AUT

PhD Thesis

Multi-omics Approaches to Investigate Responses of New Zealand Green-lipped™ Mussels to Environmental Stress and Pathogen Loads

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Multi-omics Approaches to Investigate Responses of New Zealand Green-lipped™ Mussels to Environmental Stress and Pathogen Loads

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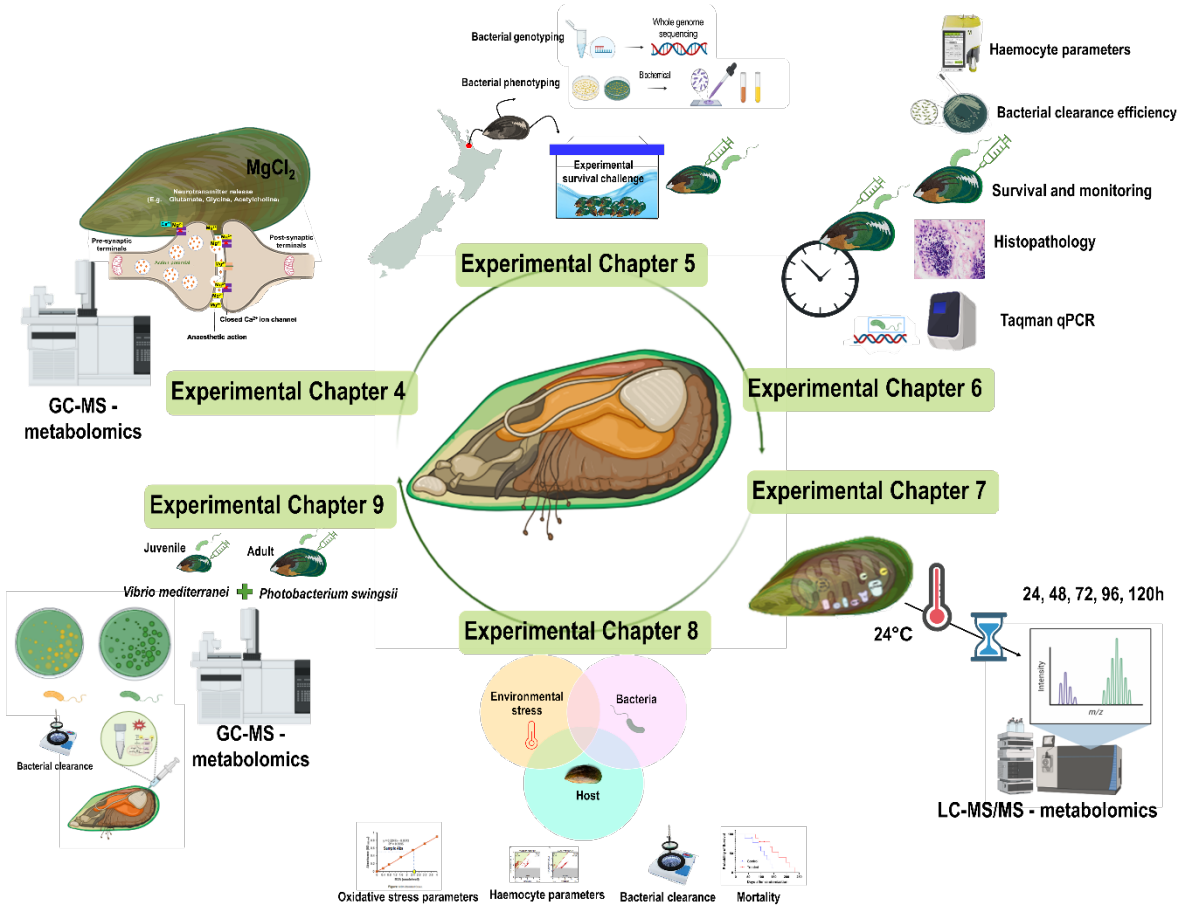
Dedication

To the pursuit of science and the wonders of marine research, which have fuelled my passion and shaped my academic journey. May our collective efforts continue to uncover the mysteries of the ocean and contribute to the conservation and understanding of our precious marine ecosystems.

To all the people dedicating their life's work to conserving and preserving our planet.

To my beloved mussels, for their ultimate sacrifices for this work.

Thesis Abstract



Greenshell™ mussels are important ecosystem engineers, with an established position in the aquaculture sector of New Zealand. Significant industry losses have been experienced, in terms of mass mortalities, on mussel farms in recent years. The exact cause of these mortalities is unknown, however association with increased water temperatures experienced during summer months have been deemed as a likely cause. Literature suggests that summer mortality involves intrinsic (immune dysfunction, gametogenesis, and spawning) and extrinsic (frequency and duration of heatwaves) factors, causing a physiological tipping point resulting in mussel death. Pathogen loads seem to proliferate during the summer months resulting in disease outbreaks. Novel research is required to study the impact of these mortalities and to mitigate disease outbreaks to assure health and sustainability for this industry. In this study, a multi-disciplinary approach, along with physiological evaluations, were employed to investigate the investigate the interplay of selected bacterial pathogens *Photobacterium* spp. and *Vibrio* spp. and temperature changes threatening the health status of adult, green-lipped

mussels, *Perna canaliculus*. This thesis consists of two literature reviews (**Chapter 2** and **Chapter 3**) and six experimental chapters (**Chapter 4** -**Chapter 9**).

Chapter 2 evaluates the immune status of Greenshell™ mussels and **Chapter 3** explores physiological biomarkers relating to *Vibrio* sp. infections in mussels. Building upon the insights gained from extensive literature reviews, the focus now shifts to the experimental chapters, aiming to illuminate potential intersections and broader implications within the research framework. The first experimental chapter of this thesis (**Chapter 4**) assesses the effect of magnesium chloride ($MgCl_2$) on adult Greenshell™ mussels' physiology and metabolic response. $MgCl_2$ significantly impacted the haemolymph metabolome in anesthetised mussels, indicating major physiological dysregulation. In **Chapter 5**, four bacterial isolates retrieved from moribund *P. canaliculus,* from a previous summer mortality event, were identified as *V. celticus*, *P. swingsii*, *P. rosenbergii and P. proteolyticum* using whole genome sequencing. Additionally, mussels injected with *P. swingsii* showed high mortality, along with expression of virulence genes (*hsp60*, *zm*, *vcpA*, *toxR*, *ompU*, *mshA*, *chi*, *lip*, and *plp*), suggesting pathogenesis of this bacterium to Greenshell™ mussels. Bacterial progression utilising *P. swingsii* was further investigated to better understand the mussel immune response and bacterial effect on mussel mortalities (**Chapter 6**). This study showed that the most profound effects of bacterial infection on mussels were seen at 48 hours post challenge (hpc) where mussel mortality, haemocyte counts and haemolymph colony forming units were the highest. The quantification of *P. swingsii* via targeted PCR showed highest levels of bacterial DNA at 12 hpc in the adductor muscle, gill, and digestive gland. Histopathological observations suggested a non-specific inflammatory response in all mussels associated with a general stress response. This study highlights the physiological effects of *P. swingsii* infection in Greenshell™ mussels and provides histopathological insight into the tissue injury caused by the action of injection into the adductor muscle.

In **Chapter 7**, the impact of temperature stress on the metabolome of mussels was investigated utilising metabolomics. Mussels were exposed to two temperatures, 16 °C and 24 °C for five-days, creating a controlled laboratory marine heatwave environment. The metabolite changes in the presence of temperature stress provided insights into several pathways involved in defence and repair mechanisms, which reallocated energy away from organismal growth towards maintenance. Since all mussels in this experiment survived, this suggests that *P. canaliculus* has the potential to adapt to heat stress up to 24°C, by regulating their energy metabolism, balancing nucleotide production, and implementing oxidative stress mechanisms overtime. In the next chapter (**Chapter 8**), an experiment was performed to investigate the effects of thermal stress, bacteria, and combined stressors on selected

immunological parameters and the survival of mussels. The total haemocyte count, viability, bacterial counts, total antioxidant capacity, and lipid peroxidation were used as indicators to measure an immune response of infected mussels to different temperatures. Water temperature at 24°C significantly affected immune functions and led to oxidative stress and reduction of immunosurveillance in the *P. swingsii* infected mussels. The combination of temperature-pathogen stress affected the survival of mussels with highest mortality at 24°C in the presence of bacteria. This chapter demonstrated that mussels have lower tolerance to the combined effects of high temperature stress and pathogen infection.

Chapter 9 investigated the effect of bacterial coinfection, on mussel size, bacterial clearance efficiency and metabolic response, considering juvenile and adult mussels. This study showed greater mortality in juvenile mussels within the bacterial coinfection group, suggesting that susceptibility of small mussels to bacterial infections are greater than in adults. Large decreases in energy metabolites were detected in mussels when exposed to multiple bacterial pathogens. Potentially due to high energy expenditure and metabolite functions to support immunity and protein synthesis during this pathogen interactions.

Collectively, these findings demonstrated a rigorous exploration of complex biological processes within New Zealand Greenshell™ mussels (*P. canaliculus*) and deepened the understanding of their general health, disease progression, transmission mechanism and host-pathogen interactions. These findings can be used to help assess *P. swingsii* transmission risk within and among Greenshell™ mussels' populations and facilitate appropriate management and restoration strategies for both wild and cultured mussel species.

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Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: _____

Date: 20.09.2023

Co-authors contribution

The co-authored literature review and experimental chapters in this thesis have been given a weighting (% time) to produce the completed output. As co-author, I hereby approve and declare that my role in this study, as indicated below, is representative of my actual contribution and I hereby give my consent that this work may be published as part of the PhD thesis of Awanis Azizan.

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Chapter 1: Introduction and thesis framework

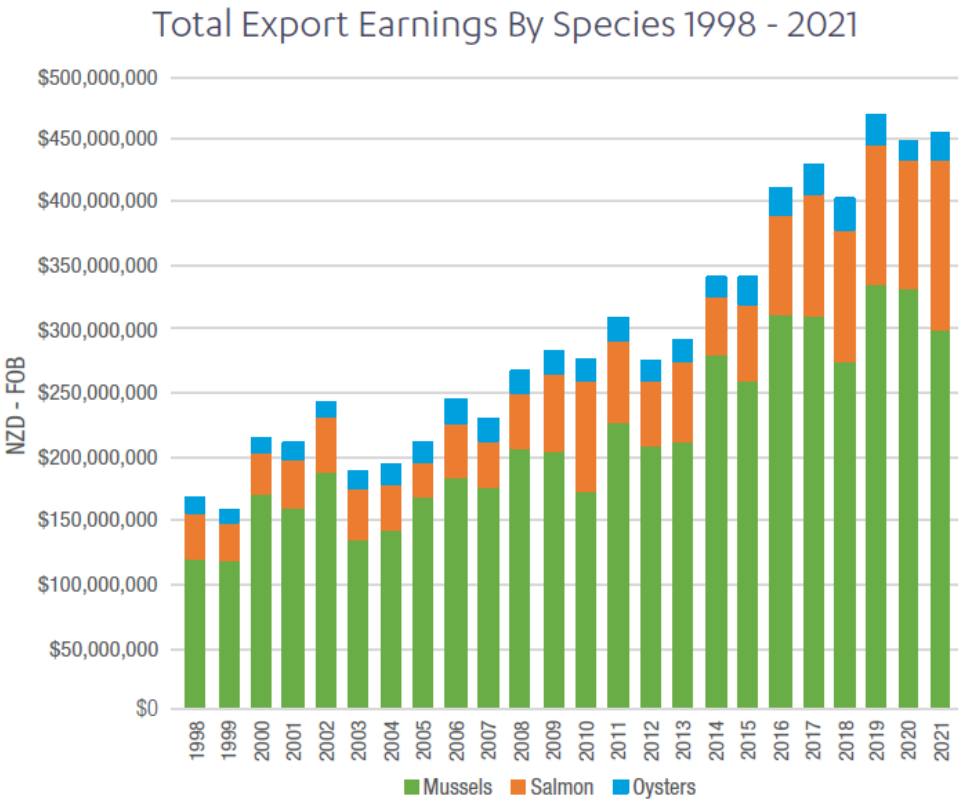
1.1 GENERAL INTRODUCTION

1.1.1 Global and New Zealand seafood production

Seafood has been a vital component of human nutrition since the beginning of time (FAO, 2016). Throughout human history, from the hunter-gatherer period through modern civilization, fish and seafood have been part of human diets. Thus, the importance of seafood remains pivotal, especially for communities living along coastlines and in river systems (Guthrie et al., 2022, Moore and Heilweck, 2022).

Aquatic foods, known as "blue foods," are gaining attention for their potential to address nutrition and sustainability. They encompass a wide range of species from both wild capture and aquaculture production (Golden et al., 2021). However, the diversity of these foods and the factors driving their production remain unclear. Aquaculture is on the rise, especially in low- and middle-income countries, but faces challenges in different regions, particularly the disparities between wild capture and farmed aquatic foods dynamics (Gephart and Pace, 2015). International trade and domestic consumption play significant roles in this complex landscape, highlighting both challenges and opportunities in the global aquatic food industry.

Global fish and shellfish production is estimated to have reached about 179 million tonnes in 2018, with a total first sale value estimated at USD 401 billion, of which 82 million tonnes, valued at USD 250 billion, came from aquaculture production (IbisWorld, 2022). New Zealand (NZ) aquaculture has followed a similar trajectory and is becoming an increasingly important economic contributor (Fig. 1.1a and Fig. 1.1b). The market size of the Fishing and Aquaculture industry in New Zealand has grown 1.6% per year on average between 2017 and 2022 (IbisWorld, 2022, Pinu et al., 2019). Despite New Zealand aquaculture being largely based on growing Greenshell™ mussels (*Perna canaliculus*) (84%), Chinook salmon (*Oncorhynchus tshawytscha*) (12%) and oyster (*Crassostrea gigas*(2%), there are excellent opportunities for the development of other species as the world's appetite for New Zealand seafood is increasing (Heasman et al., 2020). The other small commercial species in terms of value of exports included abalone (*Haliotis iris*) for meat and shell jewellery, seaweed products and freshwater crayfish (*Paranephrops* sp.), lobsters and squid (Heasman et al., 2020). The need for diversification of aquaculture species is recognised with collaborative research and development projects already underway.



	Mussel	Salmon	Oyster
Harvested Product (green weight tonnage)	**82,792**	**16,258**	**1,520**
Export Revenue (NZ$ millions)	**$302m**	**$150m**	**$16m**
Est. Domestic Revenue (NZ$ millions)	**$45m**	**$150m**	**$8m**
Est. Total Revenue (NZ$ millions)	**$347m**	**$300m**	**$24m**
Est. Total Sector Revenue (NZ$ millions)	**$671m**		

Figure 1.1. a) Total export earnings by species 1998 – 2021, b) Value of New Zealand aquaculture exports in 2022 [Source: AQNZ (2022)].

1.1.2. New Zealand Greenshell™ mussel aquaculture

The culturing of the *Perna canaliculus* is a well-established shellfish aquaculture industry (Vakily, 1989). The distribution of *P. canaliculus* stretches along the South Island, and the

northern areas of New Zealand (Fig. 1.2) where they generally inhabit rocky reefs, wharf piles, and soft muddy bottom habitats (Morton and Miller, 1973). The controlling factors for their distribution are environmentally driven, dependant on temperature and salinity parameters. *P. canaliculus* tolerates temperature ranges from 5.3°C (in the far south) to 27°C (in the north) and can survive at salinity between 30 to 35 practical salinity units (Flaws, 1975, Hickman and Illingworth, 1980). Farming of mussel uses longline systems comprised of a taut 'backbone' strung across a line of surface floats and anchored to the seafloor at each end. At the sea-surface, the backbone usually extends across about 110 m and carries dropper-ropes. The crop mussels are seeded onto the dropper, which is suspended from the backbone in a series of loops. Depending upon water-depth, each loop may extend to about 20 m. A backbone may carry approximately 2.5 – 3.5 km of dropper in total (South et al., 2022).

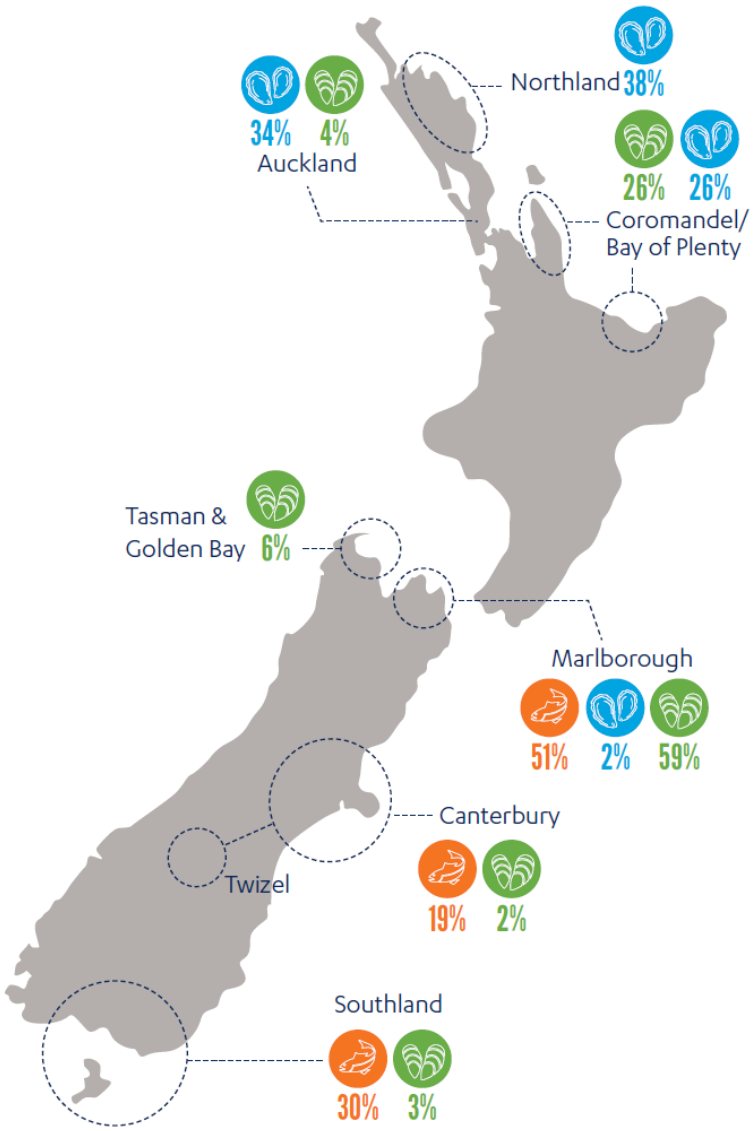


Figure 1.2. Key aquaculture areas of green-lipped mussels in New Zealand [Source: AQNZ (2022)].

1.1.3 Infectious diseases in marine mollusc and bivalve immunity

Disease outbreaks pose a significant threat to aquaculture development, resulting in significant financial losses (Lane et al., 2022). It has been reported in the last few decades that bivalve aquaculture has suffered from diseases at all stages of the lifecycle, whether it is larval, juvenile, or adult, and that some of the industries have even collapsed as a result (Castinel et al., 2019). Mortalities in bivalves have been associated with viruses, bacteria, and protozoa, leading to mortality at each developmental stage. Pathogens and parasites that may be hazards to the New Zealand bivalve aquaculture industry include abalone viral ganglioneuritis virus, two herpesviruses (abalone herpesvirus and ostreid herpesvirus 1 microvariants), parasites (*Bonamia exitiosa*, *Bonamia ostreae*, *Hexamita* spp., *Marteilia refringens, Mikrocytos mackini*, *Labyrinthuloides haliotidis, Margolisiella* (=*Pseudoklossia*) *haliotis, apicomplexan parasite X (APX), Perkinsus marinus* and *Perkinsus olseni*), infectious pancreatic necrosis-like virus and other aquatic birnaviruses, Haplosporidiosis (*Haplospordian tumefacientis, Haplospordian* sp.), Platyhelminth flatworms (flatworms, including *Postenterogonia orbicularis, Urastoma cyprinae* and a member of the family Planoceridae), trematodes (including *Cercaria* spp*., Protoeces* spp., *Bucephalus* spp.), red worm disease (*Mytilicola intestinalis, M. orientalis*), bacterium (*Xenohaliotis californiensis*), cyanobacteria including *Plectonema terebrans*, *Hyella caespitosa, Mastigocoleus testarum, Mastigocoleus* sp*.* and aggregated cyanobacterium *Pleurocapsa* sp. intracellular bacterial disease (Rickettsiales and/or Chlamydiales and/or an unidentified mycoplasma) (Georgiades et al., 2016, Webb and Duncan, 2019, Newton and Webb, 2019). Moreover, diverse strains of bacteria belonging to *Vibrionaceae* family have been implicated in bivalve mortality outbreaks in hatcheries, commercial farms, and natural habitats. Examples of common *Vibrio* species detected in New Zealand shellfish include *V. fluvialis* II, *V. harveyi, V. splendidus*, *V. tubiashii*/*coralliilyticus* and *Photobacterium swingsii* (Georgiades et al., 2016).

There have been notable mortality events associated with the detection of pathogens in adult New Zealand Greenshell™ mussels (*P. canaliculus*) (Ericson et al., 2023c). The occurrence of mortalities in Greenshell™ mussels has been linked to a condition known as summer mortality with increasing evidence that implicates the involvement of opportunistic or pathogenic marine bacteria after one or more stress events. Summer mortality has also been observed in many shellfish aquaculture industries around the world (Garnier et al., 2007, Malham et al., 2009, Newton and Webb, 2019, Ashton et al., 2020), where pathogens (e.g., *Vibrio* spp*.,* OsHV-1) are also often identified as a cause of death (Li et al., 2012). During the summer of 2018, bacteria were isolated from Greenshell™ mussels as part of diagnostic

investigations into mussel summer mortalities potentially due to increasing water temperatures experienced in the summer months around New Zealand (Nguyen and Alfaro, 2020b). Mussel cultivation zones along the Coromandel coast in the northeastern region of New Zealand, along with other northern areas of the country, have witnessed elevated sea surface temperatures (SSTs) in recent times (Stevens et al., 2021). Depending on the magnitude and duration of this warming, it has the potential to trigger marine heatwaves (MHWs), characterised by periods when SSTs surpass the seasonally varying 90th percentile for at least five consecutive days (Oliver et al., 2017). In the Marlborough Sounds, a crucial mussel farming location situated in the northern part of New Zealand's South Island, sea surface temperatures exceeding 20°C are now documented during the summer season (Broekhuizen et al., 2021). There has been a rise in the occurrences of Greenshell^{TM} mussels mortality, which can lead to significant economic losses (Newton and Webb, 2019). Such instances of mortality tend to happen when seawater temperatures rise notably during the summer months. To illustrate, during the summer of 2018, as seawater temperatures hit 25 °C, as many as 20% of cultivated mussels in the Firth of Thames detached from their lines (Peart, 2019). As a next phase of the project the isolates were subjected to follow-up tests to characterise them based on phenotypic and genotypic approach, and to compare the relative pathogenicity of the various isolates in *P. canaliculus* in a laboratory-based study. Of importance from these results is the identification of *Photobacterium swingsii*, a bacterium from the *Vibrionaceae* family, causing the highest mortality rates in mussels in a controlled experiment, which is now reported in *P. canaliculus* in association with summer mortality events, for the first time (Azizan et al., 2022).

