amplicons were slightly fainter than ideal, so a fifth gel was run with PCR products that only differed to the fourth cycle by having an annealing temperature of 55˚C (Figure 71).

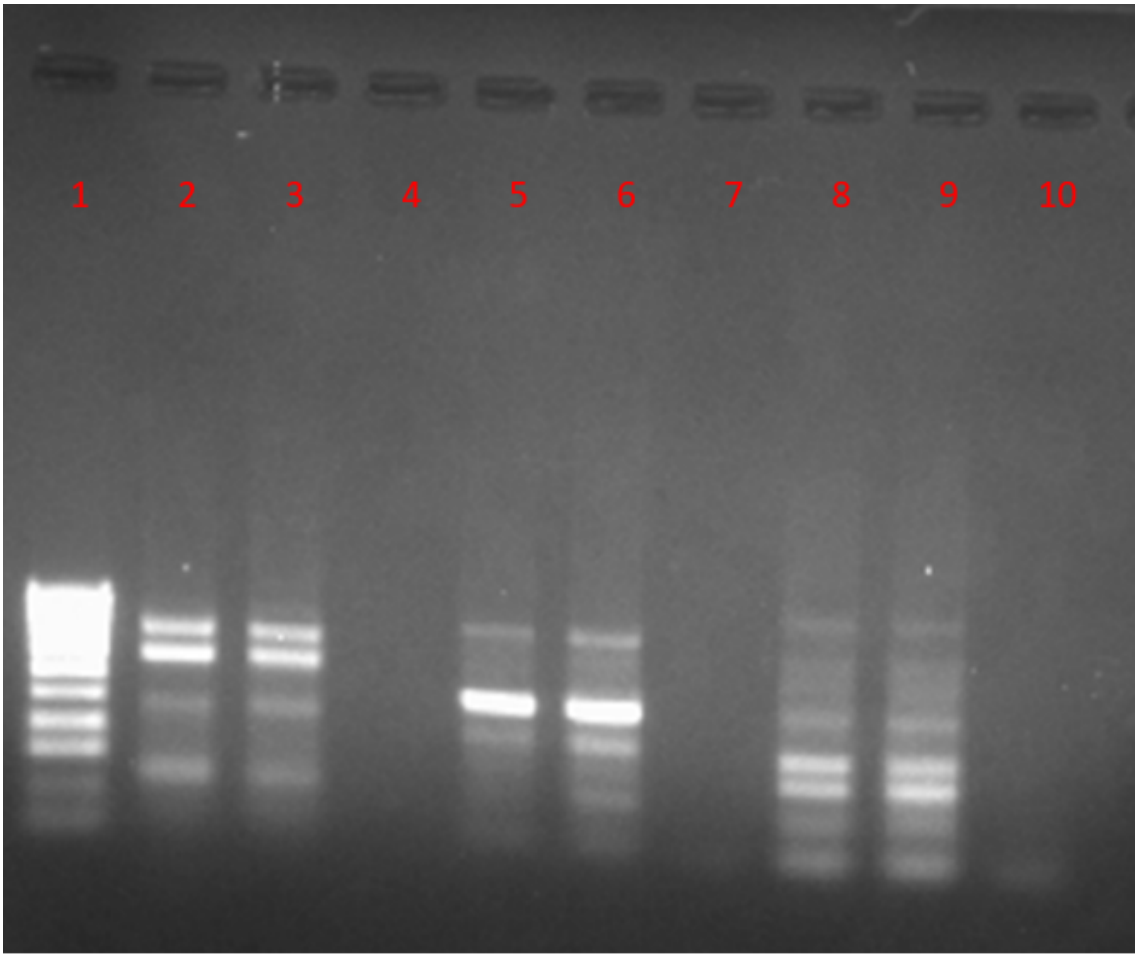

Figure 71: Results of PCR with an annealing temperature of 55˚C of kākahi DNA, using primers HSP70 K 28F, HSP70 K 465R, HSP70 K 536R, and HSP70 K 667R (Table 4). The ladder is in well 1. Wells 2-4 used primers HSP70K 28F and HSP70 K 465R. Wells 5-7 used primers HSP70 K 28F and HSP70 K 536R. Wells 8-10 used primers HSP70 K 28F and HSP70 K 667R.

This fifth gel successfully strengthened the desired amplicons (Figure 71) and three of them were cut out and sent to the Massey Genome Service for Sanger sequencing. Two amplicons were cut out of well 2, and one was cut out of well 5. Unfortunately, the sequencing results from the three bands cut out of the fifth gel did not align with the HSP70 consensus sequences from other mollusc species.

5.4.3 Results from the gel electrophoresis of the PCR products that used New Zealand green-lipped mussel DNA

When the same primers (HSP70 K 28F, HSP70 K 465R, HSP70 K 536R, and HSP70 K 667R) were run with green-lipped mussel DNA, the first PCR cycle produced very inconsistent results on the gel (Figure 72).