Due to the frequency of mortality events in molluscs, especially bivalves, and the economic importance of many bivalve species in aquaculture and coastal ecosystems, there is a growing number of studies on the detection, rapid identification, reliable differentiation of species and progression of diseases of the bacterial pathogen (Soto et al., 2010, Gudkovs et al., 2016). A histological or ultrastructural examination cannot differentiate the tested pathogen from the normal microbiota in the tissues due to the dynamic nature of disease progression. Hence, it is challenging to associate the clinical signs of Photobacteriosis in Greenshell^{TM} mussels with the impairment of Greenshell^{TM} mussel tissues and cells at the time of infection. There has been significant progress in understanding the dynamics of pathogen distribution and concentration in vivo during infection (Travers et al., 2008a, Howells et al., 2021, Wang et al., 2021b) Real-time PCR (qPCR) and omics methods are routinely used in diagnostic laboratories to detect particular pathogens, diagnose many diseases, and have been applied to study the onset and progression of Photobacteriosis in bivalve tissues (Soto et al., 2010, Reichley et al., 2015, Wang et al., 2021b). Advanced molecular methods, such as this are

useful for (1) identifying individuals at risk of disease outbreaks and (2) conducting high-throughput surveillance testing of overtly healthy mussel populations for certification and management of stocks.

Considering that marine heatwaves are lasting longer, and causing more damage to aquaculture species and threatening economic and ecological security, a better understanding of combined environmental stressors need to be researched and this research needs to be effectively transferred into farming practices (Fisheries New Zealand, 2021). Despite the well-documented physiological response of mussels to acute thermal stress, the interaction of multiple stressors has yet to be explored. Many shellfish mortalities are thought to be caused by interactions between multiple stressors, which refers to the simultaneous presence or occurrence of multiple stress factors or environmental pressures that can impact a biological system or ecosystem, but further research is needed to confirm this (Ericson et al., 2022, Muznebin et al., 2022a). Of crucial importance is the understanding and evaluation of how thermal stress and *Photobacterium* sp. infections affect Greenshell^{TM} mussels physiological stress and immune responses.

In the pursuit of a comprehensive understanding of the intricate interactions within complex biological systems, modern science has turned to 'omics' methodologies, such as metabolomics, genomics, and quantitative polymerase chain reaction (qPCR). These techniques offer a high-resolution view into the biochemical and genetic components that underpin the behaviour, response, and health of organisms. Metabolomics, for instance, aims to unravel the dynamic metabolic profiles of organisms, shedding light on physiological responses, stress mechanisms, and the subtle shifts that may herald health issues (Alfaro and Young, 2018). Genomics delves into the genetic underpinnings, helping classifies the bacterial species identity and responses to environmental stressors (Symonds et al., 2019). Then again quantitative polymerase chain reaction (qPCR), grants us the precision to quantify the expression of specific genes, allows for discrimination of congeneric pathogens (Ríos-Castro et al., 2022), and is useful to monitor the progress of an infection (Michael et al., 2015), uncovering molecular responses with unparalleled accuracy (Lacroix et al., 2014). While these methods are often associated with their intricacy and expense, their utilisation brings forth a wealth of valuable information that cannot be gleaned through traditional approaches. This could empower the aquaculture industry to monitor the well-being of farmed organisms in real-time, detect early signs of stress or disease, and take timely preventive or corrective measures.

In lieu of the New Zealand governments goals of sustainable aquaculture industry growth utilising a resilient model (Fisheries New Zealand, 2021), this research supports efforts to strength biosecurity management in a changing environment by exploring new conditions under which pathogenic bacterium *P. swingsii* affects Greenshell™ mussels. Considering the worldwide epizootics with big economic losses in Greenshell™ mussels aquaculture, the rationale underlying this research is that this information is essential for improving management strategies to establish mussel aquaculture that is less susceptible to a globally changing environment. The research described here is significant because it determined how marine heatwaves are likely to compromise mussel immunity and susceptibility to bacterial infection in combination with thermal stress. Additionally, this research developed a platform that can be extended to study the impact of these multi-stressors and other pathogens (or commensals) for other infecting shellfish hosts, hence strengthen our understanding of the causality behind the summer mortalities in the larger aquaculture context. Ultimately, the results have an important positive impact as baseline information assisting with the development of animal health strategies and policies for mitigating the risks.

1.2 THESIS AIMS

Mussels serve as a robust model organism for elucidating and investigating the physiological principles governing molluscan defence mechanisms. **This thesis aims to investigate the interplay of selected bacterial pathogens *Photobacterium* spp. and *Vibrio* spp. and temperature changes threatening the health status of adult, Greenshell™ mussels, *Perna canaliculus*.** Currently, there is limited information on bacterial pathogens of Greenshell™ mussels and few studies have described the underlying disease leading to massive mortalities. The findings provide insights into novel bacteria isolated from a mass mortality event that can negatively affect survival of Greenshell™ mussels. In addition to identification and characterisation of novel bacteria, the immune mechanisms of mussels were investigated and allow for the identification of relevant biomarkers for these processes. Bacterial typing, animal challenge tests, metabolomics, different flow cytometry assays and histopathological analysis were used to allow for a more detailed picture of the host's responses to external stressors at both cellular and molecular levels and generate accurate biomarker signatures for mitigation strategies.

The specific objectives of this thesis were to:

1. Optimise method using magnesium chloride ($MgCl_2$) as anaesthetic for sampling standardisation on the adult Greenshell™ mussels.
2. Identify and characterise bacteria isolated from a summer mortality outbreak and assess the pathogenicity.
3. Develop a targeted bacterium species-specific Taqman® based real-time PCR for *Photobacterium swingsii* for tracking the infection progression of the selected bacterial pathogen in the laboratory challenged mussel.
4. Examine mechanistic insight the response of mussel metabolome response to elevated seawater temperature.
5. Examine how thermal stress and pathogen infection can negatively affect the survival and immune responses of mussel under laboratory challenged experiment.
6. Investigate the physiological changes in the haemolymph of juvenile and adult Greenshell™ mussels infected with *Vibrio mediterranei*, *Photobacterium swingsii*, and a combination of both strains in the laboratory challenge.

1.3 THESIS STRUCTURE

In order to achieve the overall aims of this thesis, six experiments were conducted (Fig. 1.3). The results from these studies were published as peer reviewed articles and are presented in this thesis as six experimental case studies in Chapters 4-9. These case studies, in combination with two review papers, introduction, discussion and conclusion comprise the 10 chapters of the thesis, as follows:

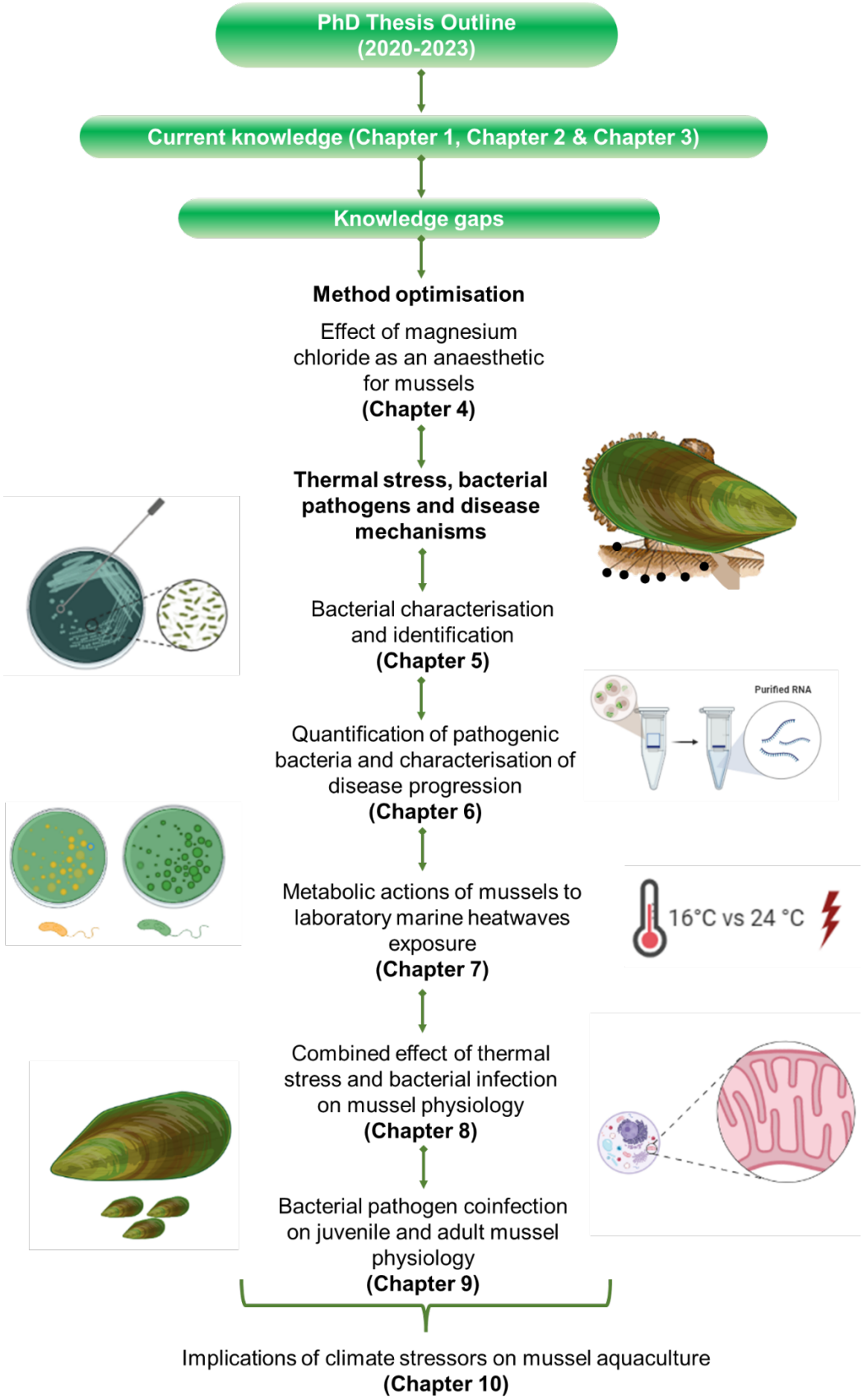


Figure 1.3. Schematic overview of the topics addressed in this thesis.

A review of the relevant literature on mussel immunity related to the Greenshell™ mussel health and disease assessment, along with some insight into mussel biology and physiology is presented in **Chapter 2**. A review of the relevant literature on biomarker research on *Vibrio*-mussel interactions, and aspects related to the mussel health and disease assessment, along with some insight into mussel biology and physiology is presented in **Chapter 3**. The specific effects of muscle-relaxing agents on baseline metabolism in invertebrates are unknown, however, it is evident that chemical applications alter molecular, cellular, and physiological parameters. **Chapter 4** investigates the effect of the muscle relaxant magnesium chloride ($MgCl_{2)}$ on the metabolic response of Greenshell™ mussels. Since no bacterial pathogens have been described (by name and species) as causative agent of mortalities in adult Greenshell™ mussels **Chapter 5**, identifies and characterises isolated bacteria pathogens belonging to *Vibrionaceae* family and tests the survivability of these isolates on mussels via an injection challenge test**.** To track the infection progression of the selected bacterial pathogen, *Photobacterium swingsii* in the laboratory challenged mussels, a targeted bacterium species-specific primer was developed and tested, to measure bacterial specific infection and concentration patterns in different mussel tissues (**Chapter 6**). Greenshell™ mussel responses to elevated water temperature, which occur during marine heatwave events are not well characterised. **Chapter 7** investigates mechanistic insights into the response of the metabolome of Greenshell™ mussels in relation to a five-day laboratory-based marine heatwave exposure. In **Chapter 8,** dual stressors of temperature and pathogen exposure, are used to determine if there is a synergistic, antagonistic, or a non-significant effect on *P. canaliculus* survival, bacterial clearance efficiency, selected haemocyte parameters and sex. The findings of this research provide novel insights into the interaction between temperature and bacteria that can be used to develop strategies and policies to mitigate the risk of animal diseases for the prevention of mass mortalities. While temperature stress and bacteria are both factors that induce oxidative stress in shellfish, it is unclear how the combination affects mussel physiology. Therefore, **Chapter 9** assesses metabolic response to effects of bacterial pathogen coinfection in mussels, utilising juvenile and adult Greenshell™ mussels exposed to a single pathogen and combination of multiple pathogens. The most important findings are highlighted and discussed in **Chapter 10** together with final conclusions and future perspectives.

1.4 THESIS OUTPUTS

1.4.1 Peer-reviewed papers

The following publications originated from this study:

AZIZAN, A., ALFARO, A. C., YOUNG, T. & VENTER, L. 2021. Beyond relaxed: magnesium chloride anaesthesia alters the circulatory metabolome of a marine mollusc (*Perna canaliculus*). *Metabolomics*, 17, 1-11.

AZIZAN, A., ALFARO, A. C., JARAMILLO, D., VENTER, L., YOUNG, T., FROST, E., LEE, K., VAN NGUYEN, T., KITUNDU, E., ARCHER, S. D. J., ERICSON, J. A., FOXWELL, J., QUINN, O. & RAGG, N. L. C. 2022. Pathogenicity and virulence of bacterial strains associated with summer mortality in marine mussels (*Perna canaliculus*). *FEMS Microbiology Ecology*, 98, 1-14.

AZIZAN, A., VENTER, L. & ALFARO, A. C. 2023. A review on green-lipped mussel, *Perna canaliculus* immunology: The drivers, virulence factors, advances, and applications. *New Zealand Journal of Marine and Freshwater Research*, Accepted.

AZIZAN, A., VENTER, L. & ALFARO, A. C. 2023. Physiological biomarkers of mussel Vibrionaceae: A review on the constraints and potentials. *Aquaculture international*, Submitted.

AZIZAN, A., ALFARO, A. C., VENTER, L., JARAMILLO, D., BESTBIER, M., FOXWELL, J., BENNET, P. & YOUNG, T. 2023. Quantification of pathogenic *Photobacterium swingsii* and characterisation of disease progression in the New Zealand Greenshell™ mussel, *Perna canaliculus*. *Journal of Invertebrate Pathology*, Submitted.

AZIZAN, A., VENTER, L., JANSEN VAN RESBURGN, P. J., ERICSON, J. A., ALFARO, A. C. & RAGG, N. L. C. 2023. Metabolite changes of *Perna canaliculus* following a laboratory marine heatwave exposure: Insight from metabolomics analysis. *Metabolites*, *13* (7), 815.

AZIZAN, A., ALFARO, A. C., VENTER, L., ZHANG, J. J., ERICSON, J. A., YOUNG, T., DELORME, N. J. & RAGG, N. L. C. 2023. Interactive effects of elevated temperature and *Photobacterium swingsii* infection on the survival and immune response of marine mussels (*Perna canaliculus*). *Science of The Total Environment*, Submitted.

AZIZAN, A., CARTER, J., ALFARO, A. C., VENTER, L., YOUNG, T., SHARMA, S. S. & CHEN, T. 2023. Investigating the effect of bacterial co-infections on juvenile and adult, green-lipped mussels (*Perna canaliculus*). *Journal of World Aquaculture Society*, Accepted.

1.4.2 Presentations at conferences, symposia, or workshops

The following conference contributions resulted from this study:

AZIZAN, A., et al. (2022). Pathogenicity and virulence of bacterial strains associated with summer mortality in marine mussels (*Perna canaliculus*). Oral presentation, Waiti Waita - for The NZMSS & NZFSS Joint Conference 2022.

AZIZAN, A., et al. (2022). Pathogenicity and virulence of bacterial strains associated with summer mortality in marine mussels (*Perna canaliculus*). Oral presentation, AUT Postgraduate research symposium 2022.

AZIZAN, A., et. al. 2021. Beyond relaxed: magnesium chloride anaesthesia alters the circulatory metabolome of a marine mollusc (*Perna canaliculus*). Poster presentation, Virtual AUT Postgraduate Research Symposium, November 2021, Virtual event.

AZIZAN, A., et. al. 2021. Beyond relaxed: magnesium chloride anaesthesia alters the circulatory metabolome of a marine mollusc (*Perna canaliculus*). Lighting talk. Asia Pacific Virtual Podium, November 2021, Virtual event.

AZIZAN, A., et. al. 2021. Pathogenicity and virulence of bacterial strains associated with summer mortality in marine mussels (*Perna canaliculus*). Oral presentation. Physiomar Conference, September 2021, Virtual event.

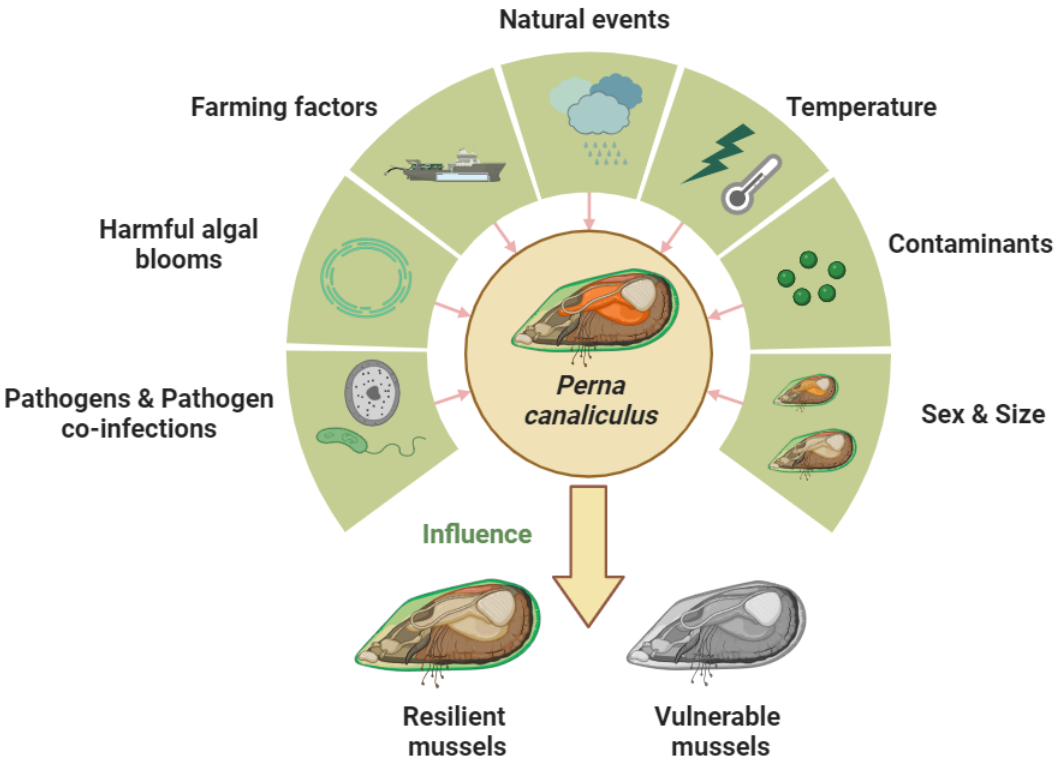
AZIZAN, A., et. al. 2021. Beyond relaxed: magnesium chloride anaesthesia alters the circulatory metabolome of a marine mollusc (*Perna canaliculus*). Poster presentation. Physiomar Conference, September 2021, Virtual event.

Awards

Best Poster Award at the AUT Postgraduate Research Symposium 2021.

Highly Commended Student Poster Presentation at Physiomar Conference 2021.

Chapter 2: A review on green-lipped mussel, *Perna canaliculus* immunology: The drivers, virulence factors, advances, and applications



In this chapter, a review of the relevant literature on mussel immunity related to the Greenshell™ mussel health and disease assessment, along with some insight into mussel biology and physiology is presented.

This chapter has been prepared for publication:

AZIZAN, A., VENTER, L. & ALFARO, A. C. 2023b. A review on green-lipped mussel, *Perna canaliculus immunology*: The drivers, virulence factors, advances, and applications. *New Zealand Journal of Marine and Freshwater Research*, Accepted.

Abstract

The endemic, green-lipped mussel (*Perna canaliculus*), trademarked as Greenshell™ mussel, contributes most to the New Zealand aquaculture industry based on tonnage and export value. Research on mussel immunity is motivated greatly by economical and biosecurity necessities. Indeed, mussel aquaculture is threatened by pathogenic micro-organisms and environmental stressors. As such there is a need to understand the mechanisms that drive mussel immune responses and the associated interactions with the environment. Specifically, this review 1) characterises *P. canaliculus* and examines the existing immunological studies conducted on this particular mussel species, 2) evaluates the literature pertaining to the cellular and humoral components of mussel immunity, 3) identifies and discusses pathogens that are relevant to green-lipped mussels, 4) focuses on the virulent factors employed by mussel pathogens likely to induce diseases, 5) provides a comprehensive analysis of the response mechanisms employed by mussels when faced with various stressors, and 6) delves into the applications of omics approaches and explores future perspectives for immunological studies in mussels. Finally, this review highlights diverse strategies proposed to bolster mussel aquaculture, drawing upon insights from immunological research. These strategies encompass gene rearrangement mechanisms, probiotics, immunostimulants, and selective breeding, all of which have shown promise in enhancing the health and resilience of mussel populations. By exploring these immunological findings and their practical applications, this review paper contributes to the advancement of sustainable mussel aquaculture practices, paving the way for improved productivity and disease management in the industry.

Keywords

Bacteria, Cellular defence, Greenshell™ mussel, Humoral defence, Immunity, Mechanisms, New Zealand, Omics, Pathogens, Virulence.