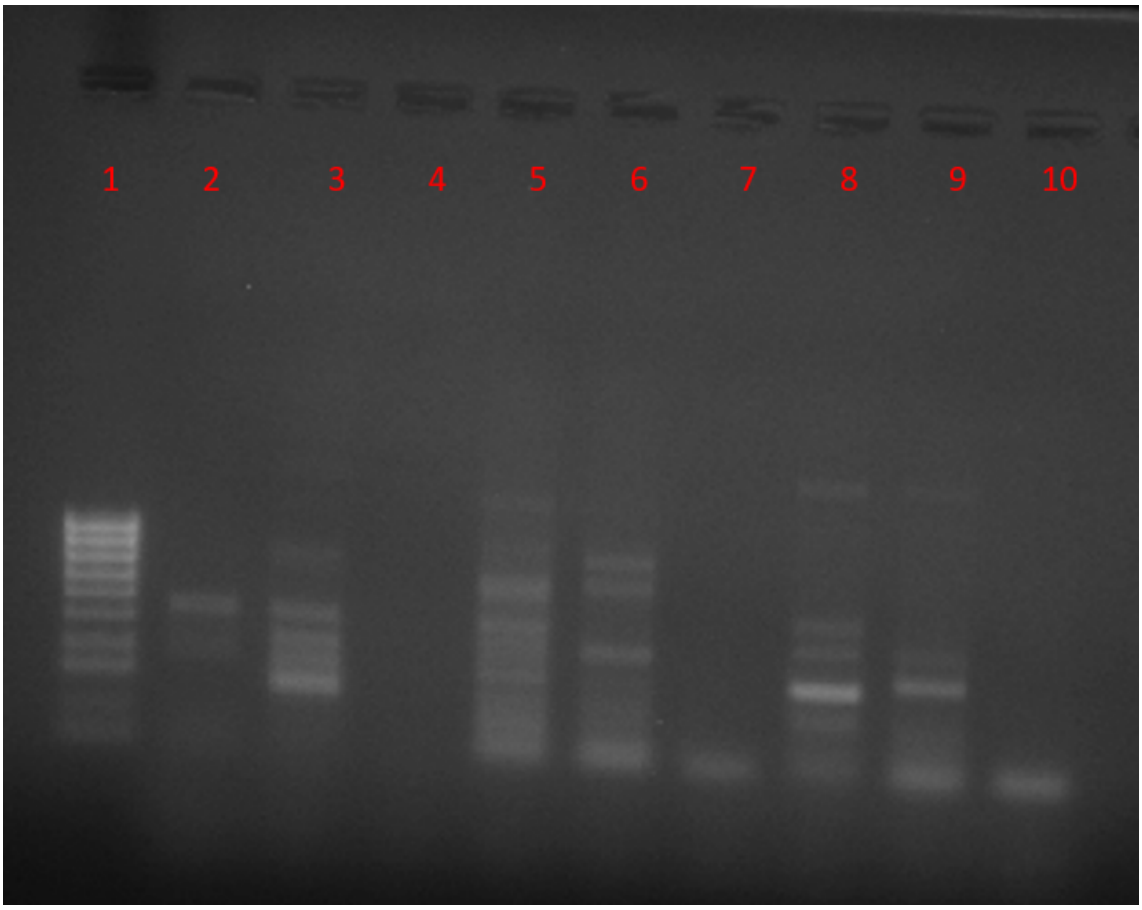

Figure 72: Results of PCR with New Zealand green-lipped mussel DNA, at an annealing temperature of 55˚C, using primers HSP70 K 28F, HSP70 K 465R, HSP70 K 536R, and HSP70 K 667R (Table 4). The ladder is in well 1. Wells 2-4 used primers HSP70K 28F and HSP70 K 465R. Wells 5-7 used primers HSP70 K 28F and HSP70 K 536R. Wells 8-10 used primers HSP70 K 28F and HSP70 K 667R.

The amplicon sizes and strengths were highly variable (Figure 72) and none of them were strong enough to cut out and send away for sequencing. Fortunately, this was remedied by increasing the annealing temperature to 60˚C, with the products of this second PCR cycle showing fewer amplicons (Figure 73).