2.1 INTRODUCTION

The Greenshell™ mussel farming industry is leading the New Zealand aquaculture sector in terms of production volumes and export revenue (Lane et al., 2020). Compared to other commercially cultivated bivalve species, *Perna canaliculus* farmed stocks have experienced relatively few disease outbreaks to date (Castinel et al., 2019). However Greenshell™ mussels are not immune to disease threats, necessitating awareness and baseline mussel health information as precautionary measure (Rolton and Ragg, 2020). More efforts are needed to acquire a detailed understanding of the immunological pathways implemented by *P. canaliculus* when exposed to biotic and abiotic factors. Stressors, such as pathogens and

environmental stressors are being investigated using multiple levels of biological organisation (e.g., molecular, biochemical, cellular, organismal) (Waller and Cope, 2019). The impacts of multi-stressors on the marine environment, and the combination of omics research approaches along with histopathological assessments, is seen as a unique opportunity to quantify and identify aspects of disease physiology. As downstream tools, omics applications can be combined with upstream phenotyping tools to characterise the profiles of different cells and tissues (Nguyen and Alfaro, 2020a). The new knowledge generated from these emerging approaches will no doubt improve our understanding of bivalve physiology, especially with regards to immune defences to pathogens and environmental stresses. Additionally, proactive disease prevention strategies, along with risk analyses of current pathogens within farmed shellfish, and surveillance and mitigation measures will be key in maintaining sustainable aquaculture farms (Fox et al., 2020).

Collectively, this review showcases the current knowledge on the New Zealand Greenshell™ mussel (*P. canaliculus*) immunology and summarises the key immunological responses of this species to the presence of stressors. The focus of this review is placed on pathogens and diseases reported within *P. canaliculus*, encapsulating factors that determine the prevalence of bacterial pathogens. Furthermore, this review explores the diverse modes of transmission employed by bacterial pathogens, unravels the underlying mechanisms driving their virulence, and provides elucidation on the intricate interplay between the host and pathogens. The effect of biotic and abiotic stressors on *P. canaliculus* immunity are also reported, along with an overview on omics approaches to study bacterial pathogens. Understanding the fundamental mechanisms that govern the immune response and stress adaptation in mussels holds significant scientific implications. This knowledge not only contributes to our comprehension of mussel biology, but also provides crucial insights for future immunological studies across various mussel species. Moreover, it enables us to anticipate and predict potential community changes. Finally, this review synthesizes the practical applications of mussel immunity knowledge within the industry and discusses some potential next steps necessary to facilitate the development of effective measures for mitigating mussel diseases and safeguarding existing healthy populations.

2.2 *PERNA CANALICULUS*

Green-lipped mussels, trademarked as Greenshell™ mussels (*Perna canaliculus*) are endemic to the inshore coastlines of New Zealand (NZ), and are classified as the most important aquaculture species in NZ. Approximately 303 million NZD was generated from

Greenshell™ mussel exports in 2021. These mussels are typically farmed for food and nutraceutical products (such as oil extracts and powders). From an indigenous context, *P. canaliculus* is a treasured species, with an active role as guardian in the natural environment (Castinel et al., 2019, Miller et al., 2023). *P. canaliculus* is also used as a model indicator species in NZ, where it serves as a reliable bioindicator of coastal contamination and supports biomarker research on the effects of multiple environmental stressors (Webb et al., 2020). *P. canaliculus* mussels are grown in shallow coastal waters, on longline systems for approximately 15-18 months until a harvestable size of 90-100 mm is achieved (Stenton-Dozey et al., 2021). In these culture systems, an increasingly diverse range of biofouling organisms (i.e., algae, worms) and potential pathogens are present, leading to escalated production costs (Soliman and Inglis, 2018) and possible threats to the aquaculture industry (Georgiades et al., 2020).

Morphologically *P. canaliculus* mussels have two shell valves hinged together by an umbo. Byssal threads often hang outside the shell and are utilised for attachment to other mussels and surfaces to improve stability and protection in the mussel bed (Zhao et al., 2021). As a suspension-feeding bivalve, *P. canaliculus* feeds by pumping water through their gill filaments (i.e., ctenidia) to filter out particles onto the surface of ctenidium, while also eliminating the ingested particles as pseudofaeces. The gills are the main site of interaction with the surrounding environment, acting as an important organ for oxygen uptake, bioaccumulation site for contaminants or bacteria and evacuation of waste (Gui et al., 2016). The gills are composed of various epithelial cells, mucous glands, cuboidal respiratory epithelium, trabecular cells and infiltrated haemocytes, which contributes to the recognition or agglutination of filtered pathogens (Saco et al., 2020). Like the gills the mantle is covered by mucus and constantly exposed to microbes from the external environment (Gerdol, 2017). The digestive gland is composed of basophilic cells supporting enzyme production and secretion and digestive cells with lysosomal content important for intracellular digestion, detoxification (Dimitriadis et al., 2004) and immunity (Allam and Raftos, 2015).

Mussel tissues, such as haemolymph, gills, mantle, and hepatopancreas (digestive gland), are frequently utilised to monitor immune function as they serve as major sources of immune molecules (Nguyen et al., 2019c). Furthermore, mussel haemolymph have been well-studied for understanding aspects of cellular and humoral immune response (Green et al., 2019). Haemolymph contains haemocytes, which are responsible for main cellular defence mechanisms such as pathogen phagocytosis, encapsulation, infiltration, and production of reactive oxygen and nitrogen species. Additionally, haemocytes are rich in hydrolytic enzymes and express proteins involved in pathogen recognition and agglutination (Campos et al.,

2015). Haemocytes are found in all internal spaces of mussels, circulating the haemolymph, surrounding all tissues, and migrating into the pallial and extrapallial spaces (Saco et al., 2020). Considering the ease of haemolymph extraction and its crucial role in innate immunity, most immunological studies or research on the effects of infections in *P. canaliculus* have focused on haemolymph as the primary biological sample (Fig. 2.1). Previous studies have also utilised multiple tissues including gills, adductor muscles, hepatopancreas, digestive glands, and mantle tissues for a comprehensive assessment of the overall immune system and its responses to various stressors and pathogens (Nguyen et al., 2019c). Mussels from various life stages, such as larvae and spat, as well as sections of the whole animal itself (histological sections) has also been document within immune studies.

In this review, a comprehensive literature review and analysis of immunological studies conducted on *P. canaliculus* were conducted to provide insights (Table 2.1) with the following criteria implemented: 1) the study had to measure an immune response following the presence of a stressor, 2) in either field or laboratory study; 3) the methods had to clearly indicate which sample and tests (assays) were used to detect the immune response; and 4) the study had to be evaluated on *Perna canaliculus*. Searches were carried out in the following online bibliographic databases: Scopus, Google Scholar, JSTOR, Web of Science, PubMed and Elsevier. The keywords used for manual searches were bacteria, cellular defence, greenshell^{TM} mussel, humoral defence, immunity, mechanisms, New Zealand, omics, pathogens and virulence.

Interestingly, immunological studies on *P. canaliculus* have increased in the last five years (30 out of the 36 studies were published between 2018-2023, ordered by year of publication). Method-wise the use of bioassays, flow cytometry and gas chromatography-mass spectrometry (GC-MS) have been mainly used to investigate *P. canaliculus* immunology, while microscopy, in situ hybridisation, polymerase chain reactions (PCR) and genotyping also being incorporated in various studies. Compared to other mytilid species, data from genomic and protein databases, are largely unavailable for *P. canaliculus*. Yet, the molecular data from other species enables comparative immunological studies to confer findings and update knowledge on *P. canaliculus* immunity. Yet, the molecular data from other species enables comparative immunological studies to confer findings and update knowledge on *P. canaliculus* immunity. Significant research effort characterising immune systems of bivalves (Allam and Espinosa, 2016, Zannella et al., 2017, Grinchenko and Kumeiko, 2022) and particular oyster immunity (Wang et al., 2018b, Adzigbli et al., 2020, Petton et al., 2021), have significantly enriched our understanding of immunological processes implemented by mussels. Mussel

immunity is briefly discussed, with a focus on the functions of the immune processes, key genes, and pathways relating to significant diseases and stressors. Importantly, we also highlight the application of immunological research or the lack thereof in *P. canaliculus*.

Table 2.1 Immunological studies performed on *P. canaliculus* electing an immune response following exposure to a stressor or detection of a pathogen.

Year	Tissues	Methods	Stressors	Field study	Laboratory study	Cellular defence	Humoral defence	Bacterial characterisation	Immune responses	References
2009	Larvae	1) TCD bioassays 2) Histology 3) 16S rRNA sequencing	Pathogen		X	X		X	Identified *Vibrio splendidus* and a *V. coralliilyticus* / *neptunius*-like isolate. Bacterial treated group showed: • Detachment of cilia • Aggregation of bacterial cells around the velum • Tissue necrosis	(Kesarcodi-Watson et al., 2009a)
2013	Haemolymph Gill	1) Differential cell counts 2) Commet assay	Cadmium		X	X			Cadmium contaminated group showed: • ↑basophils and eosinophiles • ↑formation of micronuclei, nuclear buds, fragmented-apoptotic cells, and bi-nuclei • ↑DNA damage	(Chandurvelan et al., 2013)
2013	Larvae	Bioassays	Thermal stress		X		X		HSP70 induction	(Dunphy et al., 2013)
2015	Gill	GCMS metabolomics	Thermal stress		X		X		Metabolite changes	(Dunphy et al., 2015)
2016	Gill Haemolymph Mantle Digestive gland Muscle	1) ICP-MS 2) Microscopic observations 3) Bioassays	Earthquake disturbances	X		X	X		Mussels from the Port of Lyttelton: • ↓metallothionein-like protein • ↓catalase activity • ↑lipid peroxidation • ↑alkaline phosphatase • ↓hyalinocytes • ↑eosinophils, basophils • ↑micronuclei, binuclei, fragmented-apoptotic cells, nuclear buds	(Chandurvelan et al., 2016)
2016	Whole animal	16S rRNA genotyping	Pathogens	X				X	Detected *Vibrio vulnificus*	(Cruz et al., 2016)

2018	Haemolymph	PCR analysis	Pathogens	X				X	Detected *Toxoplasma gondii, Giardia duodenalis*	(Coupe et al., 2018)
2018	Gill	GCMS metabolomics	Thermal stress		X		X		Metabolite changes	(Dunphy et al., 2018)
2018	Haemolymph	1) GCMS metabolomics 2) Flow cytometry	Copper		X	X	X		Copper contamination in mussels resulted in: • ↑haemocyte mortality • ↑ROS production • ↑apoptosis • Metabolite changes	(Nguyen et al., 2018a)
2018	Haemolymph	1) GCMS metabolomics 2) Flow cytometry	Pathogen		X	X	X		Injection with *Vibrio splendidus* and a *V. coralliilyticus* / *neptunius*-like isolate resulted in: • ↑ROS production • ↓haemocyte viability • ↑THC • Metabolite changes	(Nguyen et al., 2018c)
2018	Haemolymph	1) GCMS metabolomics 2) Flow cytometry	Pathogen		X	X	X		Injection with *Vibrio splendidus* and a *V. coralliilyticus* / *neptunius*-like isolate resulted in: • ↑ROS production • ↓haemocyte viability • ↑apoptosis • Metabolite changes	(Nguyen et al., 2018b)
2019	Haemolymph	1) GCMS metabolomics 2) Flow cytometry	Lipopolysaccharide		X	X	X		LPS stimulation resulted in: • ↑ROS production • ↑apoptosis • Metabolite changes	(Nguyen et al., 2019a)
2019	Haemolymph Gill Hepatopancreas	1) GCMS metabolomics 2) Flow cytometry	Pathogen		X	X	X		Injection with *Vibrio splendidus* and a *V. coralliilyticus* / *neptunius*-like isolate resulted in: • ↑THC • ↓haemocyte viability • Metabolite changes	(Nguyen et al., 2019c)
2019	Mantle Gill Muscle Hepatopancreas	1) GCMS metabolomics 2) Flow cytometry	Pathogen		X	X	X		Injection with *Vibrio splendidus* and a *V. coralliilyticus* / *neptunius*-like isolate resulted in: • ↑ROS production • ↑apoptosis • Metabolite changes	(Nguyen and Alfaro, 2019)

2019	Larvae	Bioassays	Hatchery stress		X	X	X		At 4.5 calcium carbonate saturation (compared to control) larvae showed: • 8-hydroxydeoxyguanosine • lipid hydroperoxides • ↑protein carbonyls • ↑superoxide dismutase • ↑catalase • ↑glutathione reductase • ↑glutathione peroxidase • ↑glutathione S-transferase	(Ragg et al., 2019)
2020	Whole animal	Bioassays	1) Starvation 2) Thermal stress		X	X	X		• 24h fasting did not affect protein carbonyls and lipid hydroperoxide accumulation or protein carbonyls and lipid hydroperoxide accumulation after exposure to heat stress. • 54h increased oxidative damage and decrease antioxidant enzyme activity. • HSP70 was affected by heat shock	(Delorme et al., 2020a)
2020	Gill	1) GCMS metabolomics 2) Label free proteomics analysis	Mortalities	X			X		Metabolite changes Under expression of cytoskeleton proteins	(Li et al., 2020)
2020	Haemolymph Hepatopancreas	GCMS-metabolomics	1) Harvesting 2) Transport	X			X		Metabolite changes	(Nguyen et al., 2020)
2020	Haemolymph	1) GCMS metabolomics 2) Flow cytometry	Mortalities	X			X		Stressed samples showed: • ↓ROS • ↑Apoptosis • Metabolite changes	(Nguyen and Alfaro, 2020b)
2020	Haemolymph	Flow cytometry	Sampling stress		X	X			Effects of sampling time of haemocyte aggregation, haemocyte populations, haemocyte counts, haemocyte viability, phagocytoses, ROS production described.	(Rolton and Ragg, 2020)
2020	Whole animal	Bioassays	Microplastics Triclosan		X	X			Triclosan exposure resulted in: • ↑Superoxide dismutase and lipid peroxidation activity • ↓glutathione-S-transferase activity	(Webb et al., 2020)

2021	Whole animal	Bioassays	1) Emersion 2) Relative Humidity		X	X	X		Mussels subjected to relative humidity during emersion showed: ↑protein carbonyls, lipid hydroperoxides, 8-hydroxy-deoxyguanosine. Re-immersion resulted in: ↑superoxide dismutase, catalase, glutathione peroxidase and reductase.	(Delorme et al., 2021a)
2021	Haemolymph Gill	1) GCMS metabolomics 2) Flow cytometry 3) Bioassays	Thermal stress		X	X	X		Mussels treated with severe heat stress (30°C for 1h) showed: • ↑THC • ↓ROS • ↓Total antioxidant capacity • Metabolite changes	(Delorme et al., 2021b)
2021	Haemolymph	1) GCMS metabolomics 2) Flow cytometry	Transport stress	X		X	X		Total haemocyte counts and haemocyte viability performs better when mussels are transported in water. Metabolite changes	(Venter et al., 2021)
2022	Haemolymph	1) GCMS metabolomics 2) Flow cytometry	1) Pathogen 2) Temperature		X	X	X		*Vibrio* sp. *DO1 (V. coralliilyticus/neptunius-like isolate)* injection showed ↓THC and temperature showed ↑apoptosis. ROS Metabolite changes	(Ericson et al., 2022)
2022	Haemolymph	1) Bioassays 2) Flow cytometry 3) GCMS metabolomics	1) LPS 2) Temperature		X	X	X		Mussel kept for 48h at 26°C injected with LPS displayed: • ↓Phenol oxidase and acid phosphatase activity • ↑THC • ROS, haemocyte viability • Metabolite changes	(Muznebin et al., 2022a)
2022	Histology tissue section	1) In situ hybridisation (ISH) 2) Microscopic observations	Pathogens	X				X	Detected *Perkinsus olseni,* apicomplexan parasite X (APX), copepods *(Pseudomyicola spinosus* or *Lichomolgus uncus*), *Microsporidium rapuae*	(Muznebin et al., 2022b)
2022	Histology tissue section	Microscopic observations	Season	X		X			↓Phagocytosis in winter Various differential haemocyte counts and characterisations.	(Muznebin et al., 2022c)

2023	Haemolymph	1) Flow cytometry 2) Bioassays	1) Pathogen 2) Temperature		X	X			Injection with *Photobacterium swingsii* at 24°C after 120h resulted in: • ↓THC • ↑haemocyte viability • ↑TAC • ↑lipid peroxidation	(Azizan et al., 2023b)
2023	Haemolymph Gill Muscle Digestive gland	1) qPCR 2) Flow cytometry 3) Histopathology	Pathogen		X	X	X		Injection with *Photobacterium swingsii* caused: • ↑THC • Haemocyte viability • Positive amplification signals muscle, gill, and digestive gland • Haemocyte infiltration • Digestive gland atrophy • Tissue degeneration	(Azizan et al., 2023a)
2023	Haemolymph	GCMS-metabolomics	Pathogens		X		X		*V. mediterranei* and *Photobacterium swingsii* injection altered various metabolites.	(Azizan et al., 2023c)
2023	Haemolymph	1) GCMS metabolomics 2) Flow cytometry	Thermal stress		X	X	X		Mussels at 24°C for 13 months showed: • ↓THC • ↑superoxide positive haemocytes • ↑total apoptotic cells • Metabolite changes	(Ericson et al., 2023c)
2023	Spat	Bioassays	Seeding density	X		X			TAC varied ↑Oxidative stress late summer	(Reyden et al., 2023)
2023	Haemolymph	1) GCMS metabolomics 2) Flow cytometry 3) Bioassays	Thermal stress		X	X	X		Mussels at the highest temperature ramp for 11 weeks showed: • ↓THC • ↓superoxide positive haemocytes • Metabolite changes	(Venter et al., 2023)

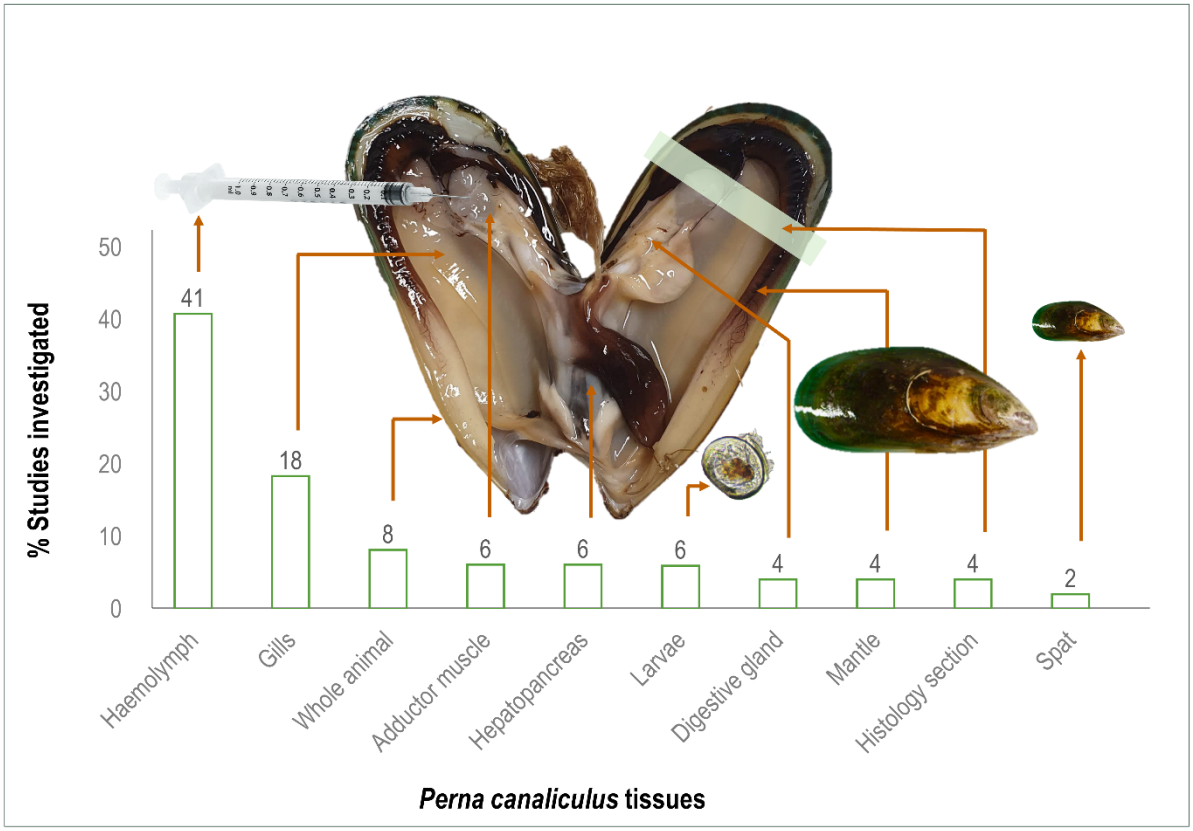


Figure 2.1. Summary of the different tissues and samples utilised in *Perna canaliculus* research when investigating mussel immunity or associated factors or threats.

2.3 MUSSEL IMMUNITY

Mussel immunity refers to the complex defence mechanisms and responses exhibited by mussels, which enable them to protect themselves against various stressors and maintain their overall health. The mussel immune system outlined in Figure 2.2 consists of: 1) an efficient cellular and humoral innate immune system (internal defence), 2) physical barriers (shell and mucus) and 3) behavioural avoidances (external defence). These are generally implemented during unfavourable conditions or in the face of pathogen infections (Gerdol et al., 2018). The first part of the external defence system is the shell, protecting soft tissue from physico-chemical insults. Next, the skin and mucosal layer, traps microbes and facilities the elimination thereof via ciliary activity (Allam and Raftos, 2015). Mucosal surfaces play a key role in activating systemic immune responses, containing various cells and bioactive molecules that trap foreign invaders before reaching the soft tissue (Allam and Espinosa, 2016). From an internal defence system point of view, cellular and humoral defence mechanisms, along with innate immunity help to control the proliferation of pathogens in mussels (Gerdol and Venier, 2015, Bouallegui, 2019). The cellular and humoral defence

systems will be discussed individually along with their various components in the following sections.

2.3.1 Cellular immunity

The bivalve cellular immune response is achieved by haemocytes, which are present in haemolymph in an open vascular system. In *P. canaliculus*, haemocytes can be divided into granulocytes and hyalinocytes (Rolton and Ragg, 2020), with various sub-types often classified as eosinophilic granulocytes, basophilic granulocytes, and small and large hyalinocytes (Muznebin et al., 2022c). Granulocytes typically represent the major cells involved during defence reactions (Bouallegui, 2019). In the presence of pathogens (foreign particles) bivalve haemocytes implement different cellular defence mechanisms, such as haemocytosis, phagocytosis, encapsulation, apoptosis, and autophagy (Nguyen and Alfaro, 2020a).

During haemocytic infiltration (**haemocytosis**), haemocytes are activated, which leads to an observable increase in their circulation and subsequent movement towards infected or injured tissues, guided by chemo-attractant substances (Labreuche et al., 2006). Chemoattractant, such as cytokines, chemokines, and other soluble molecules, are chemical signals released by damaged tissues, pathogens, or other immune cells. These substances create a concentration gradient in the surrounding environment, serving as guidance for immune cells, such as haemocytes, to migrate towards the site of injury or infection (Labreuche et al., 2006). In *P. canaliculus*, haemocytosis has been reported in the mantle, the connective tissue around digestive tubules, digestive epithelium and gonads as a response to the presence of the parasite *Perkinsus olseni* (Muznebin et al., 2022b).

Phagocytosis is the process by which cells recognise and ingest nonself molecules and cell debris. During phagocytosis, the phagocyte usually attaches to the target particle with specialized receptors on its surface, facilitating adherence. This initial attachment is mediated by receptor-ligand interactions, enabling the phagocyte to recognise and bind to the pathogen or foreign material with specificity and selectivity. Within the phagosomes, cytoskeleton modification, internalisation and destruction occur, whereafter the phagosome and lysosome fuse together and destroy the target particle using lysosomal enzymes, reactive oxygen species (ROS), nitric oxide or antimicrobial factors (Song et al., 2010). Using microscopic observations as well as flow cytometry analyses, the process of phagocytosis has been reported within *P. canaliculus* following exposure to stressors (Rolton and Ragg, 2020, Muznebin et al., 2022b). Additionally, the production of ROS has been seen in *P. canaliculus*

studies in response to *Vibrio* sp. (Nguyen and Alfaro, 2019), thermal stress (Delorme et al., 2021b) and immunostimulant exposure (Muznebin et al., 2022a).

When foreign bodies are too large to be phagocytosed, they will be **encapsulated**. Here a capsule of haemocytes encloses the pathogen and cytotoxic products are released by the haemocytes in an attempt to destroy the invader (Allam and Raftos, 2015). The process of encapsulation to eliminate foreign particles have not been reported within *P. canaliculus* studies. Yet linkage to the phenoloxidase pathway responsible for melanisation activation following recognition and encapsulation of pathogens (Coaglio et al., 2018), has been reported in *P. canaliculus* exposed to an endotoxin (Muznebin et al., 2022a).

Apoptosis is the final defence response for an infected cell that is unable to clear the infectious agent. This programmed cell death involves a series of coordinated events that lead to cell morphological alterations and biochemical changes (Romero et al., 2015). Generally during apoptosis, the cell shrinks, rapid blebbing occurs (the process in which a cell's membrane forms rounded protrusions or bulges, known as "blebs," on its surface), the nucleus collapses, DNA fragmentation takes place, and the cell breaks into apoptotic bodies, which are often phagocytised before they lyse in an attempt to prevent further spread (Sunila and LaBanca, 2003). In bivalves two major apoptotic pathways exist. In the first pathway, the intrinsic apoptotic signalling pathway is triggered by the initiator caspase 9, which is activated by the mitochondrial release of cytochrome c. The second, the extrinsic or death receptor pathway, involves initiator caspase-8, which is activated by binding of several death receptors (Wang et al., 2018b). In *P. canaliculus* mechanisms of apoptosis have been induced by lipopolysaccharides' (LPS) (Nguyen et al., 2019a), heat stress (Ericson et al., 2023c) and cadmium exposure (Chandurvelan et al., 2013).

Autophagy is characterised by the presence of many autophagosomes, which fuse with the cellular lysosome system and initiate the degradation of the phagocytised material (Carella et al., 2015). Different autophagy categories are described, such as microautophagy (involves the direct engulfment of small portions of the cytoplasm and cellular components by the lysosomal membrane), chaperone-mediated autophagy, and macroautophagy (involves the formation of double-membraned structures called autophagosomes, which engulf portions of the cytoplasm, organelles, or other cellular components), regulated by serval autophagy related proteins (ATGs) (Picot et al., 2020). Various autophagy related genes have been described in *Crassostrea gigas* (Liu et al., 2022), the formation of autophagosomes and autolysosomes (proof that the autophagic pathway was affected) have been reported in *Mytilus galloprovincialis* (Balbi et al., 2018) and autophagic enzymes were stimulated in *M.*

edulis (Falfushynska et al., 2019). Data linking autophagy processes with *P. canaliculus* are lacking and creates an interesting opportunity for future research.

2.3.2 Humoral immunity

In synchronisation with behavioural and cellular defence systems, humoral immunity is the molecular system, which can be triggered by physical injury, pathogens, or biochemical compounds (Bassim et al., 2015). The humoral components of immunity are carried out by humoral defence factors, produced by haemocytes, which are the key players of mussel defence mechanisms when released into the haemolymph (Allam and Raftos, 2015, Gerdol and Venier, 2015). In broad terms, the humoral immunity consists of 1) the pathogen associated recognition system, 2) activation of signalling pathways and 3) the synthesis of specific reactive effectors (Bassim et al., 2015). Each of these have their own components, as summarised in figure 2.2, and will be discussed next.

2.3.2.1 Pathogen associated molecular patterns (PAMPS) via pathogen associated pattern recognition receptors (PRRs)

Pathogen recognition receptors (PRRs) are the molecular motifs found in haemocytes and tissues of bivalves. PRRs sense potential danger, activate intracellular signalling pathways and finally react by synthesising the immune effective molecules including the release of effector molecules, mediators, and intermediate elements (Kaloyianni et al., 2009, Burgos-Aceves and Faggio, 2017). In mussels, there are several homolog genes related to key immune functions, such as peptidoglycan recognition proteins, lectins, toll-like receptors, gram-negative binding proteins and scavenger receptors as showcased below.

Peptidoglycan recognition proteins (PGRPs), bind selectively to peptidoglycans (PGNs), which can indicate growth-promoting conditions to non-growing bacteria, mediate processes such as selective recognition of bacteria and allow the sensing of a nonself cells in the host (Venier et al., 2016). PGRPs can be classified into three classes depending on their length or extracellular characteristics. The first class consists of short/extracellular PGRP-S, followed by the second class of intermediate/transmembrane PGRP-I, and the third class of long/intercellular PGRP-L. (Liao et al., 2022). PGRPs are involved in immune signal transduction and recognition of non-self-peptidoglycans, as well as assisting with agglutination stimulation and phagocytosis (Dziarski and Gupta, 2006, Liao et al., 2022). In mussels, PGRPs have been identified in vent mussels *Bathymodiolus septemdierum* (Ikuta et al., 2019), deep-sea mussels *Bathymodiolus azoricus* (Détrée et al., 2017) and pearl mussels *Hyriopsis*

cumingii (Yang et al., 2013, Tao et al., 2014). Due to comprehensive genome data published for *Mytilus* species, at least 35 PGRPs have been identified for *Mytilus* mussels (Liao et al., 2022). It remains unclear if *Perna* mussel species share the same PGRPs and genetic characteristics as other mussel species, as only a draft genome of Greenshell™ mussel is currently available (Ashby, 2019).

Lectins are sugar binding proteins that interact specifically and reversibly with membrane glycoproteins or glycolipids of bacteria. They play an important role in recognition and eradication of microorganisms entering the living organisms (Chellapackialakshmi and Ravi, 2022). An array of lectin families have been identified in molluscs, as reviewed previously (Gerdol et al., 2018). In mussels C-type lectins (CTLs) have been associated with agglutination and opsonisation of pathogens and parasites (Venier et al., 2009, Bettencourt et al., 2014, Gerdol and Venier, 2015). The lectin, fibrinogen-related proteins (FREPs) have been described to assists with agglutination, antibacterial effects, and developmental processes (Gerdol and Venier, 2015). Additionally, R-type lectins have been reported to bind to different types of ligands (Venier et al., 2009, Kovalchuk et al., 2013). There are some uncertainties about the function of galectin lectins, which has previously been detected in *M. galloprovincialis* (Venier et al., 2009). Currently, data concerning lectins in *P. canaliculus* and *Perna* species in general, are absent, with no data reported to confirm the presence or describe the functions of lectins in *Perna* species.

Toll-like receptors (TLRs) are membrane-spanning proteins which play a role in the detection of pathogens along with an immune activation role to defend against microorganisms (Brennan and Gilmore, 2018). These molecules can bind to a variety of pathogen-associated molecular patterns (PAMPs) of bacteria, including LPS and flagellin, and activate transcription factors that trigger the production of pro-inflammatory cytokines and chemokines. In *M. galloprovincialis,* 23 TLRs have been identified (Gerdol and Venier, 2015), while three TLRs (McTLR1-3) have been found in *M. coruscus* (Li et al., 2019b). Also, different myeloid-differentiation primary response genes 88 (MyD88) used by TLRs to activate transcription factors via a complex cascade have been identified in *M. galloprovincialis* and *M. edulis* (Toubiana et al., 2013). Even though there have been many studies that identify TLRs in other species of mussels, there is still a gap in the understanding of whether *Perna* species possess these.

Glucan (or gram)-negative binding proteins (GNBPs) includes proteins that bind gram-negative bacteria, LPS and ß-1,3-glucan. They identify and bind to specific targets on the surface of microorganisms and trigger a variety of defence reactions through the actions of

protease cascades and intracellular immune signalling pathways (Song et al., 2010). In *P. virdis,* β-1,3-glucan binding protein has been previously characterised to support prophenoloxidase activity (Jayaraj et al., 2008). Following the generation of a *M. edulis* transcriptome database, researchers have detected, three different glucan binding proteins and four lipopolysaccharide-β-1,3-glucan binding protein fragments, without clear conclusions on which proteins are responsible for bacterial recognition (Philipp et al., 2012). Apart from these studies research on this topic within mussels remains scarce, with no contributions from *P. canaliculus* recorded.

Scavenger receptors (SRs) are endocytic receptors that bind and internalise a variety of microbial pathogens and endogenous host molecules (Wang et al., 2018b). There are several functions associated with the receptor family, including lipoprotein binding, cellular transport, and clearing pathogens (Canton et al., 2013). The number of scavenger receptor cysteine-rich superfamily (SRCR-SF) domains in bivalve transcriptomes are relatively high, but these receptors remain poorly characterised (Gerdol and Venier, 2015). In *M. chilensis*, a transcriptome overview showed scavenger-like receptors as upregulated following injection with saxitoxin (Detree et al., 2016). While in *M. galloprovincialis* transcriptome profiles revealed the scavenger receptor (macrophage receptor with collagenous structure) within the muscle of mussels (Moreira et al., 2015). Moreover, these receptors are found in other invertebrate models (such as *Drosophila melanogaster* and *Caenorhabditis elegans*) (Gerdol and Venier, 2015), but not in *P. canaliculus* mussels.

2.3.2.2 Activate intracellular signalling pathways

Once foreign compounds have been successfully recognised, signalling cascades initiate cellular defence, to enable transmission signals to move from extracellular to intracellular targets (Bassim et al., 2015, Wang et al., 2018b). Typically, phagocytosis is activated, followed by the release of ROS, enzymes and antimicrobial molecules, along with transcription of immune and stress response genes (Canesi and Pruzzo, 2016). Several immune signalling pathways have been investigated in bivalves such as mitogen-activated protein kinase (MAPK), janus kinase / signal transducer and activator of transcription (JAK-STAT), nuclear factor kB (NF-kB), toll-signalling pathways and complement component pathways (Song et al., 2010, Gerdol and Venier, 2015).

Mitogen-activated protein kinases (MAPK) are proteins which can transduce extracellular stimuli into cellular responses important for immune response, cell damage and apoptosis (Tian et al., 2020). MAPKs are grouped into three sub-families including, extracellular signal-

regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs) and p38-MAPKs (Bassim et al., 2015). Typically, ERKs are activated by mitogens and differentiation signals, while the JNK and p38 MAPKs are activated by stress stimuli. Tumour necrosis factor α (TNFα) is a master cytokine that mediates inflammatory responses and innate immunity and can activate all three groups of MAP kinases (Sabio and Davis, 2014). Within the haemocytes of *M. galloprovincialis*, TNFα, was found to induce a decrease of ERK phosphorylation, suggesting the down regulation of anti-apoptotic MAPK signalling (Betti et al., 2006). Furthermore, the activation of p38-MAPK and JNK has been confirmed in *M. galloprovincialis*, when exposed to temperatures above 24°C, highlighting the involvement of these signalling cascade during thermal stress (Anestis et al., 2007). Also, in *M. galloprovincialis*, the presence of *V. splendidus*, activated immune signalling components (p38 MAPK and protein kinase C), confirming that *V. splendidus* affects bivalve haemocytes through dysregulation of immune signalling (Ciacci et al., 2010). Research on *M. californianus* and *M. galloprovincialis* has demonstrated that heat and cold stress activated JNK and p38-MAPK signalling, which might be important for subsequent molecular responses to stress (Yao and Somero, 2012). In *P. viridis*, MAPK was annotated following transcriptome sequencing while studying the effects of endocannabinoids on mussel attachment (Dai et al., 2021). While in *P. perna*, phosphorylated p38 MAPK was activated following heat, osmotic, and hypoxic stress (Zilberberg et al., 2011). To date no research has confirmed MAPKs in *P. canaliculus*.

Janus kinase / signal transducer and activator of transcription (JAK/STAT) is an important pathway for intracellular signal transduction of cytokine receptors, and is known known to be triggered as an immune response in bivalves (Bassim et al., 2015). Three types of STATS have been identified in the shell pearl mussel (*Hyriopsis cumingii*) and showed expression following a challenge experiment with *Staphylococcus aureus* or *Aeromonas hydrophilia*, linking the STAT functions to defence following bacterial infection (Dai et al., 2017). In *M. galloprovincialis* cytokine interferon gamma (IFNγ), stimulated tyrosine phosphorylation of different members of STAT-like proteins, suggesting that haemocyte functions can be modulated by cytokines and bacterial signals (Canesi et al., 2003). The roles of the JAK/STAT pathway and their newly identified cytokines from *P. canaliculus* will be an interesting research field with promise to expand our understanding of mussel immunity.

Nuclear factor κB (NF-κB) polymerising enzymes partake in cell differentiation and immunity. Once the TLR pathway is activated, cascade reactions induce phosphorylation of kB inhibitory proteins, which lead to degradation thereof. The active NF-κB is transported to the nucleus where it binds to the relevant DNA response element and initiates the recruitment of general transcription factors (Bassim et al., 2015). The signalling mechanism for NF-κB activation is

crucial for controlling several cellular and organismal processes, such as cellular growth, apoptosis, inflammatory responses, and provide an immediate cellular immune defence (Li et al., 2015). Genes associated with NF-κB activity have been previously reported in mussels. For example, a study on the deep sea mussel (*Bathymodiolus azoricus*) confirmed the presence of the Rel-homology domain, a conserved motif present in all members of the Rel/nuclear-factor NF-κB family (Bettencourt et al., 2007). The NF-κB gene pathway was also affected in *M. galloprovincialis* following exposure to ibuprofen to infer the pharmacological effects and the possible implications on the non-target organism performance and ecological risk assessment (Maria et al., 2016). In *M. edulis*, NF-κB transcripts was used as an inflammatory marker of salinity stress (Falfushynska et al., 2023), and in *B. azoricus* as a target for copper exposure (Martins et al., 2017b). A detailed summary of how differentially expressed proteins regulate the intracellular inflammatory NF-kB pathway was obtained from proteomics analyses performed on *M. galloprovincialis* subjected to a waterborne infection with *V. splendidus*. The results demonstrated proteins that enhance the NF-kB pathway, generally showed increased expression, whereas inhibitory proteins exhibited decreased expression (Saco et al., 2021). The NF-kB pathway has been described in mussel species other than *P. canaliculus* and still lacks functional evidence of their implication in immune gene regulation.

Toll-signalling pathways are activated by all toll-like receptors (TRLs) that accumulates in the activation of NF-kB transcription factors and MAPKS (Leulier and Lemaitre, 2008). TLR signalling are largely divided into two pathways: the myeloid differentiation primary response 88 (MyD88)-dependent and Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF)-dependent pathways (Kawasaki and Kawai, 2014). Once cell signalling cascades are activated, two distinct pathways come into play. The MyD88-dependent pathway triggers to the production of inflammatory cytokines, while the MyD88-independent pathway is associated with the interferon (IFN)-β induction and dendritic cell maturation (Rauta et al., 2014). An array of 15 toll signalling pathways have been identified in *M. galloprovincialis* following gene transcription, while 11 transcripts were found on publicly available *M. edulis* ESTs. As a conclusion, that showed that in *M. galloprovincialis,* the complete toll pathway can be triggered by Gram-positive and negative bacteria (Toubiana et al., 2014). In *M. coruscus* a novel toll-like receptor (McTLRj) was identified which arguably plays a pattern recognition receptor role in toll signalling pathways (Xu et al., 2019). TLR-mediated signalling plays an important part in limiting and eliminating pathogenic infections, while also promoting tissue repair (Rauta et al., 2014). The TLR signalling pathway in *P. canaliculus* are still largely unexplored with various cytokine responses yet to be established.

Complement component pathway (system) depends on many interacting proteins to recognise and eliminate foreign microorganisms. When activated, the complement pathway promotes proteolytic reactions that function in the same way as lectins. This system partakes in the initiation of defence mechanisms, including immune cell homing and trafficking, agglutination, adhesion, opsonisation and cell lysis (Bassim et al., 2015). Typically, the complement system has more than 30 serum and cell-surface proteins which are activated by complement component 1 (C1; in the antibody-dependent classical pathway), lectin (in the lectin pathway), or C3 (in the antibody-independent alternative pathway) (Huang et al., 2016). C-type lectins (CTLs) that can trigger the lectin pathway of the complement system have been reviewed in mussels (Gerdol and Venier, 2015), including the mussel species, *Hyriopsis cumingii* (Huang et al., 2016), *M. galloprovincialis* (Venier et al., 2011, Gerdol et al., 2011) and *M. coruscus* (Chen et al., 2018, Han et al., 2021). Despite the extensive research on these immune signalling pathways, it remains uncertain whether all these pathways are conserved across *P. canaliculus* and how they are regulated in different environments.

2.3.2.3 Synthesis of antimicrobial effectors

Antimicrobial peptides (AMPs) are a group of molecules that form part of the humoral innate immune system, which contributes to the first line of defence against pathogens (Leoni et al., 2017, Bouallegui, 2019). Antimicrobial activity obtained by AMPs derives from disruption of the membrane and osmotic lysis of bacteria. Some AMPs are also said to be efficient in inhibiting viral infections (Zannella et al., 2017). Generally AMPs include (but are not limited to) defensins, mytilins, myticins, mytimysins, and mytimacins based on primary structure (Wang et al., 2013b, Gerdol and Venier, 2015). Both the diversity and structural features of mussel peptides (Mitta et al., 2000) and the potential application of AMPs in aquaculture (Cheng-Hua et al., 2009) have been reviewed elsewhere, and will not be discussed here as it goes beyond the scope of this review.

With the focus on mussel AMPs, a review by Zanella et al. (2017) describes the isolated peptides along with the mussel species of interest. In brief, *Mytilus galloprovincialis* defensin (MGD) 1 and 2; myticin A, B and C; mytimycin; mytimacin and big-defensin have been linked to antimicrobial activity in studies on *M. galloprovincialis*. Then again, mytilin A and B have been found in *M. edulis* and myticusin-1 in *M. coruscus* (Zannella et al., 2017). Additional research on, *M. galloprovincialis*, found a potential new family of AMPs, called myticalins. In vitro confirmations against a broad range of Gram-positive and Gram-negative bacteria, confirmed antimicrobial properties of seven chemically synthesised myticalins (Leoni et al., 2017). Moreover another type of AMP linked to *M. coruscus*, was described as myticusin-beta,

which is suggested to be an effective alternative to antibiotics (Oh et al., 2020). More recently, twelve AMPs representing the main AMP families were found in following characterisation of the haemocyte transcriptome (Yang et al., 2022). Research on *P. viridis* identified four mytilin-like antimicrobial peptides (called pernalins), evident by highest transcription levels in haemocytes. Moreover, pernalin genes were down regulated as immune response after bacterial infection with *Vibrio parahaemolyticus* (Zeng et al., 2022).

Advances have clearly been made in the field of mussel AMPs, yet their presence in *P. canaliculus* has not been described. Future research endeavours should focus on characterising AMPs as part of the Greenshell™ mussels' immune system and to determine the molecular mechanisms involve in protecting them from pathogenic microorganisms.

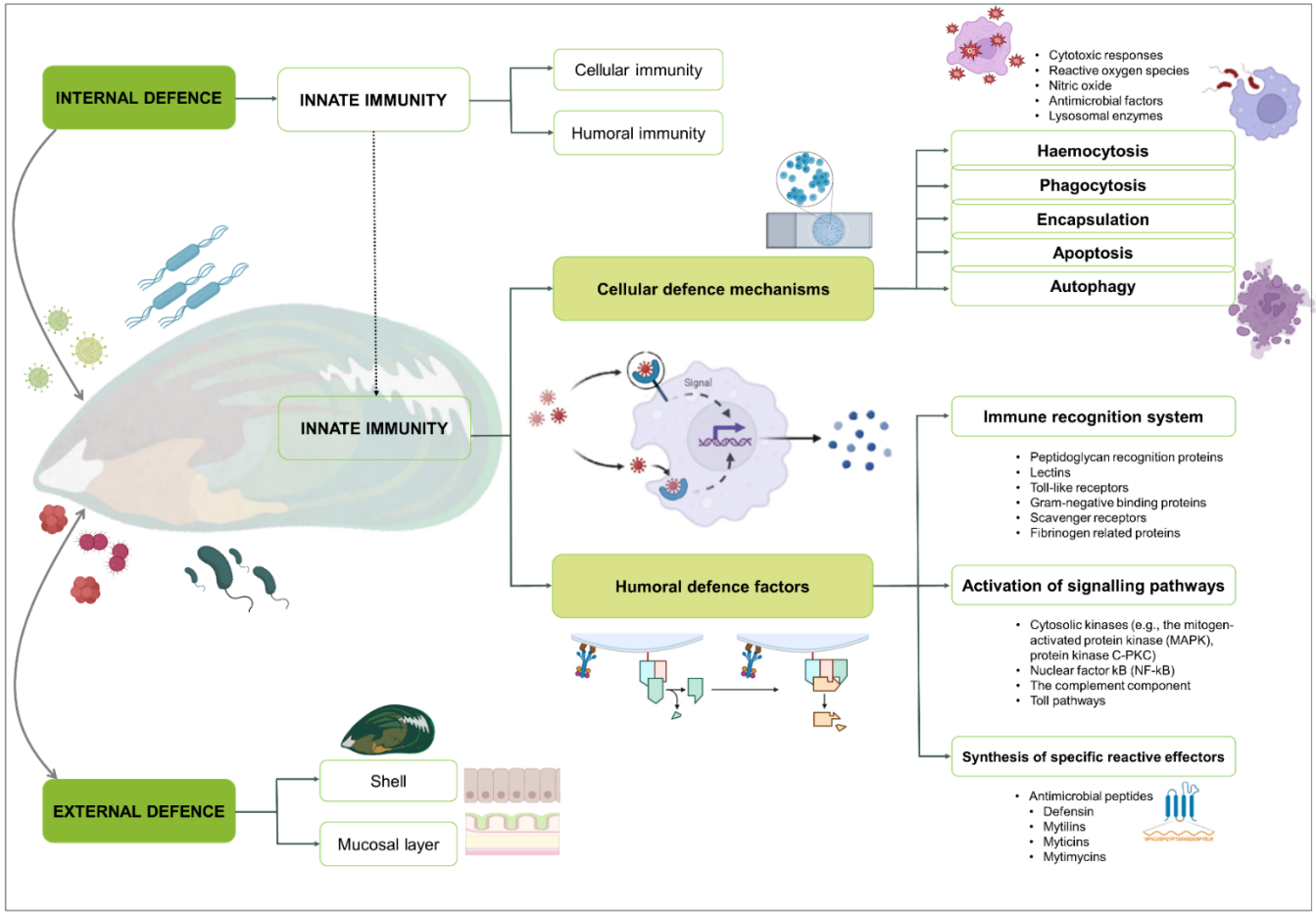


Figure 2.2. An overview of bivalve immunity, considering external and internal defences, along with cellular and humoral defence factors relating to mussel species.