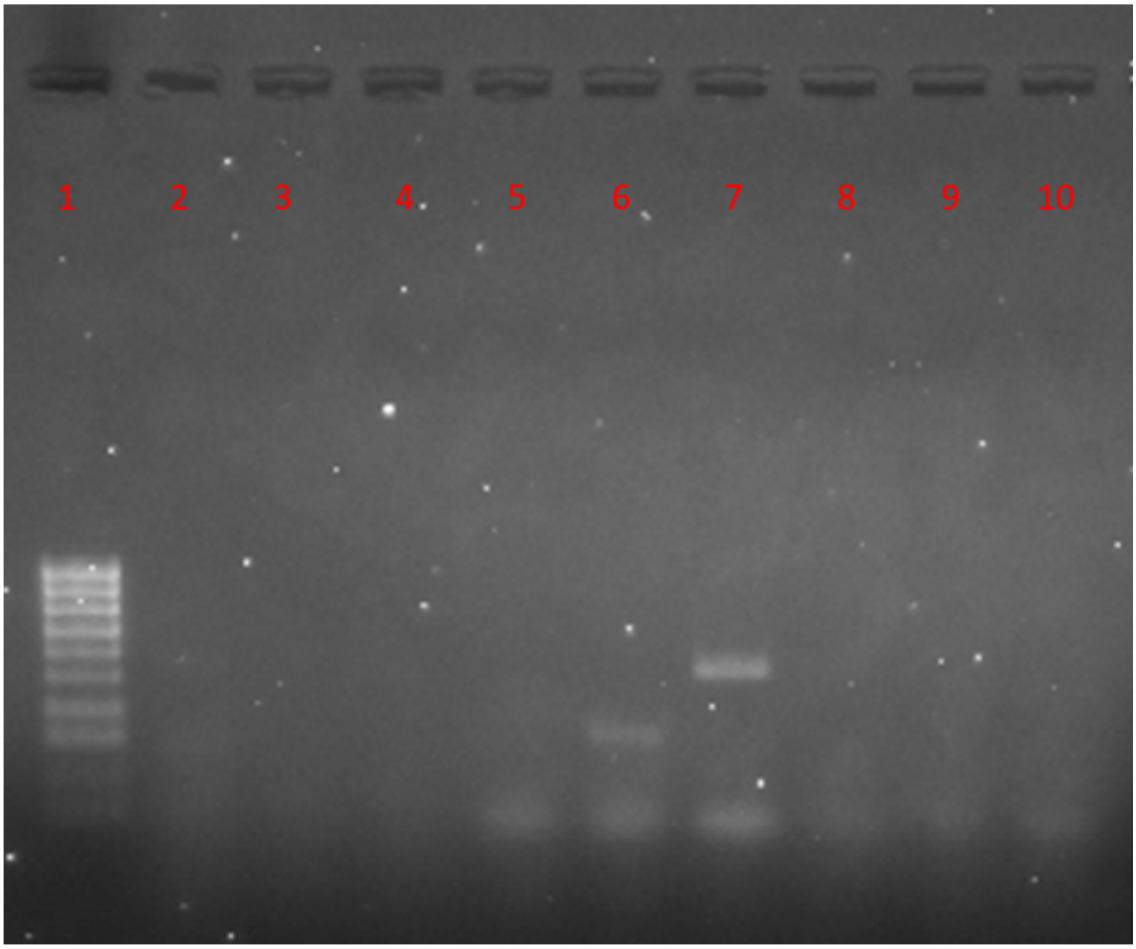

Figure 73: Results of PCR with New Zealand green-lipped mussel DNA, at an annealing temperature of 60˚C, using primers HSP70 K 28F, HSP70 K 465R, HSP70 K 536R, and HSP70 K 667R (Table 4). The ladder is in well 1. Wells 2-4 used primers HSP70K 28F and HSP70 K 465R. Wells 5-7 used primers HSP70 K 28F and HSP70 K 536R. Wells 8-10 used primers HSP70 K 28F and HSP70 K 667R.

An amplicon from well 7 was cut out and sent away for sequencing (Figure 73). Unfortunately, the sequencing results did not align with the HSP70 sequences of other molluscs.

5.5 Discussion

The first round of PCR validated the primers' ability to amplify the DNA extracted from the kākahi gill tissue. However, the gel showed very thick, multiple bands, which indicated that the primer concentration was too high. Therefore, in the second round of PCR, the primer concentration was reduced. In the second, and subsequent rounds of PCR, only one of the forward primers was used. HSP70 K 28F was used because it produced a bigger product than HSP70 K 321F in the first gel (Figure 67).

Although the primer concentration had been reduced, the second gel still showed multiple bands so were not useful for sequencing. To remedy this, the annealing temperature was

increased to 60˚C. The third round of PCR reduced the size of the bands too much (Figure 69), so the annealing temperature was reduced to 56˚C in the fourth round. This produced several, strong individual bands. However, several that we wanted to cut out were slightly too faint, so a fifth gel was run with the PCR products that had an annealing temperature of 55˚C. This had the desired effect and produced stronger bands, three of which were cut out and sent for sequencing. Unfortunately, when the results were compared to the HSP70 consensus sequences from other mussel species, there was no observable alignment. There could be several factors that caused this to happen. Firstly, the primers that were designed were not specific enough to successfully amplify HSP70 from kākahi DNA. It could be that the kākahi HSP70 sequence is less like that of other mussel species than assumed. It may be necessary to sequence the entire kākahi genome to successfully amplify and identify this gene. Unfortunately, this was not possible due to time and budget constraints. Further consultation with local iwi would also be required before undertaking genome sequencing. However, this is something that should be investigated in the future.

The final part of this research investigated whether the designed primers could amplify HSP70 in a marine mussel species. Previously extracted green-lipped mussel DNA was readily available and so was used for this process. The first cycle of PCR produced highly variable band sizes and strengths, so a second cycle was run with an increased annealing temperature. The second gel produced a band that was cut out and sequenced. However, the sequencing results gave the same outcome as the previously sequenced kākahi DNA. The green-lipped mussel sequence did not show any alignment with the HSP70 sequences of other molluscs. In future experiments, new primers will need to be designed to target the specific region of DNA. The work presented in this chapter may inform future studies, as it demonstrated the need for a whole genome sequence for kākahi in order to firstly, identify the HSP70 gene, and secondly, to measure changes in HSP70 expression during heat stress.