2.4 MUSSEL DISEASE

In the aquaculture sector, disease outbreaks can be a substantial constraint, resulting in significant losses (Naylor et al., 2021). There are three major pathogenic agents that cause diseases in mussels namely, viruses, protistans and bacteria (Travers et al., 2015, Webb and Duncan, 2019). There are also other diseases that may be caused by fungi (*Aspergillus*, *Penicillium* and *Fusarium*), porifera (*Cliona* spp.), and helminth parasites, such as trematodes, cestodes, and nematodes (Gagné et al., 2008, Carver et al., 2010, Santos et al., 2017). It is believed that the most significant protozoan pathogens come from the genera *Perkinsus*, *Haplosporidium* and *Marteilia*. Several diseases caused by *Perkinsus olseni, Haplospordian tumefacientis, Marteilia refringens* and the parasite Apicomplexan X (APX) are under surveillance and requires mandatory notifications to the World Organisation for Animal Health if detected (Georgiades et al., 2016, Muznebin et al., 2022a). Marine mussels, which are filter feeders, concentrates diverse and rich bacterial commensal microbiota made up of different Gram-negative and Gram-positive bacteria species from different genera like *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Photobacterium*, *Moraxella*, *Aeromonas*, *Micrococcus, Bacillus* and *Nocardi*a (Sugumar et al., 1998, Garnier et al., 2008, Biel et al., 2014, Prado et al., 2014, Kwan and Bolch, 2015). The effects of bacterial such as *Vibrio* species (*Vibrio alginolyticus, V. splendidus, Vibrio anguillarum, Vibrio harveyi* and other *Vibrio* spp.), *Photobacterium*, *Pseudomonas* and *Aeromonas* on mussels have been previously described (Travers et al., 2015).

A range of endemic threats have been documented in Greenshell™ mussel, such as: Apicomplexan X (APX), digestive epithelial virosis (DEV), rickettsia-like organisms/chlamydia-like organisms/endozoicomonas like organisms (RLO/CLO/ELO), *Perkinsus olseni*, *Vibrio splendidus*, *Tergestia agnostomi* and *Enterogonia orbicularis* (Castinel et al., 2019, Webb and Duncan, 2019). In most cases, bacteria survive within a temperature tolerance range, where their growth may be compromised but they remain viable. The presence of *Vibrio* spp. has gained considerable attention within *P. canaliculus* populations, as resilience to climate change stressors are being placed at the forefront of research. *Vibrio* sp., which include V*ibrio splendidus* and *Vibrio coralliilyticus/*neptunis-like isolate (Kesarcodi-Watson et al., 2009a), *Vibrio parahaemolyticus* (He et al., 2022), *Vibrio mediterranei* (Andree et al., 2021) and *Photobacterium swingsii* related to the *Vibrio* genus (Azizan et al., 2022) are largely found in aquatic environments that Greenshell™ mussel populations. *Vibrio* is one of eight genera within this large family of Gram-negative, gamma-proteobacteria (Thompson, 2006). Because of their extensive metabolic diversity and genetic variation, they have a high colonisation potential and can lead to dangerously high accumulations (Le Roux, Frédérique et al. 2015;

Le Roux, Frédérique and Blokesch 2018). For the accurate identification of *Vibrio*-like species, appropriate bacterial typing, including phenotypic and genotypic methods are usually required. Vibrio 16S ribosomal ribonucleic acid (rRNA) gene sequences contain variations specific to *Vibrio*-like species, which serves as an important tool for phylogenetic identification, taxonomic classification, and evolutionary studies of *Vibrio* bacteria (Baker-Austin et al., 2018). *Vibrio* sp. can be primary pathogens, responsible for pathological changes in healthy bivalves, or opportunistic pathogens, which cause disease when the protective barrier is breached or immunity is suppressed (Destoumieux-Garzón et al., 2020). In addition, the genus *Photobacterium* belongs to the family of Vibrionaceae, one of the oldest established genera. Primarily, this bacterial genus consists of marine luminescent Gram-negative bacteria found worldwide in marine ecosystems (Romalde et al., 2014). Several species of *Photobacterium* were isolated from different marine animals, including bivalves and fish (Urbanczyk et al., 2011). Fish were found to harbour *Photobacterium damselae* subspecies *damsela*e (Lozano-León et al., 2003). *P. swingsii*, *P. galatheae* and *P. rosenbergii* have been shown to be responsible for disease in corals, oysters, and mussels (Thompson et al., 2005b, Gomez-Gil et al., 2011, Machado et al., 2015, Eggermont, 2017).

2.5 PATHOGENICITY AND VIRULENCE FACTORS

The ability of *Vibrio* sp. to cause disease or mortality in a host is correlated with its pathogenicity. A bacterium is pathogenic when 1) it is present in large quantities within the host organism, 2) it is capable of being isolated and re-cultured from the host organism, 3) experimental reproduction is feasible in a healthy host organism, and 4) it is possible to isolate and identify the same pathogenic bacterium following use in an experiment as aligned with the principles outlined in Koch's postulates (Paillard et al., 2004). Virulence factors encoded by virulence genes, generally give rise to the pathogenicity of *Vibrio* strains (Deng et al., 2020). A virulence factor is a specific molecule secreted by a bacterium that promotes the bacterium's ability to be virulent (Lam et al., 2014). Mostly virulence factors allow pathogens to infect and damage the host, by enabling pathogenic adherence and entrance, establishment and multiplication, avoidance of host defences, damage to the host, and finally exit from the infected host (Deng et al., 2020, Muznebin et al., 2022b). Bacterial virulence factors, measured from pathogen infections within aquatic organisms with respect to motility, attachment, and colonisation, exopolysaccharide production and biofilm formation, lytic enzymes, and heat-shock proteins will be discussed in the following sections (Table 2.2, Fig. 2.3).

2.5.1 Motility

During the early stages of infection, bacterial motility is crucial for pathogenic bacteria to facilitate attachment, colonisation, nutrient acquisition and biofilm formation (McCarter, 2001, Johnson, 2013). *Vibrio* sp. are known to have two types of specialised flagella, that governs motility (Defoirdt, 2014). The first type is a sheathed polar flagellum used for swimming in aqueous environments (e.g., water), while the second type, is a lateral flagellum that enables navigation in gelatinous environments (e.g., mucus or biofilms) (Johnson, 2013). Following *Vibrio* sp. infections, flagellar motility has been identified in the bacterium (Naka and Crosa, 2011). A motility gene has been identified in the bacterial isolates from mussel larvae (Eggermont et al., 2017). Additionally, the sheathed flagellum was said to evade immune responses via their swimming motility following an oyster challenge with *Vibrio* sp. (Yoon et al., 2008).

2.5.2 Biofilm formation

A biofilm can be defined as a three-dimensional community of bacteria, predominantly in a matrix-like polysaccharide, that forms on surfaces to protect the bacteria from various stresses, including environmental fluctuations, nutrient scarcity, and chemical assaults (Alam et al., 2007, Yildiz and Visick, 2009). Bacteria secrete extracellular polysaccharides in the form of a capsule or loose slime (Costerton et al., 1981), to attach itself to the host cells (Defoirdt, 2014). Conversely, the loose slime creates a matrix enabling microorganisms to grow, adhere to hydrophobic surfaces and thrive by providing nutrients, protection from detergents and antimicrobials, as well as protection from phagocytic predators (Mah and O'Toole, 2001, Donlan and Costerton, 2002). The formation of biofilms has been reported from *V. parahaemolyticus* isolates obtained from contaminated mussel seafood (Ashrafudoulla et al., 2019) and Asian green mussels, *Perna virids* (Palamae et al., 2022). In addition, biofilm formation by *Vibrio* sp. involves specific genes (e.g., exopolysaccharide synthesis, flagella, pili) as well as regulatory processes (e.g., quorum sensing, cyclic dimeric guanosine monophosphate signalling) (Yildiz and Visick, 2009). The expression of the hemagglutinin/protease regulatory protein (*hapR*) gene in *V. cholera*, for example, was reported to increase biofilm formation and virulence (Zhu et al., 2002). Mannose sensitive hemagglutinin (*mshA*) gene found in bacterial isolates from Greenshell™ mussels (Azizan et al., 2022), has also been linked to biofilm formation (Johnson, 2013).

2.5.3 Lytic enzymes

Lytic enzymes produced by many pathogenic bacteria are often crucial to pathogenesis (Johnson, 2013). By damaging host tissues, these enzymes allow pathogens to take up nutrients and spread. There are many lytic enzymes produced by aquaculture pathogens, among them haemolysins, proteases, chitinases, phospholipases, and lipases (Ruwandeepika et al., 2012, Defoirdt, 2014).

2.5.3.1 Haemolysins

Haemolysin is a toxic substance found in many pathogenic *Vibrio* sp. and plays an important role in the infection process. Four main haemolysin families have been identified, including thermostable direct haemolysin (TDH) from*V. parahaemolyticus*, El Tor haemolysin (HlyA) from *V. cholerae*, thermolabile haemolysin (TLH) from *V. parahaemolyticus* and another thermostable haemolysin (δ-VPH) *V. parahaemolyticus* (Zhang and Austin, 2005). With relation to mussel research, thermostable haemolysin (*tdh*) and TDH-related haemolysin (*trh*) genes were detected in *V. parahaemolyticus* isolated from *M. galloprovincialis* following genomic analyses (Ottaviani et al., 2005).

2.5.3.2 Proteases

Proteases are enzymes which catalyse the hydrolysis of a peptide bond into a protein molecules (Vázquez et al., 2006). Proteases can include metalloproteases (proteases that need metal ions), serine proteases (proteases where serine is the nucleophilic amino acid), cysteine proteases (proteases with nucleophilic cysteine thiols at their active site), collagenases (proteinases that degrade collagen), caseinases (proteinases that degrade casein) and gelatinases (they degrade gelatin) (Shinoda and Miyoshi, 2011). Proteases can also help bacteria evade host immune system, for example ROS production in host cells was inhibited by *vam*, the zinc metalloprotease obtained from *V. aestuarianus* (Johnson, 2013). Secretion of extracellular protease (gelatinase and caseinase) have been positively identified in *Vibrio* sp. and *Photobacterium* sp. isolated from the blue mussel larvae, however, no correlation between in vitro expression levels of virulence-related genes in vibrio was found (Eggermont et al., 2017). Zinc-metalloprotease (*zm*) and single-zinc metalloprotease (*vcpA*) genes were identified following qPCR analysis of *Photobacterium* sp. and *V. celticus* isolated from the infected Greenshell™ mussels (Azizan et al., 2022).

2.5.3.3 Chitinases

Among the most abundant molecules in the ocean is chitin, a polymer made of N-acetylglucosamine (GlcNac) monomers, which *Vibrio* sp. use as a source of cellular energy (Aunkham et al., 2018). Chitinases are glycosyl hydrolases with the ability to degrade chitin directly to low molecular weight chitooligomers (Kumar et al., 2022). Typically, it is thought that *Vibrio* sp. sense chitin, attach to the chitin, express a large number of enzymes and proteins to degrade the chitin to GlcNac and oligosaccharides, and lastly catabolise the chitin hydrolysis products (Souza et al., 2011). Chitinases producing bacteria has been detected from *M. trossulus* (Beleneva and Maslennikova, 2005), *P. viridis* (Khantavong et al., 2009) and *P. canaliculus* (Azizan et al., 2022).

2.5.3.4 Lipases and phospholipases

Lipases are a type of lipolytic enzyme that breaks down long-chain triacylglycerols into fatty acids and glycerol molecules at the interface between water and lipids (Adetunji and Olaniran, 2021). Phospholipases directly lyses the host cells by disruption of the phospholipid membrane, while the products from cell lysis can act as signalling molecules to induce apoptosis or inflammation (Wan et al., 2019b). Both of these lipolytic enzymes have been associated with bacterial virulence linked to mussels. For example, phospholipase was detected as a potential virulence phenotype in *V. parahaemolyticus* isolates obtained from mussel samples (*Mytella guyanensis*), also lipase was detected but not reported as significant (Silva et al., 2018). Another study identified four *Vibrio* spp. (*V. diabolicus, V. alginolyticus, V. parahaemolyticus* and *V. harveyi*) from *M. coruscus*, whereafter pathogenic virulence factors were analysed among the tested isolates (Hossain et al., 2020). The results showed that all *Vibrio* spp. isolates were positive for phospholipase, and 87.5% of the isolates were positive for lipase (Hossain et al., 2020). Also in *P. swingsii* isolated from *P. canaliculus* the phospholipase (*plp*) gene was detected, which potentially attributes towards virulence in Greenshell™ mussels (Azizan et al., 2022).

2.5.4 Bacterial heat-shock proteins

The primary role of heat-shock proteins (HSPs) is to assist with protein folding, to protect cellular proteins from environmental stressors, while also ensuring cellular homeostasis. However, HSPs are also important virulence factors and often affect pathogenesis indirectly (Roncarati and Scarlato, 2017). HSPs include chaperones and proteases which are critical for overcoming protein denaturation changes (Maleki et al., 2016). Bacteria require increased production of molecular chaperones to survive the infectious process. Bacteria often express

molecular chaperones on the cell surface (acting as adhesins) and can release them into the extracellular spaces to act as signalling virulence factors (Henderson et al., 2006). The protease HSPs are responsible for removal of damaged polypeptides from stressed cells (Roncarati and Scarlato, 2017). Since HSPs can refold or degrade damaged proteins, bacteria that produce them can thrive in harsh temperatures (Maleki et al., 2016). *Hsp60* gene was identified and hypothesised to be an infection factor for bacterial isolates from Greenshell™ mussels during summer mortality outbreak (Azizan et al., 2022).

2.5.5 Membrane-localised regulatory proteins

Membrane regulatory proteins are needed for the adhesion, invasion and the release of pathogen toxins (Ye et al., 2015). An example of membrane regulatory protein is Cholera toxin transcriptional activator (ToxR), which is embedded within the inner membrane and functions as regulator of gene expression (Krukonis and DiRita, 2003). ToxR controls bacterial virulence and survival in certain pathogenic bacteria such as *V. cholera* under adverse conditions, by activating the virulence genes such as *ctx*, *tcp* encoding toxin coregulated pilus (TCP) and cholera toxin (CT) as well as biofilm formation (DiRita et al., 1991, Johnson, 2013). Furthermore, ToxR also controls the expression of outer membrane proteins (Omps), such as *ompU* and ompT, and biofilm formation, which are necessary for the survival of the bacteria under unfavourable environmental conditions (Okuda et al., 2001, Provenzano and Klose, 2000). High *toxr gene* expression in pathogenic *P. swingsii* and *P. rosenbergii* was predicted to be involved in the pathogenicity towards *P. canaliculus* (Azizan et al., 2022). Additionally, *toxR and ompK* genes were found in *V. alginolyticus* isolated from mussel, *P. viridis* (Najwaa et al., 2015).

2.5.6 Quorum sensing

A cell-to-cell signalling process called quorum sensing (QS) is used by bacteria to modulate communal behaviour and gene regulation based on cell density changes and chemical molecules called autoinducers (AIs) in the surrounding environment (Srivastava and Waters, 2012). Many Gram-negative bacteria, such as *Vibrio* sp. use acylated homoserine lactones (AHLs) as their major autoinducer molecules as signal molecules (Defoirdt, 2014, Islam et al., 2022b). Generally, QS regulates gene expression and bacterial phenotypes which are responsible for the virulence of aquaculture pathogens through downstream master regulators (Defoirdt, 2014). Among the virulence-related phenotypes controlled by these QS systems are motility, biofilm formation, and protease production (Islam et al., 2022b). For example, *V. tasmaniensis* and *V. crassostreae*, and their multi-channel quorum sensing mutants were

characterised in vitro and challenged in vivo with blue mussel larvae to investigate their impact on virulence (Islam, 2016). In a follow-up study the AI-2 autoinducer-mediated quorum sensor was found to reduce virulence of *V. crassostreae.* Also cinnamaldehyde as a quorum sensing inhibitor, was used to demonstrate that quorum sensing does not control the virulence of *V. tasmaniensis* and *V. crassostreae* towards blue mussel larvae (Islam et al., 2022b). The aromatic signalling molecule indole has also been flagged as a promising target for anti-virulence therapy in aquaculture, particularly for the control of virulence-related phenotypes (biofilm formation and motility) in various bacterial pathogens, including *Vibrio* sp. (Zhang et al., 2023a).

In summary, understanding bacterial virulence factors is of paramount importance due to its multifaceted implications. Firstly, it allows us to comprehend how bacteria cause diseases and the specific mechanisms they employ to evade mussel immune system. This knowledge paves the way for the development of highly effective strategies for combating and preventing bacterial infections. Secondly, investigation of bacterial virulence factors helps in identifying potential targets for therapeutic interventions. Profound insight into the key factors responsible for bacterial pathogenicity empowers researchers to engineer targeted therapies or formulate vaccines which selectively neutralize these factors, resulting in more refined, efficient, and precise treatments. Furthermore, unravelling bacterial virulence factors aids in the development of diagnostic tools. By identifying and characterizing these factors, scientists can devise diagnostic tests that detect their presence or activity, enabling early and accurate identification of bacterial infections in mussel.

Table 2.2 Summary of bacterial virulence factors reported to contribute to mussel mortalities.

	Categories	**Functions**	**Virulence factors**	**Pathogen spp.**	**Reference**
1	Motility	Movement	*flagellar motility* (*fla*) and swimming motility phenotype	*Vibrio* sp. *and Photobacterium* sp.	(Eggermont et al., 2017)
2	Biofilm formation	Seeding and dispersal of many cells	High log CFU/cm^2 biofilm formation	*V. parahaemolyticus*	(Ashrafudoulla et al., 2019)
		Not specified	Mannose sensitive hemagglutinin (*mshA)*	*Photobacterium* spp. (*P. swingsii, P. galatheae and P. rosenbergii*) and *V. celticus)*	(Azizan et al., 2022)
3	Lytic enzymes	Haemolysin	*tdh* and *trh* genes	*V. parahaemolyticus* isolates	(Ottaviani et al., 2005)
		Extracellular protease	*Gelatinase and caseinase*	*Vibrio* sp. *and Photobacterium* sp.	(Eggermont et al., 2017)

		Proteases	Zinc-metalloprotease *(zm)* and single-zinc metalloprotease *(vcpA)*	*Photobacterium* spp. *(P. swingsii, P. galatheae and P. rosenbergii) and V. celticus)*	(Azizan et al., 2022)
		Chitinases	Not specified	*Vibrio* sp.	(Beleneva and Maslennikova, 2005)
		Chitinases	Chitinolytic activity	*Bacillus circulans*	(Khantavong et al., 2009)
		Chitinases	*chi gene*	*Photobacterium* spp. *(P. swingsii, P. galatheae and P. rosenbergii) and V. celticus)*	(Azizan et al., 2022)
		Lipase and phospholipase	*Lipase and phospholipase activities*	*Mytella guyanensis*	(Silva et al., 2018)
		Phospholipase	*Lipase and phospholipase activities*	*Vibrio* spp. (*V. diabolicus, V. alginolyticus, V. parahaemolyticus* and *V. harveyi*)	(Hossain et al., 2020)
		Phospholipase	*lp gene*	*Photobacterium* spp. *(P. swingsii, P. galatheae and P. rosenbergii) and V. celticus*	(Azizan et al., 2022)
4	Bacterial heat-shock proteins	Hsp60	*hsp60* gene	*Photobacterium* spp. *(P. swingsii, P. galatheae and P. rosenbergii) and V. celticus)*	(Azizan et al., 2022)
5	Membrane-localised regulatory proteins	toxR and o*mpU*	*toxR* and *ompU* genes	*V. alginolyticus*	(Najwaa et al., 2015)
		toxR and ompK	*toxR and ompK genes*	*Photobacterium* spp. *(P. swingsii, P. galatheae and P. rosenbergii) and V. celticus)*	(Azizan et al., 2022)
6	Quorum sensing	Multichannel quorum sensing system	*luxM*, *luxS* and *cqsA*; l*uxM*, *luxR* and *luxS*	*V. tasmaniensis* LGP32 *and V. crassostreae* J2-9	(Islam, 2016, Islam et al., 2022b)
		Quorum sensor	AI-2 autoinducer	*V. crassostreae J2-9*	(Islam et al., 2022b)
		Quorum sensing inhibitor	Cinnamaldehyde	*V. tasmaniensis LGP32 and V. crassostreae J2-9*	(Islam et al., 2022b)
		Quorum sensing inhibitor	Indole	*V. tasmaniensis LGP32 and V. crassostreae J2-9*	(Zhang et al., 2023a)

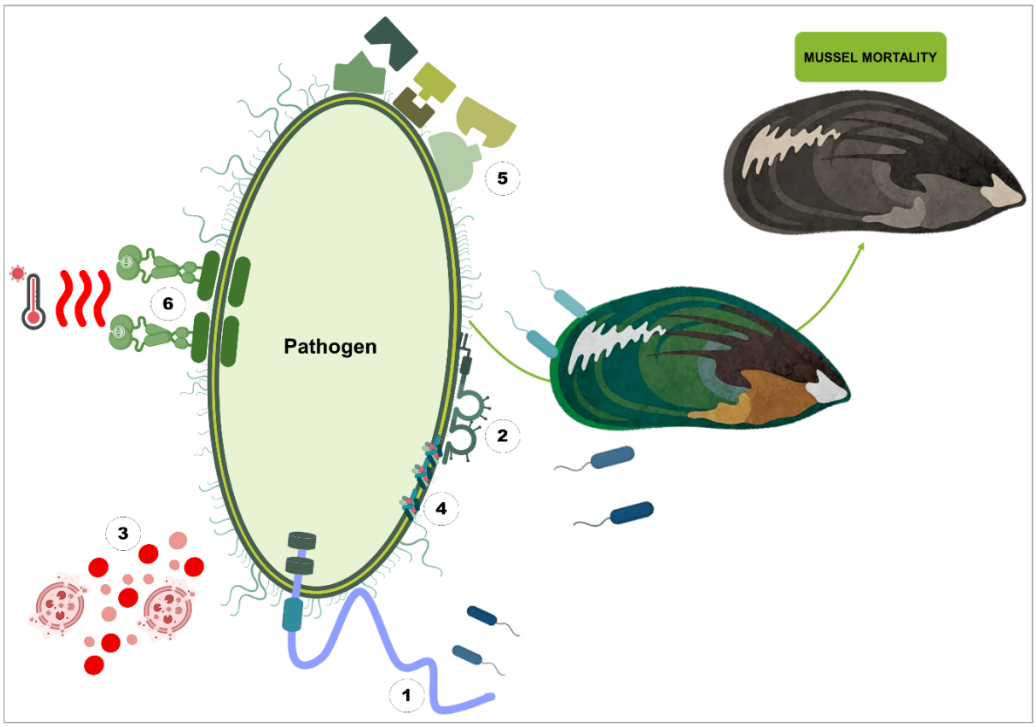


Figure 2.3. Schematic overview of different virulence factors produced by pathogenic bacteria potentially attributing to mussel mortality. Bacteria implement the following factors to promote virulence: 1) motility, 2) biofilm formation, 3) lytic enzymes, 4) heat-shock proteins, 5) membrane localised regulatory proteins and 6) quorum sensing.

2.6 FACTORS COMPROMISING *PERNA CANALICULUS* IMMUNITY

Stresses, both biotic and abiotic, trigger a costly cascade of cellular and molecular processes to maintain cellular homeostasis, which in time weakens biological defence mechanisms (Coates and Söderhäll, 2021). Pathogen and parasite infections in mussels result from a combination of biotic and abiotic drivers. Among biotic drivers, such as host size, immunity status, density of the host population, physical injury, predators, other pathogens, malnutrition etc., have been demonstrated to facilitate the evolution and spread of pathogens and the occurrence of disease outbreaks due to immune-compromised animals (Bondad-Reantaso et al., 2005, Skein et al., 2018, Bommarito et al., 2022). Abiotic drivers, including several environmental factors, influence pathogen prevalence and abundance. Climatic events, such as storms, droughts, aquatic and atmospheric heatwaves, have a significant impact on water quality. These events cause changes in salinity, pH, introduce pollutants (chemicals, pharmaceuticals, plastics), and lower dissolved oxygen levels. Consequently, these factors contribute to animal stress and compromise their immune systems. (Babarro and de Zwaan, 2002, Mydlarz et al., 2006, Lane et al., 2020, Reverter et al., 2021, Bommarito et al., 2022). The majority of studies in *P. canaliculus* within the last 14-years have investigated the

influence of biotic or abiotic factors on mussel immunity (either directly measured specific immune parameters or detected changes in immune-related markers) in either wild population of mussels (field study) or in a laboratory experiment (Fig 2.4). These biotic or abiotic factors are next discussed.

2.6.1 Pathogens

As mussels lack immunological memory, the innate immunity has the responsibility to secure protection to pathogenic microorganisms (Campos et al., 2015). Yet the effect of pathogens on mussel health (immunity) has been understudied (Waller and Cope, 2019). Although Greenshell™ mussels cultured in NZ are generally free from significant production diseases (Lane et al., 2022), a number of pathogens with effects on the immune system have been reported in *P. canaliculus*. For example, the use of *Vibrio* sp. DO1 (*V. coralliilyticus/neptunius*-like isolate), to infect adult *P. canaliculus* in various laboratory studies resulted in: 1) altered metabolites suggestive of oxidative stress and changes in protein synthesis linked to immune function (Nguyen et al., 2019b); 2) changes in metabolites involved in major perturbations on the host's innate immune system (Nguyen et al., 2018c); 3) increased itaconic acid as antimicrobial metabolite and anti-inflammatory marker (Nguyen and Alfaro, 2019) and 4) alterations within the hosts oxidative stress and inflammation processes and disruption of the tricarboxylic acid cycle (Nguyen et al., 2018b). *Vibrio splendidus* and a *V. coralliilyticus/neptunius*-like isolate) were reported pathogenic in Greenshell™ mussel larvae, causing mortalities, and histopathological changes in the digestive system (Kesarcodi-Watson et al., 2009a). The use of this same *V. coralliilyticus*/neptunius-like isolate in a subsequent *P. canaliculus* experiment showed a higher proportion of dead haemocytes and lower overall haemocyte counts than uninfected controls (Ericson et al., 2022). An increase in metabolites linked to the immune supportive metabolite pathways (glutathione pathway and branched chain amino acids) were observed, when infecting mussels with *V. mediterranei* (Azizan et al., 2023c). Reports on other pathogenic micro-organisms, threating *P. canaliculus* immunity included: the protozoan parasites *Toxoplasma gondii* and *Giardia duodenalis* from commercially sourced mussels, detected via specific gene targets (Coupe et al., 2018). Also detected in *P. canaliculus* is, *Perkinsus olseni*, apicomplexan parasite X (APX) (78%), copepods (*Pseudomyicola spinosus* or *Lichomolgus uncus*) and *Microsporidium rapuae* collected from a commercial mussel farm, characterised by histology and confirmed by in situ hybridisation (Muznebin et al., 2022b). Disease dynamics are constantly changing, necessitating continued research on host-parasite interactions. Moreover, the empirical data generated can be combined modelling methods to assist in forecasting future disease events (Lane et al., 2022).

2.6.2 Pathogen co-infections

Co-infection is defined as the simultaneous infection of one host with multiple pathogens which may be causative agents of different diseases or variants of the same microbes (Martcheva and Pilyugin, 2006). Many pathogens, such as bacteria, microparasites, and viruses often co-occur within the same individual host, largely with a harmful outcome to the host (Toews et al., 1993, Morley, 2010, Dong et al., 2015, Figueroa et al., 2017, Shen et al., 2019). It is notable that the co-infecting pathogens can be homologous (interactions occur between pathogens of the same types or species i.e., two different strains of bacteria) or heterogeneous (interactions occur between pathogens of different types or species i.e., involving a bacteria and a virus or parasite) (Kotob et al., 2017). In mussels co-infected pathogens may interfere with the host immune response and compete for nutrients, either by synergistic or antagonistic actions between the pathogens (Künili et al., 2021). In *P. canaliculus*, a laboratory-based bacterial co-infection study, using *Vibrio mediterranei* and *Photobacterium swingsii*, resulted in higher mortalities, increased bacterial colonies for a longer period, and a decreased metabolite response largely influencing amino and fatty acid metabolism (compared to mussels receiving a single pathogen) (Azizan et al., 2023b). Co-infections on *P. canaliculus* larvae using *V. splendidus* and *V. coralliilyticus/neptunius*-like strains resulted in high mortality rates (Kesarcodi-Watson et al., 2009a). Co-infections can have an important impact on the development and severity of disease and more research is needed to improve our understanding on the interactions between pathogens and how they interact with the immune response of the mussel host.

2.6.3 Temperature

Fluctuations in water temperature can significantly alter immune functions in mussels (Rahman et al., 2019), as demonstrated within *Perna canaliculus*. In a study where green-lipped mussels were subjected to 26°C for 48h an increase acid phosphatase and phenoloxidase activity was reported, along with increased metabolites linked to the glutathione metabolism and an increased realise of ROS by the haemocytes (Muznebin et al., 2022a). Chronic exposure (13 months) of *P. canaliculus* to 24°C resulted in 100% mortality towards the end of the experiment, and haemocytes showing increased respiratory burst (superoxide-positive) and apoptosis after 6 months (Ericson et al., 2023c). Furthermore, *P. canaliculus* subjected to a severe heat shock (30°C for 60 min) showed increases in non-viable haemocytes, and metabolites which support antioxidant molecules. Decreases in the generation of ROS production and total antioxidant capacity were also observed (Delorme et

al., 2021b). During a simulated high marine heatwave experiment (18 - 24°C, using a +2°C per week ramp) on *P. canaliculus*, the metabolomics data indicated the activation of molecular defence mechanisms, along with an increase in antioxidant metabolites. Additional evidence for immune functions was seen within the cytology results where high temperature stress affected the haemocyte counts and the percentage of superoxide-positive haemocytes (Venter et al., 2023). Mussels stressed at 33°C showed reduced GABAergic synapse activity after 3 hours (Dunphy et al., 2018) and mortality within two days (Dunphy et al., 2015). Larve of the Greenshell™ mussel induced significant amounts of HSP70 when experiencing temperatures of 40°C or more (Dunphy et al., 2013).

Pathogen infection, often encountered in the field, usually do not occur in isolation, but act in collection with other factors such as temperature, pH level, dissolved oxygen levels, nutrition availability and water quality. The collective effect of multiple drivers can be either a simple addition of the effects from individual drivers, greater (synergistic) or less (antagonistic) than the sum of isolated effects (Baag and Mandal, 2022). Immune parameters of *P. canaliculus* have been affected due to temperature in combination with bacterial infections and food limitation. Mussels infected with *Vibrio* sp. had less haemocytes and lower antioxidant capacity when kept at a higher temperature (24°C) for 24h (Ericson et al., 2022). Lower haemocyte counts were also seen when infecting *P. canaliculus* with *P. swingsii* at 24°C. Moreover, higher total antioxidant counts, and lipid peroxidation levels were seen at 24°C (Azizan et al., 2023b). When subjecting *P. canaliculus* to 54 h of fasting followed by heat stress (27°C), an increase in oxidative damage and decrease in antioxidant enzymes were seen (Delorme et al., 2020a). It is crucial to have a robust and accurate understanding of the effects of temperature (and associated stressors) on mussel immunity to be able to predict the future effects of rising temperatures.

2.6.4 Contaminants

Contaminants are often detected in coastal areas and represent a potential threat to bivalves, from direct toxic actions or from alterations of the homeostatic mechanisms including the immune system (Renault, 2015). Environmental pollutants can suppress mussel immunity, resulting in an elevated parasite infection rate. Additionally, parasites can interact with both natural and anthropogenic stressors, compromising mussel health and increasing mortality rates (Sures et al., 2017). The effects of heavy metals, pesticides, polycyclic aromatic hydrocarbons, nanoparticles, polychlorinated biphenyls, and pharmaceuticals on mussel immunity has been reviewed (Renault, 2015). In *P. canaliculus* the effects of cadmium, copper, microplastics and triclosan on the immune system have been investigated. Cadmium

significantly altered the proportional composition of haemocytes, induced DNA damage in haemocytes and increased nuclear aberrations in *P. canaliculus* (Chandurvelan et al., 2013). *P. canaliculus* exposed to copper resulted in increased haemocyte production, production of ROS and haemocyte apoptosis. Additionally metabolites linked to oxidative stress and apoptosis were affected (Nguyen et al., 2018a). Triclosan increased mussel oxidative stress markers including SOD and LPO, while microplastics enhanced the uptake of triclosan within the tissue of mussels (Webb et al., 2020). Ultimately it has been demonstrated that mussels living in contaminated areas are more vulnerable to infections due to immunosuppression caused by pollution (Ordás et al., 2007), highlighting contaminants as an important variable to examine in the evaluation of mussel immune responses.

2.6.5 Natural events

Mussel immunity can show seasonal fluctuations, driven by complex interactions between endogenous host factors and environmental factors (Balbi et al., 2017). Differential haemocyte counts of *P. canaliculus* reported higher phagocytosis in summer and lowest in winter months (Muznebin et al., 2022c). Post-earthquake biomarker measurements of *P. canaliculus* showed reduced metallothionein-like protein and catalase activity levels and increased lipid peroxidation and alkaline phosphatase levels in mussel gill and digestive gland tissues collected from affected sites post-earthquake period (in relation to a reference site) (Chandurvelan et al., 2016). These biomarkers have been suggested as indicators of general stress and could be used to follow recovery of mussels following exposure to natural disasters.

2.6.6 Farming factors

Biomarker approaches have been proposed as measurable indicators for quality control of farmed shellfish (Matozzo et al., 2018). The quality of aquaculture products are an outcome of the positive interactions between good environmental factors and correct farming procedures (Moschino et al., 2010). Immune parameters-based biomarkers in *P. canaliculus* have been used with connection to various farming practises on larvae, spat, juveniles and adults. For example, elevation of the aragonite saturation state to 4.5 Ωarag, to enrich pre-veliger incubation water, increased superoxide dismutase, glutathione reductase, and peroxidase levels, thereby minimising oxidative stress during this process (Ragg et al., 2019). Seeding density had no effect on oxidative stress markers (total antioxidant capacity and lipid peroxidation) in spat during summer months (Reyden et al., 2023). Additionally, subjection to low relative humidity during emersion, following by re-immersion, as a medium of transport or shoreline exposure, resulted in increased oxidative damage biomarkers (protein carbonyls,

lipid hydroperoxides, 8-hydroxydeoxyguanosine) in juvenile *P. canaliculus* (Delorme et al., 2021a). Metabolite biomarkers of cultured *P. canaliculus* showed that mechanical harvesting associated with commercial processing of farmed mussels resulted in anaerobic metabolism and affected amino and fatty acid metabolism, which plays vital roles in mussel immunity (Nguyen et al., 2020). This research area provides farmers with a comprehensive understanding of the importance of minimising intense physical stress, such as handling, to reduce potential immunosuppression in farmed mussels.

2.6.7 Sex

For the most part animals of opposing sex respond differently to stressful environments due to energetic trade-offs between reproduction (e.g., gamete production, mating behaviours, parental care, and offspring development) and stress resistance (e.g., changes in temperature, salinity, pH, nutrient availability, or exposure to toxins or pathogens) (Petes et al., 2008). This has been seen in the blue mussel *Mytillus edulis* with males being more affected by reduced seawater pH, increased temperature, and a bacterial challenge than the female (Ellis et al., 2014). In *P. canaliculus*, males showed higher mortality, oxidative stress, and apoptosis after pathogen exposure (Nguyen et al., 2018c). As a result, sex also affects disease susceptibility and immunological function within the host (Klein and Flanagan, 2016). This observation emphasises the need to study on sex-differences when investigating environmental stress and immunological studies.

2.6.8 Mussel life stage

Mussel life stage has been shown to have a significant effect on the expression of stress related biomarkers, highlighting the importance of including size as a parameter when performing an experiment (Zilberberg et al., 2011). It is believed that juvenile mussels are more susceptible to pathogenic infections (Benabdelmouna et al., 2018), as smaller mussels have a lower tolerance to pathogens, due to an undeveloped immune systems (Pruzzo et al., 2005, Lattos et al., 2020). However, haemocyte parameters were not influenced by the size of green mussels, *Perna viridis* (Donaghy and Volety, 2011). In a study on *P. canaliculus*, juvenile mussels showed higher mortalities, compared to adults when infected with *V. mediterranei, P. swingsii* and a combination of both (Azizan et al., 2023c). Additional research also is needed to determine if juvenile mussels exhibit an inefficient immune response when subjected to other stressors, consequently leading to mortality.

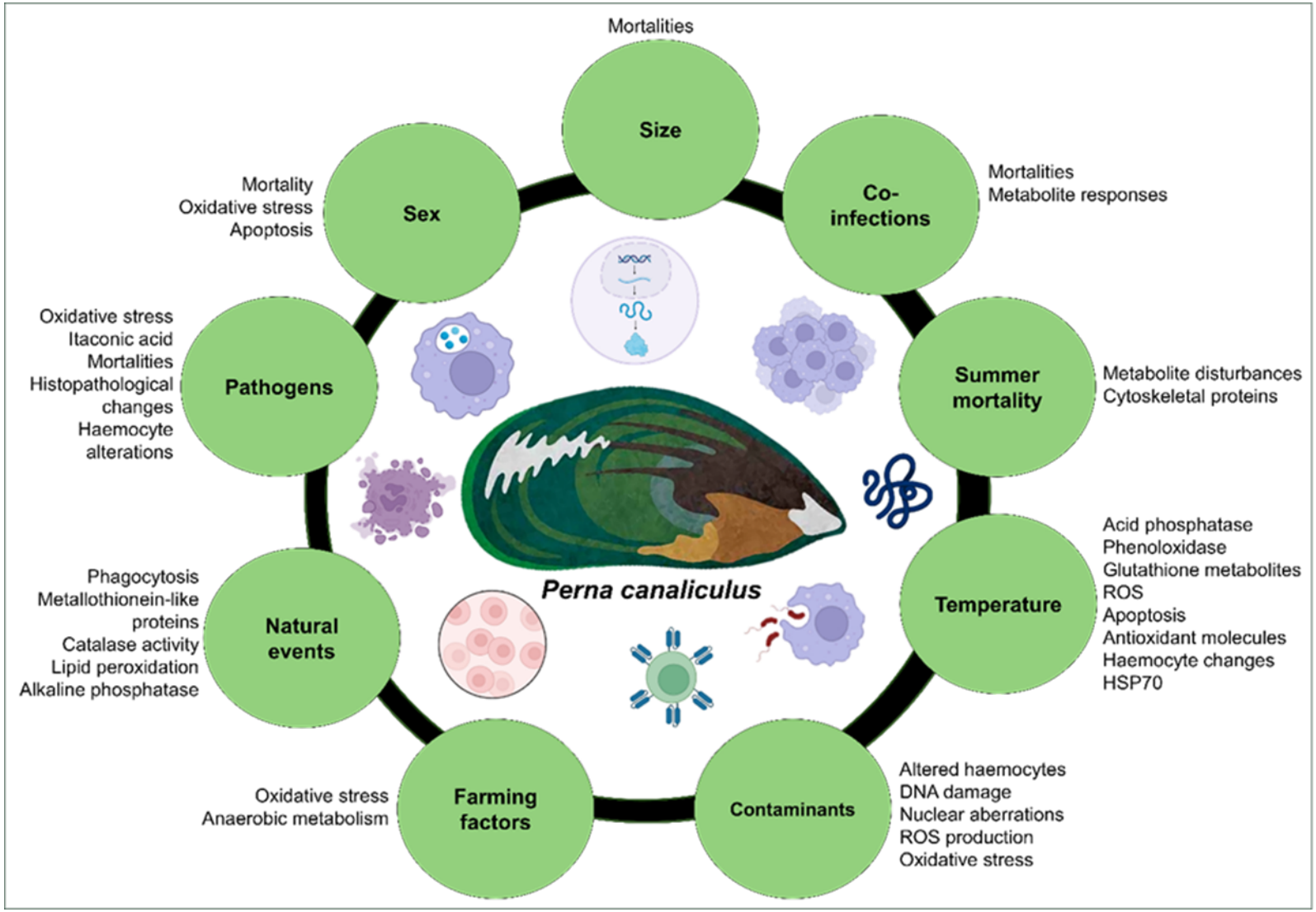


Figure 2.4. Biotic and abiotic factors recorded to produce an immune response in *P. canaliculus*.

2.7 APPLICATIONS, FUTURE PERSPECTIVES, AND CONCLUSIONS

Research on Greenshell™ mussel showcases the substantial worth of this marine organism across diverse scientific domains, encompassing aquaculture, human nutrition, environmental monitoring, and medicine (Table 2.1). Despite the lessons learned from pathogen infections around the globe, scientific gaps still exist, especially when considering *P. canaliculus* as research model. The direct impacts of diseases for example mortality are easy to monitor or quantify for farmers. However, indirect effects, such as a cellular response, are potentially more significant but more difficult to measure (Castinel et al., 2019). Utilisation of multi-omics approaches allows researchers to obtain more inclusive information of indirect effects of disease. Omics approaches involve high throughput techniques that have greatly increased the ability to characterise the function and dynamics of genes (genomics), expressed genes (transcriptomics), proteins (proteomics), low molecular weight metabolites (metabolomics) (Alfaro and Young, 2018). The sensitivity and specificity of most omics techniques makes them powerful tools in immune studies. Characterisation of bivalve haemocytes and tissues via transcriptomes, proteomes and metabolomes in response to pathogenic infections and/or environmental stressors have provided useful information on the mechanisms that drive the innate immune system in response to stress challenges, as well as understandings of complex host-pathogenic-environmental interactions across bivalve species (Nguyen and Alfaro, 2020a).

2.7.1 Omics potential

In brief, genomics technology (e.g., whole genome sequencing) can identify pathogens associated with disease, and or assist in characterising a particular aetiology. Additionally, sequence analyses (i.e., sequence-dependent, e.g., 16S clone or amplicon analysis or metagenomics), detection or quantification of a target sequence [sequence-independent; e.g., in-situ hybridisation (ISH) and quantitative polymerase chain reaction (PCR)] and multiple target sequences (e.g., DNA microarrays) can be used to generate a complete catalogue of genes that are involved in host–pathogen interactions (Burge et al., 2016b). Transcriptomic research can play a significant role in mussel research, as a tool to assess mechanisms involved in biological processes, such as responses to environmental stressors, new nutritional diets, or pathogens (Chandhini and Rejish Kumar, 2019). Expressed sequence tags can be utilised for the detection of differential expression and regulation of certain genes (Tanguy et al., 2008). RNA-Seq using next-generation sequencing allows exploration of the transcriptome of non-model organisms (Rey-Campos et al., 2019b), such as the Greenshell™ mussel, to better understand pathogenic evasion strategies. Proteomics allows for the

identification, localisation and quantification of proteins, as well as the analysis of protein modifications and the elucidation of protein-protein networks (Carrera et al., 2020). Techniques such as two-dimensional gel electrophoresis and mass spectrometry are mostly used used to provide valuable information for protein analysis in proteomics research (Campos et al., 2012). Proteomic approaches can be widely used in aquaculture to support the identification of new biomarkers and assess mechanisms involved in responses of mussels for example, to environmental stressors (Tomanek and Zuzow, 2010) or pathogens and accumulation of algal toxins (Puerto et al., 2011). Metabolomics analyses show how metabolic entities within a cell, tissue, or biofluid respond to external stressors or stimuli, at a certain time (Alfaro and Young, 2018). Utilisation of nuclear magnetic resonance (NMR) and mass spectrometry (MS) based techniques allows the measurement of changes in metabolites due to the presence of stressors (Young and Alfaro, 2018). In *P. canaliculus*, metabolomics have been previously used to investigate pathogen infections (Nguyen et al., 2019c, Ericson et al., 2022, Azizan et al., 2023c). Consequently, the integration of omics technologies together with physiology, behaviour and biology research for stressor-specific, species-specific and tissue-specific studies will be helpful for understanding various immune responses. The response can be significantly amplified if adequate and enough monitoring tolls are performed and combined together to understand the links between different pathways (Eissa and Wang, 2016).