Chapter 6: General Discussion

The first aim of this thesis was to increase our knowledge of the thermal physiology of kākahi (*Echyridella menziesii*), a freshwater bivalve mollusc endemic to Aotearoa New Zealand. The results from Chapters 3 and 4 contribute to achieving this aim by investigating if current summer temperatures (Chapter 3) or temperature treatments of 26 or 32˚C (Chapter 4) cause measurable changes in haemolymph concentrations of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in kākahi. The second aim was to provide insight into the vulnerability of kākahi to possible future water temperatures and the implications this may have for the survival of the species and the health of freshwater ecosystems in Aotearoa New Zealand. Chapter 4 addresses this second aim while Chapters 3 and 4 both address the hypothesis, which predicted that kākahi exposed to increased water temperatures will display changes in the haemolymph concentrations of biomarkers that are indicative of the metabolic disturbances that occur during heat stress.

This general discussion (Chapter 6) provides a synthesis of the changes in heat stress biomarkers that were observed in kākahi in the field (Chapter 3) and laboratory (Chapter 4) studies. A synthesis of the additional research into the presence of the heat shock protein 70 gene in kākahi is also provided. A discussion of how the results from Chapters 3 and 4 inform us about the thermal resilience or vulnerability of kākahi under ACC is then provided, including the physiological, ecological and cultural implications of the findings. Finally, limitations and shortcomings of this research are discussed and suggestions for future research are given.

6.1 Synthesis of the changes in heat stress biomarkers observed in kākahi haemolymph in response to environmental temperature

Chapter 3 demonstrated that current (2022) summer temperatures do not have a significant effect on the haemolymph concentrations of LDH, ALT, and AST in the samples collected from two kākahi populations. There were no significant differences in the concentrations of these markers in the summer samples, compared to the winter samples. Unfortunately, it was not possible to compare the summer and winter concentrations of the markers at the remaining three sites. Unusually high levels of rainfall occurred in the Manawatū and Rangitīkei regions between May and August of 2022 which made it impossible to collect winter samples from Simpson's Reserve, Moonshine Valley, and Lake Horowhenua. The results from these samples will be presented later, in the published manuscript of this thesis. Those data will enable us to determine whether the findings from the final three sites are similar or different to those from the Mauriceville and Taueru sites.

Summer water temperatures at the Mauriceville and Taueru field sites were between 18 and 20˚C. The results from these sites suggest that summer water temperatures of 18-20˚C do not cause an increase in heat stress in these kākahi populations. No elevation in haemolymph concentrations of LDH at summer temperatures indicated that metabolism was not supressed in kākahi, and normal aerobic production of energy could be

maintained. No elevation in ALT and AST haemolymph concentrations at summer temperatures suggests that 18-20˚C water does not cause an increase in oxidative stress and subsequent tissue damage in kākahi. This information expands on current knowledge of kākahi thermal physiology and may benefit kākahi conservation and freshwater ecosystem health, which is discussed in section 6.3.

The findings from Chapter 3 are dissimilar to those from previous studies, due to the lack of studies that specifically measured changes in these heat stress markers in wild bivalves between summer and winter temperatures. As mentioned in Chapter 3, haemolymph samples have been taken from other bivalve species in a field setting to measure the impacts of various environmental variables on stress marker concentrations (Fritts et al., 2015b; Steinagel et al., 2018). However, quantifying heat stress was not the focus of most of these previous studies (Steinagel et al., 2018; Jahromi et al., 2020). Fritts et al. (2015b) measured the impact of summer temperatures on haemolymph concentrations of heat stress markers, including ALT and AST. Summer temperatures in this study did cause a significant increase in AST and ALT haemolymph concentrations in two species of freshwater mussel (*Villosa vibex* and *Villosa lienosa*). However, extensive modelling techniques were used in (Fritts et al., 2015b), and there were 21 potential variables that contributed to the changes in biomarker concentrations, of which temperature was only one. The significant increases in ALT and AST in the haemolymph were dependent on other variables such as shell size, which was not accounted for in my field study. The results are, therefore, not comparable to the results of Chapter 3 in this thesis, due to the significant differences in methodology and scope.

Physiological responses to elevated temperatures have most commonly been measured in bivalves in a laboratory setting. LDH, ALT, and AST are not heat stress specific markers, but can also change in response to other stressors such as disease or changes in environmental pH, and salinity (An & Choi, 2010; Fritts et al., 2015a; Liao et al., 2019). Therefore, it is difficult in a field setting, to attribute changes in these markers to an increase or decrease in water temperature, without controlling for all the other potential causes of stress in that environment.

In summary, the results of this chapter are not in alignment with previous research, due to a lack of similar studies and significant differences in stressor/s investigated and overall scope. The field study in this thesis aimed to contribute to the gap in the literature of how haemolymph concentrations of heat stress biomarkers may change in different seasons of the year. I was successful in this aim, by showing that in two kākahi populations, current summer water temperatures do not have a significant effect on haemolymph concentrations of three known biomarkers of heat stress.

Chapter 4 provided new information about several aspects of kākahi thermal physiology and their vulnerability or resilience to increasing water temperatures. The kākahi in the lab that were exposed to 26˚C (a 6˚C increase in temperature) for seven days did not show any significant differences in the concentrations of LDH, ALT, or AST in their haemolymph, compared to the control groups. This provides evidence that a seven-day exposure to 26˚C does not cause increased heat stress in kākahi. There was no evidence of metabolic depression (no change in LDH) or increased tissue damage (no change in ALT or AST). Therefore, my results suggest that kākahi may be thermally resilient when exposed to a change in temperature from 20 to 26˚C for seven days. In Falfushynska et

al. (2014), LDH was elevated in tissue of the duck mussel (*Anodonta anatina*) after exposure to 25˚C, a 7˚C increase in temperature from control conditions, which is not in alignment with my results. However, this is not unexpected, as tolerance to a specific temperature (in this case ~26˚C) depends on many factors including life history traits and the typical thermal regime of the population's habitat (Buckley & Kingsolver, 2021). ALT and AST were typically measured in previous studies at temperatures above 26˚C (Park et al., 2009; Fritts et al., 2015a).