2.7.2 Using immune responses to manage disease occurrences and potential application to the aquaculture industry

The lack of existing knowledge on basic immune defences of marine invertebrates is highlighted when problems arise in the aquaculture industry (Mydlarz et al., 2006). By investigating mussel immunity, relevant information on the quality of the marine environment is obtained, while also facilitating the understanding of occurrence of infectious diseases (Burgos-Aceves and Faggio, 2017). To this end, knowledge on immune responses is being applied to manage disease occurrences. Discoveries such as gene rearrangement mechanisms are implemented to protect mussels against infectious diseases (Gestal et al., 2008). Probiotics have also been applied as a means to protect against disease (Kesarcodi-Watson et al., 2012). The addition of immunostimulants to diets of farm-raised invertebrates, has shown to promote immune function and prevent against disease (Mydlarz et al., 2006). Learning from past events also allows for valuable insight. For example, natural selection of shellfish which survived a mortality event can be implemented to produce seed with a greater resistance to disease by utilising genome-based biotechnologies (Fox et al., 2020). Moreover, the implementation of a standard set of biomarkers or diagnostic tools (obtained from

immunological studies) can help to assess mussel health can facilitate the rapid detection of pathogens and provide early warnings of unfavourable farm conditions (Aldridge et al., 2023).

Greater knowledge on mussel immunity can serve the aquaculture industry in various ways. For example, a strong scientific basis can be used to support biosecurity programmes, enabling risk assessments and accompany policy development and identify priorities for future research (Bondad-Reantaso et al., 2005). Surveillance (confirm that a population is free from a disease) and monitoring (determining the level of disease) programs will also benefit from immunological research, as it informs on responses implemented by the host (Cameron, 2002), and highlights which factors should be closely investigated. Ultimately, the implementation of aquatic health programs will lead to the establishment of diagnostic services, such as reference laboratories. With such advances also comes opportunities where these centres can facilitate research and collaboration, develop quality control programs and act as contact centres for advice and training to regional experts who can provide diagnostic assistance and answer technical question in the field (Bondad-Reantaso et al., 2005).

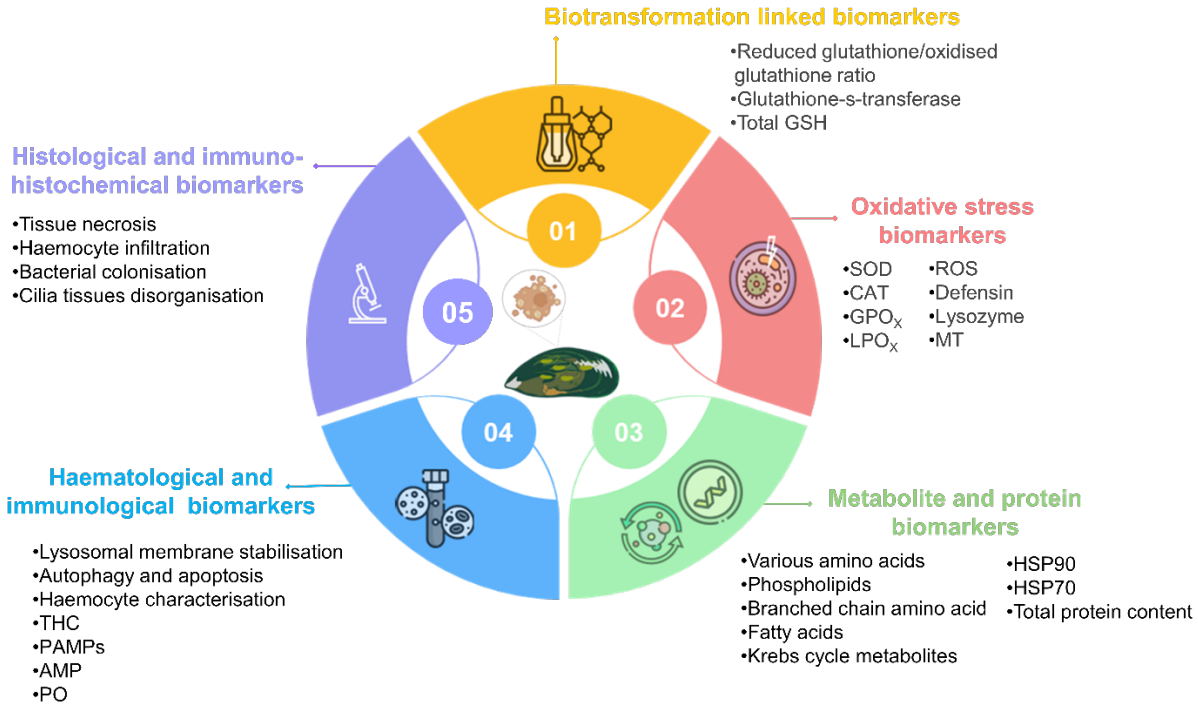
2.7.3 Going forward

Environmental stressors enables pathogenic bacteria to proliferate resulting in bacterial infections of mussels (Babarro and de Zwaan, 2002). Thus, the close link between mussels and their environment remains a key research area, as demonstrated in the examples provided above where temperature, contaminants, farming factors and natural events have been shown to affect the immune system of Greenshell™ mussels. More can still be done to understand biological responses at physiological, evolutionary and ecological levels (Boyd et al., 2018). The mechanisms underlying innate immunity depend on functional proteins, such as effector proteins, regulatory pathways and related genes, which are largely under studied (Renault, 2009). In particular factors within Greenshell™ mussels' humoral immunity, are scarce. Specific information on the immune recognition system, activation of signalling pathways and synthesis of specific effectors are needed for *P. canaliculus,* creating a research area with various opportunities. Moreover, virulence factors associated with pathogens of *P. canaliculus* are poorly recognised and not extensively examined. To improve our understanding of pathogenesis more efforts will need to be invested into the characterisation of pathogen virulence factors. While investigating the pathogen itself it is also important to understand the pathogen life history (i.e., how they spread, or what time of the year the flourish), as this will be key to predict the risk of pathogens successfully establishing elsewhere (Brian et al., 2021). A further investigation is warranted to elucidate the impact of immune system variation on natural population susceptibility to infection (Mydlarz et al., 2006). To date

only a handful of studies have accessed natural field populations of Greenshell™ mussels. Yet, to meet the resilient outcomes of on farm biosecurity plans, as outlined by the implementation plan of the NZ governments aquaculture strategy (FISHERIES`NEW`ZEALAND, 2022), more efforts will be needed.

For the purpose of providing management tools and strategies for mussel diseases and/or protection of already healthy populations, it is necessary to understand how infections and diseases interact comprehensively. Consequently, an integrated approach that considers the entire ecosystem's impact is needed. Furthermore, high-throughput technologies and rapid developments in bioinformatics and artificial intelligence are enabling omics tools to provide new methods and approaches for understanding these complex health processes. Furthermore, the ability to harness the potential of large-scale data sets effectively and derive comprehensive insights requires the strengthening of collaborations between researchers from a variety of disciplines. Science remains a huge enabler of shellfish aquaculture success in New Zealand. Scientific advances relating to *P. canaliculus* immunology is a good example hereof, where great progress has been made in identifying and characterising various immune molecules and disease from Greenshell™ mussels. These molecules act against various biotic and abiotic stressors and allows us as researchers to better understand immune response mechanisms implemented by the host and the relationship with the pathogen. Additionally, the understanding of immune responses in mussels holds substantial economic, ecological, and public health importance, both locally in New Zealand and worldwide.

Chapter 3: Physiological biomarkers of mussel Vibrionaceae: A review on the constraints and potentials



In this chapter, a review of the relevant literature on biomarker research on *Vibrio*-mussel interactions, and aspects related to the mussel health and disease assessment, along with some insight into mussel biology and physiology is presented.

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Abstract

Bacterial infections caused by *Vibrio* spp. have affected various species of mussels around the world, with limited insights into the responses implemented by mussels against these infections. A combination of chemical analyses and carefully selected biological endpoints from haemolymph and tissues can help protect aquatic ecosystems and determine the welfare status of mussels. Recent developments in biomarker identification tools, such as omics and bioinformatics have been successfully applied evaluate the effect of environmental pollutants and other chemicals on mussels. However, the application of biomarkers to assess mussel health is limited. This review describes the available scientific literature on biomarker research for *Vibrio*-mussel interactions, and those aspects related to mussel health and disease assessment; grouped as biomarkers of exposure, effects, and susceptibility. This review revealed that, when integrated, such biomarkers can provide a deeper understanding of the relative health and potential susceptibility of mussels for better management practices. Furthermore, such health biomarker data could be used to build resilient in mussels against deleterious climate change conditions, strength biosecurity management programs, improve farming and processing efficiency and add value in terms of market-desirable traits.

Keywords

Environmental stressors; biomarkers, omics, mussels; *Vibrio*; vibriosis.

3.1 INTRODUCTION

Mussels have been long recognised as a valuable economic resource, with around 94% of mussels being produced through aquaculture worldwide (Avdelas et al., 2021). This is supported by mainly mussels belonging to the marine family *Mytilidae* or to the freshwater family *Unionidae* (Bayne et al., 1976, Davenport, 2012). There are different mussel species distributed worldwide in various ecosystems, including rocky reefs, wharf piles, amongst seaweed beds and soft-muddy bottom habitats (Vakily, 1989). In addition to being valuable aquaculture resources, mussels have ecological importance, since they play a key role in the flux of energy in the environment and in the maintenance of water quality and pollution sensing (Figueras et al., 2019).

The increasing aquaculture production of mussels globally has the potential to produce high-quality protein for human and animal nutrition with less environmental impacts compared to terrestrial food production systems (Suplicy, 2020). However, mussel production is increasingly affected by rising temperatures due to climate change stressors (San Martin et al., 2020), ocean acidification (Ren et al., 2020), diseases (Castinel et al., 2019), the spread

of pathogens (Ritzenhofen et al., 2021), and general loss of mussel seed from longline systems (South et al., 2022). In particular, opportunistic pathogens such as *Vibrio* spp. have been implicated in mussel mortality events (Li et al., 2020, Nguyen and Alfaro, 2020b), requiring a better understanding of the health parameters of both aquaculture and wild mussel populations (Lupo et al., 2021). *Vibrionaceae*, is a family of proteobacteria containing the genus *Vibrio*, which include endemic inhabitants of marine microbial communities, and are responsible for approximately 20% of all disease outbreaks in mussel populations (Potasman et al., 2002). Some *Vibrio* species, such as *V. anguillarum* biovar I, *V. splendidus*, *V. diabolicus*, *V. corallyticus*, *V. aestuarianus* and *V. harveyi* are known to be highly pathogenic to mussels (Le Roux et al., 2005). However, investigations regarding how these pathogens affect mussel health are limited, and crucially needed to understand infection mechanisms and host susceptibility and resilience to inform surveillance programs.

Previous studies on the impact of vibriosis on mussel populations have focused on multiple levels of biological organisation, including biochemical, cytological, physiological, and auto-ecological indices (Newton and Cope, 2006, Sures et al., 2017, Waller and Cope, 2019, Zare Jeddi et al., 2021). This holistic approach is important as laboratory-based studies with different *Vibrio* species have revealed a complex interaction between molecular, cellular, biochemical, physiological, and behavioral responses in bivalves (Dong et al., 2017, Liu et al., 2014b, Wu et al., 2013, Nguyen and Alfaro, 2020a, Moreira et al., 2015) . Techniques, such as genomics, transcriptomics, proteomics, and metabolomics (Alfaro and Young, 2018, Nguyen and Alfaro, 2020a) are now being applied to characterise biological pathways and mechanisms involved in *Vibrio* spp. infections. By integrating analytical approaches with carefully selected biological endpoints (biomarkers), it becomes possible to gain a deeper understanding of *Vibrio* spp. infections. Multi-omic approaches may also lead to the identification of biomarkers, which can be used in surveillance programs and management strategies across aqaculture farms and wild populations. Although the use of biomarkers is not a new paradigm, such approaches have been under-utilised in the context of mussel health assessment and the potential to build resilient in light of rapidly changing climatic conditions.

In this chapter, we present a comprehensive review of previously identified biomarkers and their applicability to mussel health assessment, following exposure to *Vibrio* spp. This work includes 91 peer-reviewed articles which included five required criteria. The paper had to 1) report on a *Vibrio* spp. that had been found to infect and/or inhabit a mussel species; 2) report *Vibrio* spp. infection concentrations (CFU/mL) which the mussel was exposed to; 3) include a robust experimental design with replication of control and bacterial treatments; 4) utilise

analytical methods to measure the mussel's response to infection, and 5) report physiological responses following infection.

3.2 BIOMARKER USES AND CHARACTERISTICS

The response of mussels to pathogenic bacterial infections can range from a single response of immune cells to the complete breakdown of physiological processes, tissue damage and death (Oliver and Fisher, 1999, Paillard et al., 2004). The measurement of responses to capture what is happening in a cell or organism at a given time can be achieved by using biomarkers (Califf, 2018). Biomarkers can be physiological, cellular, histological, biochemical, or molecular changes seen within a host (i.e., mussel) in response to a stressor (i.e., *Vibrio spp*.). used as indicators of host responses (Oliver and Fisher, 1999, Brooks et al., 2009, Dalzochio et al., 2016). Successful applications of biomarkers to monitor aquatic ecosystems have been reported in molluscs, crustaceans, and fish (Van der Oost et al., 2003, Newton and Cope, 2006, Hook et al., 2014). To ensure consistency within the literature, the following definitions for sets of biomarkers are used in this review, as derived from (Kroon et al., 2017). Firstly, **biomarkers of exposure**, are markers that indicate a response following exposure, generally as an early response signal. These are markers which tell us what happens as soon as mussels are exposed to *Vibrio* spp. Secondly, **biomarkers of effect** are markers representative of changes due to exposure, typically associated with a specific condition. These markers showcase the physiological response of mussels due to infection with *Vibrio* spp. Thirdly, **biomarkers of susceptibility** are markers indicative of an organism's likeliness to develop a disease, based on the organism's inherent ability to respond to a challenge. These markers may be used to determine, for example, if mussels from selective breeding programs will be able to survive *Vibrio* spp. infections (Fig. 31). Considering that biomarkers lie on a continuum, it may be difficult to distinguish between biomarkers of exposure and effect (Hook et al., 2014).

In mussel health assessment, biomarkers can be detected in haemolymph, extrapallial fluid and soft tissues, such as gills, digestive glands, gonads, and adductor muscle. The samples may then be used to form a biosignature profile describing the biochemical and physiological state of the host as a whole (Newton and Cope, 2006, Brooks et al., 2009, Waller and Cope, 2019). Linking biomarkers with mussel health can be of great value for diagnosis of disease (e.g., vibriosis), as it can be used to confirm infections, screen for vibriosis and monitor disease progression. Biomarkers can also be used to guide decision making for efficient curative treatments to eliminate the pathogen once it is present, while also limiting the spread of the

disease (World Health and International Programme on Chemical, 1993, Paillard et al., 2004, Gestal et al., 2008, Brosset et al., 2021).

For a biomarker to be used in risk assessments linked to ecosystem and organismal health, they need to meet certain criteria (Oliver and Fisher, 1999, Newton and Cope, 2006, Moore et al., 2007, Ryan et al., 2007, Hook et al., 2014, Kroon et al., 2017, Brosset et al., 2021). Even though the criteria are fluid, with large variations for discovery to validated biomarkers, the following qualities are desired when looking for biomarkers (Fig. 1). An ideal biomarker would: 1) be easy and safe to collect, transport and store; 2) be easy to measure and analyse; 3) possess a long half-life, with clarity on the time-relationship from exposure to response and persisting effects; 4) be relatively cheap to monitor; 5) be robust and rapid to analyse; 6) be representative of a large sample size; 7) show sensitivity to the variations in populations (e.g., from different geographical regions, seasonal trends, gender differences); 8) be distributed over wide spatial and temporal ranges of health outcomes; 9) allow quantifiable results; and 10) result in reproducible results.

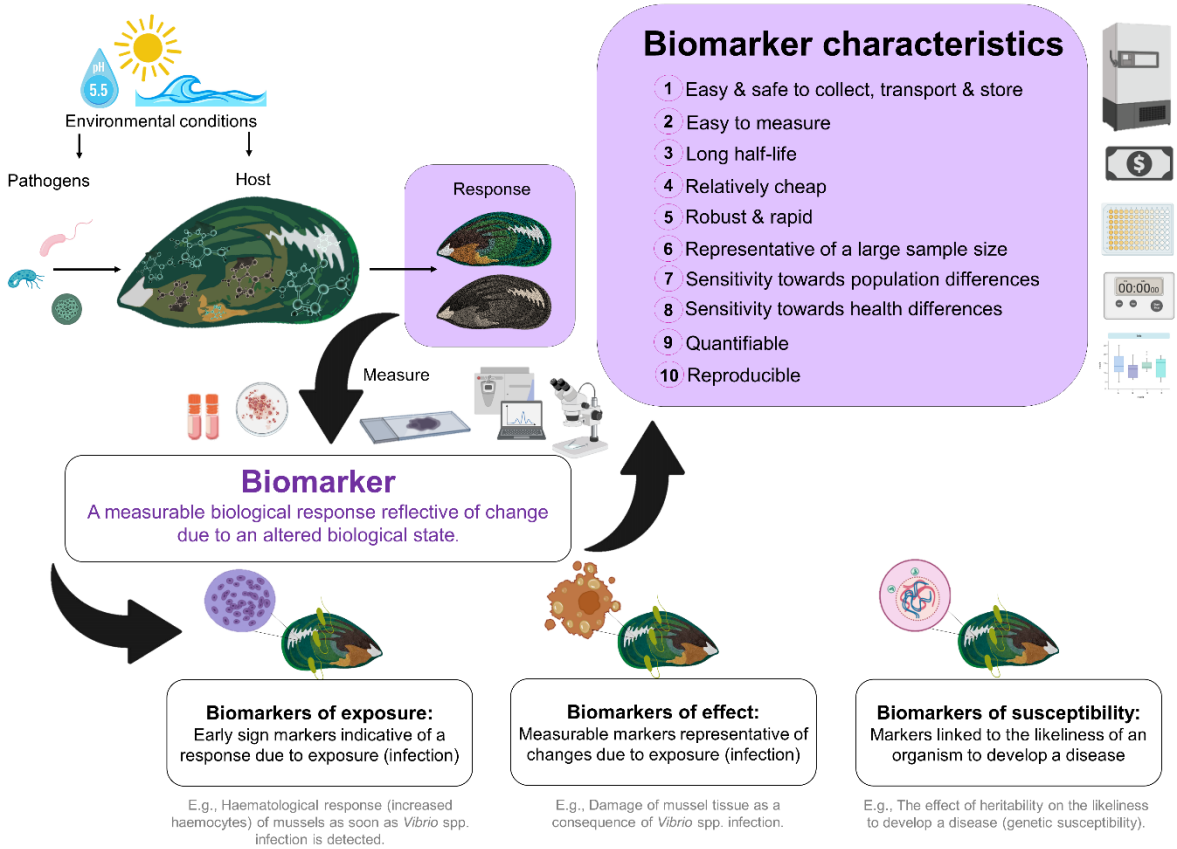

Figure 3.1. Types and characteristics of biomarkers to be used in risk assessments linked to ecosystem and organismal health.

3.3 VIBRIONACEAE

The earliest reports of shellfish-transmitted bacterial diseases were documented in the late 19th and early 20th century, with *Vibrio* species classified as the most notable examples, responsible for about 20% of all disease outbreaks (Potasman et al., 2002). A family of heterotrophic bacteria called *Vibrionaceae* (commonly abbreviated to *Vibrios*), are rod-shaped, gamma-proteobacteria which inhabit ocean environments, brackish water and freshwater environments (Thompson et al., 2004, Takemura et al., 2014). These bacteria encompass eight genera, including: *Aliivibrio*, *Echinimonas*, *Enterovibrio, Grimontia*, *Photobacterium*, *Salinivibrio*, *Vibrio*, and the newly described *Thaumasiovibrio*, with more species yet to be found (Sawabe et al., 2013, Amin et al., 2017). Their metabolic flexibility and genetic variability contribute to their high colonisation potential, with Vibrios commonly found in plants, algae (Takemura et al., 2014, Le Roux and Blokesch, 2018), zooplankton molluscs and more (Kesarcodi-Watson et al., 2009a, McFall-Ngai et al., 2013, Destoumieux-Garzón et al., 2020). Molluscs have shown to promote bacterial survival in aquaculture systems and trigger infections by serving as hosts and providing a favourable environment for pathogenic bacteria (Miccoli et al., 2019). Typically, *Vibrio* spp. are potential mussel pathogens, as they damage the host upon infection, severely compromising shellfish farming operations (Travers et al., 2015, Destoumieux-Garzón et al., 2020). The study of factors associated with an increase in *Vibrio* abundance is therefore of considerable public health and economic interest.

Reports of pathogenic *Vibrio* diseases in mussels are less known than in other bivalve species. Typically, mussels are considered to be highly resilient to microbial infections, in particular *Vibrio* spp. infections (Stabili et al., 2005), with low numbers of reported mussel mass mortalities due to Vibrios. Environmental conditions (e.g., non-optimal temperature, pH, and salinity), and chemical contaminants, intraspecific competition and predation have been at the forefront of mass mortalities in mussels (Domeneghetti et al., 2014, Charles et al., 2020a, Romero et al., 2014). Despite other stressors deemed as the main causes of mussel mortalities, Vibrio spp. strains are often found in affected mussel population. For example, *V. splendidus* has been reported in diseased *Mytilus edulis* adults (Ben Cheikh et al., 2016, Ben Cheikh et al., 2017), *V. splendidus* and *V. coralliilyticus*/*neptunius*-like strains were associated with high mortality rates in larval *Perna canaliculus* (Kesarcodi-Watson et al., 2009b), *V. coralliilyticus* was toxic in in-vitro mussel studies (Wilson et al 2013; Balbi et al 2019), and it was shown that mussels were more susceptible to pathogenic *Vibrio* spp. of the Splendidus clade (Charles et al., 2020b). Despite the various mussel mortality and *Vibrio* spp. associated studies, it is still uncertain whether *Vibrio* spp. causes mussel mortality under normal

conditions and/or in the presence of additional climate stressors (Destoumieux-Garzón et al., 2020, Lupo et al., 2021, Richard et al., 2021, Harrison et al., 2022, Eggermont, 2017).