The group of kākahi exposed to 32˚C (a 12˚C increase in temperature) also did not show any significant change in LDH, ALT, or AST concentrations in the haemolymph compared to the control groups. However, several individual kākahi in this group did have elevated AST and ALT haemolymph concentrations, highlighting the individual variation that is often observed in the physiological stress response. The lack of a significant difference in mean concentrations of these markers at 32˚C is generally not in alignment with previous studies. For example, AST concentrations were significantly elevated in Pacific oysters (*Crassostrea gigas*) exposed to 30˚C (a 10˚C increase in temperature), but there was no significant change in ALT concentrations at this temperature (Park et al., 2009). In Fritts (2015a), both ALT and AST were elevated after seven days of exposure to 30˚C (a 5˚C increase in temperature) in the elephant ear (*Elliptio crassidens*) and southern rainbow (*Villosa vibex*). It has been established that increases in ALT and AST concentrations in bivalve haemolymph in response to elevated temperature is a time-dependent response (Park et al., 2009; An & Choi, 2010) which may account for the differences in results from my experiment and previous studies. However, the increases in AST and ALT in individual kākahi exposed to 32˚C, may indicate that rates of tissue damage from oxidative stress were higher at this temperature in some kākahi, which is a similar finding to that of previous studies. A larger sample size in future studies would likely strengthen the conclusions drawn from these results and more confidently determine whether AST and ALT concentrations do significantly increase in kākahi exposed to 32˚C. It would also be useful to measure the dimensions of each kākahi to investigate if size is a factor in the physiological response to a 12˚C temperature increase.

No increase in LDH haemolymph concentrations in the 32˚C group indicated that a seven-day exposure to 32˚C does not cause suppression of the aerobic energy system in kākahi, or that this is not detectable in haemolymph. This suggests that these kākahi were not experiencing a significant heat stress event after seven days at 32˚C, as this stressor is known to downregulate the aerobic glycolysis metabolic pathway and upregulate the reactions that produce energy anaerobically, one of which is catalysed by LDH (Figure 74).

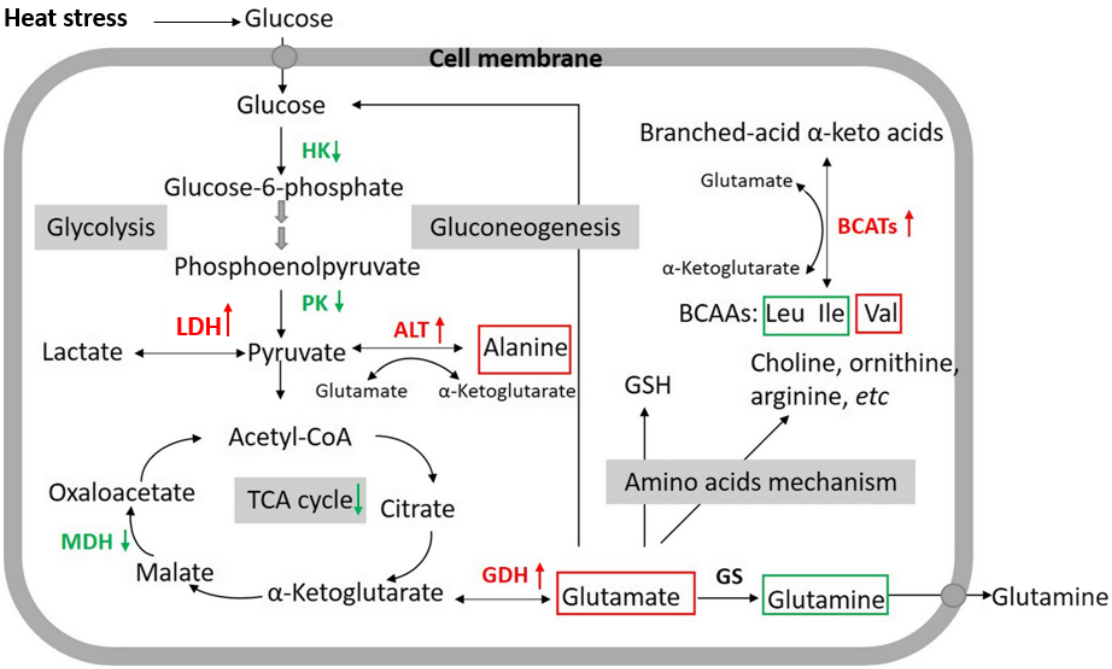

Figure 74: Carbohydrate (left) and amino acid (right) metabolism during heat stress in invertebrates. During heat stress, the enzymes involved in aerobic glycolysis (green) are downregulated, leading to downregulation of the TCA cycle. In contrast, LDH, a key enzyme in the anaerobic glycolytic pathway is upregulated. ALT is also upregulated during heat stress (Adapted from Xu et al., 2017).

As previously discussed (Chapter 2, section 2.6.2), when cells are experiencing heat stress, there is often a switch from primarily aerobic production of ATP to primarily anaerobic production. In the metabolic pathway, this switch involves the downregulation of enzymes involved in aerobic glycolysis, such as hexokinase and pyruvate kinase, and downstream suppression of the TCA cycle (Figure 74). LDH is simultaneously upregulated to catalyse the conversion of pyruvate to lactate, which is a key step in the anaerobic glycolytic pathway, an essential metabolic process to maintain ATP production during moderate and extreme heat stress (Xu et al., 2017). It is important to note that ALT also increases in this metabolic pathway, as this is a stress-responsive gene (Xu et al., 2017). Therefore, expression of ALT is upregulated during heat stress (Figure 74). This often leads to higher levels of ALT in the circulation, as tissue damage and cellular separation during heat-induced oxidative stress increases the inflow of cells (containing higher ALT levels) into the haemolymph (Park et al., 2009). Although AST in not represented in this metabolic pathway (Figure 74), concentrations of this enzyme also increase during heat stress due to increased inflow of cells into the haemolymph (Park et al., 2009).

The lack of a significant increase in LDH in kākahi exposed to 32˚C is not in alignment with previous studies, one of which found significant increases in LDH activity in the gills and digestive gland after an exposure to 25˚C and 30˚C in the duck mussel (*Anadonta anatina*) (Falfushynska et al., 2014). However, this temperature challenge continued for 14 days, so it is possible that a longer exposure to 32˚C may cause an increase in LDH

haemolymph concentrations in kākahi, as observed in other species. LDH was also significantly elevated in the pearl oyster (*Pinctada fucata*) after seven days of exposure at 33˚C (Zhang et al., 2022). However, LDH was measured in gill and digestive gland tissues in both studies, rather than in the haemolymph, which may have been a factor in the different results. It may be that changes in LDH are not detectable in kākahi haemolymph. It would be useful in future laboratory experiments to measure LDH in gill or digestive gland samples of kākahi exposed to 32˚C, to confirm that LDH remains low at this temperature in these tissues, rather than in the circulation alone.

None of the apparent differences in AST, ALT, or LDH in the 32˚C group of kākahi were statistically significant (p >0.05) meaning that no heat stress was observed under the conditions tested. Given the increase in AST and ALT haemolymph concentrations in several individual kākahi, it is possible that exposure to 32˚C may cause some degree of sub-lethal heat stress in some kākahi. Repeating this experiment with a greater sample size and also recording kākahi dimensional information might allow greater confidence in the conclusions made. In future studies, I would also suggest taking multiple samples across the seven-day period to account for potential time-dependent changes in ALT and AST, as it is possible that any of these markers may have peaked and returned to baseline within seven days. Extending the experimental period beyond seven days may also improve the strength of the conclusions made about how a 32˚C temperature challenge affects kākahi physiology.

The lack of change in the haemolymph concentrations of the chosen heat stress markers after exposure to increased water temperatures showed that the hypothesis was not supported by the results from Chapters 3 and 4. However, I was successful in achieving the aims of the thesis, which were to increase our knowledge of kākahi thermal physiology and provide insight into their thermal vulnerability or resilience to ACC. The results from Chapters 3 and 4 suggest that kākahi may be resilient to short periods (seven days) of the elevated water temperatures expected under a moderate warming scenario. The physiological ecological, and cultural implications of this are discussed in section 6.3.

6.2 Presence of the heat shock protein 70 gene in kākahi tissue

The aim of Chapter 5 was to determine whether the heat shock protein 70 gene could be identified in kākahi tissue using primers based on the HSP70 consensus sequences of other molluscs. This additional work lay outside the aims and hypothesis addressed in the previous chapters. Instead, it was completed as baseline work in an attempt to enable future studies to measure changes in HSP70 expression as another biomarker of the physiological response of kākahi to elevated water temperatures. The results from this chapter showed that primers designed from consensus HSP70 sequences were unable to target the HSP70 gene in kākahi DNA, or in New Zealand green-lipped mussel DNA. It is unlikely that this is because the gene is not present in kākahi, because HSP70 is highly conserved across the animal kingdom (Mukhopadhyay et al., 2003). Instead, it is likely due to the primers not being able to target the desired HSP70 sequence. In the future, either further design work of more specific primers should be attempted, or sequencing of the whole kākahi genome should be undertaken. This would highly increase the

likelihood of correctly identifying the HSP70 gene. Once that is established, future studies could measure changes in HSP70 expression in kākahi exposed to elevated water temperatures, increasing our knowledge of their vulnerability to different potential warming scenarios under ACC.

6.3 Resilience or vulnerability to ACC, the physiological, ecological, and cultural implications

Several freshwater mussel species have been deemed either resilient or vulnerable to ACC based on their physiological responses to different warming scenarios simulated in a laboratory (Falfushynska et al., 2014; Payton et al., 2016). One method of measuring vulnerability, which was adopted in this thesis, is to measure haemolymph concentrations of heat stress biomarkers. Mussels that show no significant increase in the concentrations of markers associated with heat stress at predicted future water temperatures are deemed to have a high thermotolerance and therefore, be thermally resilient to ACC. Mussels that show significant elevations in heat stress biomarker concentrations are said to be thermally vulnerable to ACC, due to the greater impact that increasing water temperatures have on their physiology.

The results from Chapter 4 suggest that kākahi may be resilient to a moderate warming scenario (SSP2-4.5) due to the minimal change observed in haemolymph concentrations of LDH, ALT, and AST after a seven-day exposure to 26˚C (+6˚C above pre-challenge water temperature). Kākahi may also be resilient to an extreme warming scenario (SSP5-8.5) given the lack of a significant increase in the mean heat stress biomarker concentrations at 32˚C (+12˚C). However, because several kākahi exposed to 32˚C did show an increase in ALT and AST concentrations, further research with a greater sample size is required before this conclusion can be made confidently.

Physiological resilience to ACC has positive ramifications for the conservation of kākahi populations and for the health of freshwater ecosystems in Aotearoa New Zealand. When increased temperatures have a significant impact on physiological function, metabolism is suppressed and energy is redirected towards the stress response, rather than functions that are not essential for immediate survival (Sokolova et al., 2012). Therefore, thermally vulnerable species are at risk of physiological barriers to maintaining fitness-enhancing functions such as growth and reproduction. In contrast, thermally resilient species do not need to spend a major portion of their energy budget on alleviating damage caused by heat stress, and can continue to invest energy into growth and reproduction, which is vital for the ongoing survival and fitness of the species (Sokolova et al., 2012). Resilience to moderate warming increases the likelihood of kākahi populations persisting over the coming decades.

As previously discussed in Chapter 2, kākahi is an ecological keystone species, whose filtering and bioturbation activities improve the health of the freshwater habitats they reside in. Persistence of the species therefore promotes the ongoing health of freshwater ecosystems, due to the continuation of their ecological roles. Further, their resilience to moderate warming suggests that kākahi will be able to maintain efficient rates of filtration and bioturbation, due to the lack of metabolic depression observed during exposure to

26˚C water. Metabolic depression is associated with shell valve closure in bivalves (Anestis et al., 2007), reducing filtration capacity and, therefore, the positive impact the population can have on water quality. In addition to there being no evidence of metabolic depression (no change in LDH) in kākahi exposed to 26˚C, siphoning behaviour was consistently observed in kākahi exposed to both 26˚C and 32˚C throughout the seven-day experiment. The combination of these results strengthens the conclusion that thermal resilience to a seven-day exposure of 26˚C in kākahi may allow the species to continue to contribute to freshwater health via filtration and bioturbation as water temperatures increase under ACC. Thermal resilience therefore not only has direct benefits for kākahi physiology, but also the long-term health of streams, rivers, and lakes in Aotearoa New Zealand.

Being a taonga (treasured) species, the conservation of kākahi is also of cultural importance. Persistence of kākahi populations in the face of climate change has positive implications for Māori iwi and hapū. Thermal resilience may increase the likelihood of kākahi being maintained as an extant taonga species for future generations of tangata whenua in Aotearoa New Zealand. Further, freshwater ecosystems are also considered taonga for Māori. Thermal resilience of kākahi may promote ongoing freshwater health, contributing to the maintenance of the mauri (life force) of these culturally important ecosystems (NZ, 2020).

6.4 Limitations of the research presented in this thesis and suggestions for future research into the vulnerability of kākahi to increasing water temperatures

A major limitation of both Chapters 3 and 4 was a small sample size. This contributed to large standard errors when analysing the concentrations of haemolymph biomarkers and may have contributed to the lack of statistical significance in the results. In future, sample sizes of both the field and lab haemolymph samples should be increased, ideally to ≥30 samples per site in the field study and per treatment group in the laboratory study. A power analysis based on the results of this study would be advantageous. Future experiments should also consider increasing the sampling frequency over the seven-day period, to account for potential time-dependent changes in the biomarker concentrations.

More detailed environmental monitoring of field sites for various measures over the course of the year would provide more insights into the range of conditions experienced by each study site. Only spot recordings of temperature were made in our field study and we did not monitor temperature throughout the summer and winter. Conditions in one sampling year may not reflect the full range of conditions that kākahi might experience in other years. As kākahi are typically found in small streams or the littoral zones of lakes, some populations may well be exposed to much greater temperature extremes (e.g. water temperatures >30°C when climatic events such as scarce rainfall and increased sunshine allow temperatures to increase).

All kākahi in future laboratory experiments should be individually identifiable so that their dimensions (length, depth, and width) can be included in the results, enabling the investigation of size as a significant factor in the physiological response to a temperature

challenge. In Chapter 5, having to rely on consensus HSP70 sequences when designing primers was a limitation. In future studies, obtaining an entire genome sequence for kākahi would remove this barrier and improve the specificity of the primers able to be designed.

Physiological responses to temperature are not only species specific, but also population specific (Collins et al., 2020). Like any aquatic species, the thermal performance curves and limits of each population of kākahi have evolved based on the thermal history of the habitats they have lived in (Buckley & Kingsolver, 2021). The scope of this research limited the laboratory experiment to measuring the responses of only one kākahi population and using the results to predict species-wide responses to ACC. However, the responses of this population may be different to that of other populations. Therefore, future research should expose several populations to the same temperature treatments to account for potential differences in thermal physiology at the population level.Chapter 4 of this thesis was successful in investigating the physiological impacts of a chronic seven-day exposure to a constant, elevated water temperature. However, in wild habitats, temperatures fluctuate considerably over the course of a day, week, and month. Recent studies have found that physiological performance under fluctuating conditions cannot always be accurately predicted by performance under constant conditions (Williams et al., 2016; Marshall et al., 2021). Therefore, future studies should consider adjusting the experimental design, to measure the same biomarkers in kākahi haemolymph after exposure to fluctuating temperatures.

My research focused solely on temperature as a stressor when considering the vulnerability of kākahi to ACC. However, as discussed in Chapter 2, ACC is causing a series of changes to the aquatic environment, including changes in pH, oxygen concentration, and salinity. This combination of stressors is expected to have synergistic impacts on aquatic animal physiology (Pörtner, 2012). Therefore, future research should expand upon the research presented here to include co-stressors such as water pH and oxygen concentration for local kākahi habitats. This would enable a greater depth of understanding into the vulnerability of kākahi to ACC, in turn increasing the information available for use in kākahi conservation policy and management.

Due to the length of the experiments in this research and what was measured, the effects of the temperature treatments on long term fitness were not established. Due to the important interactions between heat stress, physiological performance, and fitness (see Chapter 2.2.3), future research should consider extending the length of the experiments to investigate the impacts of the same temperatures on long term fitness metrics such as growth and reproduction. This would strengthen the conclusions made about how future predicted water temperatures may affect the persistence of kākahi populations and therefore, the health of aquatic ecosystems in Aotearoa New Zealand.

This research provides important baseline knowledge of how water temperature affects metabolism and physiological performance in kākahi. The results presented in this thesis suggest that kākahi (*Echyridella menziesii*) may be a thermally resilient species to moderate warming under ACC. However, further research is required to provide a deeper understanding of their vulnerability or resilience to increasing water temperatures, and the other environmental changes expected due to anthropogenic climate change.

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Appendix

Mollusc species whose HSP70 sequences were used to design the consensus primers (see Chapter 5) and their associated NCBI accession/s. Most species names are given, but in some instances only the genus name was available on the NCBI database.

Mollusc species or genus	NCBI accession/s
Mytilus galloprovincialis	AJ783713.1 AJ783712.1 AJ783711.1 AB180909.1 AJ585375.1 AB180908.1 AY861684.1
Scapharca broughtonii	KX383795.1 KY660263.1
Ruditapes philippinarum	KJ569079.1
Lottia gigantea	XM_009053469.1 XM_009053468.1 XM_009058212.1 XM_009047345.1
Tegillarca granosa	JN936877.1
Paphia undulata	JX885711.1
Bathymodiolus nancyschneiderae	MN986910.1 MN986911.1 MN986908.1 MN986909.1
Bathymodiolinae genus	KF720591.1
Gigantidas mauritanicus	KF720565.1
Nipponacmea fuscoviridis	LC383932.1
Mytilus coruscus	KF322135.2
Hyriopsis cumingii	KJ123764.1
Idas	KF720581.1 KF720583.1 KF720568.1 KF720573.1 KF720557.1 KF720587.1 KF720586.1 KF720574.1
Vulcanidas insolatus	KF720558.1
Biomphalaria glabrata	XM_013236359.1
Vulcanidas	KF720592.1 KF720578.1 KF720582.1
Bathymodiolus azoricus	KF720580.1
Bathymodiolus boomerang	KF720579.1
Biomphalaria glabrata	AC233578.1 XM_013226102.1

	XM_013240932.1 XM_013240930.1 XM_013240929.1
Idas iwaotakii	KF720569.1 KF720559.1
Donax longissimus	KF720564.1
Cellana toreuma	JX169849.1
Bathymodiolus billschneideri	MN986906.1
Haliotis fulgens	MH220526.1
Bathymodiolus thermophilus	MN986912.1
Bathymodiolinae	KF720590.1 KF720577.1 KF720588.1 KF720561.1
Gigantidas crypta	KF720563.1
Haliotis diversicolor	FJ812177.1
Mytilisepta virgata	LC076447.1
Terua	KF720585.1
Solen grandis	JX462605.1
Pecten maximus	LR736841.1 LR736851.1
Idas simpsoni	KF720571.1
Idas modiolaeformis	KF720570.1
Gigantidas	KF720562.1 KF720555.1
Crassostrea gigas	LR761641.1 NM_001308924.1 AB122064.1 AB122063.1 AF144646.1 AJ318882.1 XM_034461664.1 XM_011457656.3 LR761643.1 XM_034457811.1 CP048844.1
Diplodon chilensis	MF774028.1
Conus miles	KY039343.1
Sinanodonta woodiana	KT923183.1
Corbicula fluminea	KJ461738.1
Crassostrea hongkongensis	GU586491.1 FJ157365.1
Crassostrea ariakensis	AY172024.1
Gigantidas tangaroa	KF720572.1
Conus frigidus	KY765612.1
Terua arcuatilis	KF720584.1
Sinonovacula constricta	JF748730.1
Conus monile	KU881653.1
Crassostrea virginica	XM_022459720.1

	AJ271444.1 XM_022472393.1
Benthomodiolus	KF720560.1
Pteria penguin	EF011060.1
Cyclina sinensis	JF740030.3