Despite recent studies on the interactions between pathogenic *Vibrio* spp. and mussels, more research is needed before the implantation of biomarkers as a measure of mussel health becomes a reality (Newton and Cope, 2006, Charles et al., 2020b, Destoumieux-Garzón et al., 2020). Future studies targeting *Vibrio* spp. infections in mussels should: 1) Consider that *Vibrio* spp. infections are one of the many stressors affecting mussel physiology, at all life stages; 2) Note that *Vibrio* spp. are distributed everywhere in the environment (e.g., in suspended particles, sediments, biofilms, aquaculture infrastructure such as tanks and pipes, feed, water from upstream farms and the bivalves themselves), with different species of Vibrios growing at the same time; 3) The presence of Vibrio spp. can be affected by water parameters, such as temperature, pH, salinity, and nutrients. Furthermore, many Vibrio species are found in higher concentrations during summer months (Vezzulli et al., 2013, de Souza Valente and Wan, 2021), while other species remain inactive during the winter months (Croci et al., 2001). Ultimately, *Vibrio* spp. can be used as a microbial barometer of climate change (Baker-Austin et al., 2017); 4) Be aware that *Vibrio* spp. spread mainly through, horizontal contact and waterborne transmission; 5) Pathogen responses in nature are more complex than in the laboratory; 6) Baseline biomarker data should be well established to distinguish between natural variability and bacteria-induced stress; 7) Keep in mind that studies that rely on simplistic pathogenicity tests are more likely to mis-detect Vibrio spp. if concentrations are low; 8) Target disease progression from early to pathological stages, to provide a direct measurement of the *Vibrio* spp. exposure. Therefore, biomarkers play a vital role in environmental monitoring and risk assessment, particularly in the detection of Vibrio spp. on mussels. For biomarkers to serve as reliable and useful tools for detecting and monitoring aquatic disease risks, these criteria must be established for each candidate biomarker.

3.4 BIOMARKERS DETECTED IN MUSSELS IN RESPONSE TO *VIBRIO* SPP. INFECTION

Mussels are constantly being challenged by changing environments, making them more susceptible to diseases (Delorme et al., 2021b). As a result various tools and techniques exist to assess the relative health of mussels. Indeed, there has been an increased interest in research that assesses the physiological responses (biomarkers) of mussels exposed to stressful conditions. Assessment of various biomarker research are next summarised with

relation to *Vibrio* spp. infections in mussels, using exposure, effect, susceptibility, and the combined effects as biomarker classifications.

3.4.1 Biomarkers of exposure

As mentioned above biomarkers of exposure can show an early response to infection (exposure) and are generally indicative of the extent to which an organism is exposed to a given stressor. While focusing on *Vibrio* spp. infections in mussels, biomarkers of exposure are classified as: biotransformation intermediates, oxidative stress markers, metabolites and proteins, haematological parameters, and immunological parameters.

3.4.1.1 Biotransformation intermediates

The glutathione antioxidant system plays a fundamental role in the cellular defence mechanism against free radicals and other oxidants (Meister, 1988). Glutathione (GSH) scavenges free radicals and other reactive oxygen species (ROS) through enzymatic reactions where GSH is oxidised to form glutathione disulphide (GSSG), which is then reduced to GSH by the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent glutathione reductase. GSH also reacts with electrophiles to form glutathione-S-transferase (GST) during biotransformation processes to eliminate detoxification products. Furthermore, GSH has been classified as essential for the activation of immune cells when an organism is immunologically challenged (Wu et al. 2004). These and other functions of GSH and the associated pathways make GSH products and enzymes popular biomarkers, readily used in environmental monitoring research (Laitano and Fernández-Gimenez, 2016). Laboratory-based studies focused on infecting mussels with *Vibrio splendidus*, *V. anguillarum* and *V. harveyi* have found increased glutathione-S-transferase enzyme activity following exposure (Canesi et al., 2010, Liu et al., 2015). Metabolites linked to GSH: GSSH and total GSH were also found to be increased in mussels (*P. canaliculus*) infected with *Vibrio* spp. DO1 (*V. coralliilyticus*/*neptunius*-like isolate) (Nguyen et al., 2019a). In addition, GST has been detected in *M. galloproviancialis*, and *Eliptio complanata* following infections with *V. anguillarum* (Canesi et al., 2010, Wu et al., 2013, Ji et al., 2013, François et al., 2015). These studies highlight GST as a strong biomarker for evaluation of *Vibrio* spp. Table 3.1 indicates that GSH and GST could be successfully applied to monitor changes in mussel biotransformation markers. Changes in biotransformation pathways are closely linked with the antioxidant defence systems, with the production of ROS as an end-product, highlighting the importance of monitoring oxidative stress markers together with biotransformation markers. However, care should be taken when measuring biotransformation intermediates and

oxidative stress markers, as these are generally not stable compounds (or can be changed to other forms), complicating the pre-analytical disposition of samples prior to measuring the response (Ho et al., 2013).

3.4.1.2 Oxidative stress markers

Biomarkers related to oxidative stress are frequently used in mussel assessment after exposure to pathogenic bacteria, as summarised in Table 3.2. Oxidative stress results from an imbalance in the production of oxidising species (e.g., ROS and oxygen free radicals) or a significant decrease in production of antioxidants (such as glutathione) (Kroon et al., 2017, Delorme et al., 2021b). Prolonged oxidative stress can lead to enzyme deactivation, lipid peroxidation (LPOx), deoxyribonucleic acid (DNA) damage and eventually apoptosis or cell necrosis. It has been well established that mussels have an effective antioxidant system, enabling a balance of basal level free radical production (Kroon et al., 2017). Oxidative stress markers, such as enzymatic superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPOx), LPOx, ROS, defensin, lysozyme and metallothionein, have been investigated as oxidative stress indicators in mussels exposed to *Vibrio* spp.

Superoxide dismutase (SOD): This is an enzyme that catalyses the reduction of superoxide anions into hydrogen peroxide during oxidative stress, which is detoxified by catalase (CAT) to keep a balance between the generation and elimination of hydrogen peroxide (H_2O_2) (Kroon et al., 2017), and prevent the generation of highly toxic hydroxide ions (OH^-) (Fridovich, 1973). In effect, SOD eliminates superoxide anions and serves as a first line of defence against ROS. Three main SOD forms based on metal active sites are known, including copper/zinc (Cu/Zn)-SOD (in the cytosol of eukaryotes), manganese (Mn)-SOD (in the mitochondria of both eukaryotes and prokaryotes) and iron (Fe)-SOD (in both eubacteria and archaebacteria) (Geret et al., 2004). SOD activity was largely increased in *Mytillus edulis* when exposed to *V. tubiashii*, *V. splendidus*, *V. parahaemolyticus, V. tubiashii, V. splendidus*, and *V. alginolyticus*, suggesting a protective response from mussels against the free radical damage from bacterial infection (Parry and Pipe, 2004, Tanguy et al., 2013a, Hernroth et al., 2016). Other laboratory studies support an increase in SOD activity in *M. galloproviancialis* exposed to *V. harveyi* and *V. splendidus LGP32*, and in *Pinna nobilis* exposed to *V. harveyi* and other *Vibrio* spp. (Künili et al., 2021). Copper zinc superoxide dismutases (Cu/Zn-SODs) and manganese superoxide dismutases (Mn-SODs*)* are believed to play a central role in preventing oxidative injury in tissues, and they have been observed in *M. coruscus* under *V. parahemolyticus* infection (Wu et al., 2017). It has been found that Cu/Zn-SOD is important in SOD superfamily, especially in relation to mussel metabolism, since it is one of the most effective free radical scavengers

during a oxidative stress response (Wu et al., 2017, Sendra et al., 2020). On the other hand, Mn-SOD is regarded as a precursor protein, produced in mitochondria, which plays a vital role in the cytoplasm after it cleaves signal peptides (Wang et al., 2010, Wu et al., 2017). The monitoring of SOD is a versatile biomarker and might play a pivotal role in maintaining ROS concentrations in mussels.

Catalase (CAT): This is an enzyme which catalyses the decomposition of H_2O_2 into water and oxygen, thereby maintaining optimum H_2O_2 which is essential for cell signalling processes (Nandi et al 2019). The role of CAT as a defence against H_2O_2 induced by pathogen infection has been identified in various mussel tissues and haemolymph samples, as seen in Table 3.2. In a study on *M. galloproviancialis* catalase activity was stimulated in the digestive gland following infection with *V. splendidus* and *V. anguillarum*, respectively (Canesi et al., 2010). The use of CAT as a biomarker against *V. splendidus LGP32*, has been highlighted by Sendra et al. (2020), who reported up-regulation of catalase messenger RNA (mRNA) expression following infection in *M. galloproviancialis*. In a study by Bao et al. (2018), explicit expression of a *McCAT* gene was found in the hepatopancreas of *M. coruscus* following infection with *V. parahemolyticus* and *A. hydrophila*. In tissues of *P. nobilis*, co-infections between *V. harveyi* and other *Vibrio* spp. showed increased CAT activity revealing that the organism was facing oxidative stress due to the bacterial co-infection (Künili et al., 2021). These reports show that pathogenic bacteria could induce high expression of CAT in mussel tissues and CAT is a typical inducible protein, which plays an important immune defence role on the invasion of bacteria (Bao et al., 2018), and highlights CAT as a biomarker when concerned with prevention of oxidative stress damage.

Glutathione peroxidases (GPOx): In the mitochondria (and sometimes in the cytosol), GPOx reduces H_2O_2 to water, and lipid peroxides to their corresponding alcohols. Specifically, this enzyme inhibits the lipid peroxidation process and thus protects cells from the harmful effects of oxidation (Vidal-Liñán et al., 2015). Currently, there are only two studies which report GPO_X activity in mussels (*P. nobillis* and *Bathymodiolus azoricus)*, mainly focused on detection of their presence or variation in response to *Vibrio* spp. infection. In the first study, GPox activity increased significantly in the gills and digestive glands of *Pinna nobilis* following co-infection of *Haplosporidium pinnae* and multiple *Vibrio* infection (Künili et al., 2021). In the second study, glutathione peroxidase I (GPOx1) was up-regulated in digestive gland and gill tissues after mussels were challenged with *V. alginolyticus, V. anguillarum* and *V. splendidus* suspensions (Martins et al., 2014). The analysed studies reveal increased GPO_X levels, which may be associated with a decrease in the harm caused by H_2O_2 generated after invasion by the pathogen (Ren et al., 2009). However, future studies will have to consider the measurement

of superoxide radicals and hydrogen peroxide concentration alongside GPOx activity in mussels to better understand the impact of *Vibrio* spp. infections. A potential constraint of GPOx as biomarker is the fact that is can be influenced by seasonal variation (Martins et al., 2014), emphasising that specific guidelines should be in place when measuring this marker.

Lipid peroxidation (LPO_X): LPO_X occurs as a result of oxidation of polyunsaturated fatty acids as a consequence of oxidative stress, and can be quantified by measuring the LPOx degradation products, such as lysosomal lipofuscin, aldehydes, acetone and malondialdehyde (MDA) (Kroon et al., 2017). Only a few studies have identified LPO_X activity in mussels following *Vibrio* spp. challenges. In one example, it was found that both *V. splendidus* and *V. anguillarum* induced the accumulation of lysosomal lipofuscin, an end-product of lipid peroxidation, in the digestive gland of *M. galloproviancialis* (Canesi et al., 2010). Another study showed freshwater mussels (*Eliptio complanate*) co-exposed to municipal effluents and *V. anguillarum* infection which had increased level of MDA-TBA_2 adducts (or called as thiobarturic acid reactants (TBARS)) in the digestive glands (François et al., 2015). MDA levels were also observed to be higher in the gill of *P. canaliculus* under 24 and 120 hours pathogenic bacteria (*P. swingsii*) challenge at different temperatures (16°C and 24°C) (Azizan et al., 2023a). These findings suggest dysfunction of the antioxidant system in mussels, as a measure of lipid peroxide performance is a consequence of bacterial infection. Future research of LPOx biomarkers should include measured concentrations of the LPOx products, i.e., malondialdehyde (MDA) and, lysosomal lipofuscin, to produce further insights on this biomarker.

Reactive oxygen species (ROS): ROS production is necessary for the elimination of microorganisms, such as viruses, bacteria, fungi, and protozoa within the host, via the activation of respiratory burst (Deretic et al., 2013). ROS over-production tends to induce oxidative damage to various cellular components, such as lipids, proteins, and nucleic acids. In several studies, where mussels were infected with *Vibrio* spp., increased ROS was mainly reported (Table 3.2). The bacterium *V. splendidus* enhanced significant up-regulation of ROS in haemolymph samples of *M. edulis* (Tanguy et al., 2013a). Increased ROS in *M. gallloproviancialis* were reported following infected with *V. anguillarium*, *V. alginolyticus* and *V. splendidus* LGP32 (García-García et al., 2008, Costa et al., 2009, Wang et al., 2013c, Sendra et al., 2020). *P. canaliculus* also showed increased ROS production in response to *Vibrio* spp. *DO1* (*V.coralliilyticus*/*neptunius-like isolate*) infections (Nguyen et al., 2018c, Ericson et al., 2022). From these papers, it can be concluded that *Vibrio* spp. infections trigger oxidative burst, leading to the rapid production of ROS to combat and destroy invading bacteria. Along with higher ROS comes impaired cellular functions following oxidative

damage, highlighting the need to measure oxidative stress markers in tandem to characterise the cellular redox status of mussels following *Vibrio* spp. infections.

Defensin & lysozyme: These are important defence molecules which play a vital role in anti-oxidative stress and immune defence systems (Zhao et al., 2010a, Zhao et al., 2010b). Both are antibacterial components that have been characterised in marine molluscs (Oliver and Fisher, 1999). After challenging *M. galloproviancialis* with *V. harveyi*, mRNA expression level of these two stress-responsive genes were significantly up-regulated in the hepatopancreases of both male and female mussels (Liu et al., 2014b). Varying responses of defensin were further detected in different *V. harveyi* infected tissues, with the digestive gland defensin mRNA more upregulated than gill samples (Liu et al., 2014b). Lysozyme-like activities were also reported in *M. galloprovincialis* following injection with *V. alginolyticus*, *V. aestuarianus* 01/032 (*V.a.*), *V. splendidus* LGP32, V. *anguillarum* and in a second study injection with *Micrococcus lysodeikticus*, and exposure to temperature stress (Li et al., 2009a, Balbi et al., 2013, Laith et al., 2021). Up-regulated defensin and low levels of lysozyme were also found in 2- day old D-larvae of *M. edulis* following a 48-hour *Vibrio* challenge, supporting involvement of these compounds in immune functions in the early development phase of mussels (Van Hung et al., 2019). Both defensin and lysozyme are antibacterial components that have been characterised in marine molluscs (Liu et al., 2013) and can serve well as indicators of immune stress induced by *Vibrio* spp. infections. Additionally these biomarkers, showed consistent changes due to a number of *Vibrio* species infections (Van Hung et al., 2019), making it a strong potential biomarker for assessing mussel health.

Metallothioneins (MTs): MTs are highly conserved, low-molecular-weight, cysteine-rich non-inducible enzymatic proteins that participate in metabolism of essential metals, their detoxification, and scavenging of oxyradicals (Sigel et al., 2015). Aquatic pathogen or oxidative stress-producing chemicals trigger the expression of MT as a defence response. Canesi et al. (2010) reported that mussels (*M. galloprovincialis)* infected with both *V. splendidus* and *V. anguillarum* resulted in oxidative and immune stress with significant over-expression of MTs involved in antioxidant and immune functions. In a study by Ge et al. (2020), after infection by *V. parahemolyticus*, the expression of *Mytilus coruscus* metallothionein (*McMT*) in hepatopancreas tissue increased until 24 hours later, and then showed a slow decline. Bacterial endotoxins, as produced by *Vibrio* spp. are known to generate oxygen-derived products and inflammation within mussels, with induced expression of MT to follow. In response to the MT findings of Table 3.2, MT are increased for cellular protection, scavenging free radicals and acting as an anti-inflammatory mediator (Ge et al., 2020).

3.4.1.3 Metabolites and stress proteins

Various metabolomic studies have characterised metabolic changes linked to endogenous (effect biomarkers) and exogenous (exposure biomarkers) metabolites during disease progression and infection as shown in Table 3.3. *Vibrio* spp. exposure is generally associated with severe disruptions of the hosts energy metabolism, osmotic regulation, oxidative stress, signalling pathways, and respiratory mechanisms. The results of recent metabolomic studies investigating the immune responses of mussels to infection with various *Vibrio* spp. illustrate that the metabolite biomarkers differ depending on the *Vibrio* strain (Ji et al., 2013, Liu et al., 2014c, Liu et al., 2014b, Nguyen et al., 2018b), the different tissues (Nguyen et al., 2019c, Liu et al., 2014a), the sex of the host (Ellis et al., 2014, Liu et al., 2014c, Nguyen et al., 2018b) and environmental conditions (e.g. thermal stress, pollution) (François et al., 2015, Frizzo et al., 2021). Various metabolites affected by *Vibrio* spp. infections in mussels include glucose, glycine, betaine, homarine, threonine, alanine, aspartate, taurine, succinic acid, itaconic acid and branched chain amino acids (BCAAs), as well as other metabolites listed in Table 3.3. Metabolites, specifically amino acids, are crucial nutrients for pathogens and for the host which depends on amino acid metabolism to support defensive responses against pathogens (Ren et al., 2018). *Vibrio* spp. infections in mussels have been associated with significant changes in amino acid and fatty acid metabolism, as well protein synthesis linked to immune functions as seen in *Perna canaliculus* infected with *Vibrio* sp. *DO1* (*V. coralliilyticus/neptunius-like isolate*) (Nguyen et al., 2018b, Nguyen et al., 2018c, Nguyen et al., 2019c). Following targeted analyses, the metabolite itaconic acid was suggested as a biomarker in mussels following infection with *Vibrio* sp. *DO1 (V. coralliilyticus/neptunius*-like isolate), functioning as an antimicrobial metabolite and supporting mussel immune functions (Nguyen and Alfaro, 2019). The osmolyte function of amino acids in molluscs have been demonstrated where increased osmolytes (hypotaurine, homarine and glycine) and decreased of osmolytes (dimethylglycine, taurine and betaine) were linked to osmotic stress induced by *V. harveyi* in *M. galloprovincialis* (Liu et al., 2014b). Furthermore, altered metabolic biomarkers (glutamine, succinate, aspartate, glucose, ATP, homarine and tyrosine) indicated that *V. anguillarium* could induce disturbances in osmotic regulation and energy metabolism, along with cellular injury in *M. galloprovincialis* (Wu et al., 2013). Whenever assessing metabolite data linked to *Vibrio* spp. infections, one should keep in mind that the metabolome of a mussel is not only the composition of its own metabolites, but also reflective of the bacterial metabolites and metabolites from the diet, and that metabolites are conditioned by different factors such as age, sex and environment (Barber et al., 2019).

Fatty acids (lipids) are important for energy storage, development, growth and survival, and have now been classified as a potential physiological indicators for evaluating pathogen (*Vibrio* spp.) induced mortalities (Su et al., 2004), as seen in *Perna canaliculus* (Nguyen and Alfaro, 2020b, Ericson et al., 2022). Decreased concentrations of fatty acids, tridecanoic acid, myristic acid, palmitic acid, linoleic acid, were found in the haemolymph of *P. canaliculus* infected with *Vibrio* sp. DO1 potentially due to utilisation of energy sources required to simulate and immune response (Nguyen et al., 2018b, Nguyen et al., 2018c, Nguyen et al., 2019c). In contrast, analyses of *P. canaliculus* hepatopancreases showed increases in the free fatty acids (e.g., myristic acid, linoleic acid, palmitic acid, tridecanoic acid), as increased fatty acid synthesis was required to meet energy demands following infection (Nguyen et al., 2019c). The results listed in Table 3.3 are good starting points for summarising fatty acid biomarkers in mussels in response to *Vibrio* spp. infection, yet it should be considered that fatty acids are not exclusive to an organism (De Carvalho and Caramujo, 2018), and are highly affected by diet (Zhukova, 2019) and season (Silva et al., 2021), making the responses seen by mussels highly affected by various factors, not only the bacterial infection.

Stress proteins protect and regenerate cells in response to stress and harmful conditions (Kroon et al., 2017). Generally, this group of proteins consist of heat shock proteins (HSPs), which respond (by increasing synthesis) to heat and other physical and chemical stresses, the glucose-regulated proteins (GRPs), which respond to oxygen or glucose deficiency, and the stressor-specific stress proteins, which include heme oxygenase proteins (Fabbri et al., 2008). Proteins from the HSP70 family are the most analysed stress proteins when it comes to *Vibrio* infections in mussels, as shown in Table 3.3. Generally HSPs can be used to protect cells from stresses, such as pathogens (Fabbri et al., 2008). Two studies using real-time polymerase chain reaction (PCR) assays of samples from Mediterranean mussels exposed to *V. anguillarum*, observed that as infection progresses, this bacterium can produce more pronounced expression of HSP70 in mussel tissues (Cellura et al., 2006, Cellura et al., 2007). In another study, the immune gene expressions in two mussel species (*B. azoricus* and *M. galloprovincialis*) were investigated after infection with *Vibrio alginolyticus*, *V. anguillarum* and *V. splendidus* and a mixture of these *Vibrio* suspensions (Martins et al., 2014). The authors observed different results for HSP70 expression, due to differences in cis-regulatory DNA binding sequences or timescales of transcription of HSP70 in these species (Martins et al., 2014). Additionally, the conserved molecular chaperone, heat shock protein 90 (HSP90) involved in cell cycle control, organism development, and the regulation of cytosolic proteins, has been noted as a biomarker for infections of *V. parahemolyticus* in *Mytillus coruscus* (Liu et al., 2016). In *M. galloprovincialis* infected with *V. harveyi*, increased HSP90 mRNA was seen in gill and digestive gland samples, suggesting oxidative and immune stress responses

due to the infection (Liu et al., 2014b). The same researchers also studied gender-specific metabolic changes in mussels challenged with *V. harveyi* (Liu et al., 2014c). In both, male and female mussel hepatopancreas samples, the mRNA expression levels of HSP90 was increased (Liu et al., 2014c). Most recently, Castillo et al. (2017) examined the single and combined effects of ocean acidification (OA) and bacterial infection using reverse transcriptase - quantitative polymerase chain reaction (RT-qPCR) on the transcription expression of *hsp70* and *hsp90* genes related to antioxidant systems in *M. chilensis* (Castillo et al., 2017). Under controlled carbon dioxide (CO_2) conditions, HSP70 and 90 were down-regulated, but only HSP90 was differentially expressed in response to *V. parahaemolyticus, V. tubiashii, V. splendidus, V. alginolyticus* infections under OA stress, probably due to modulation of the stress-related proteins (e.g., hypercapnic acidosis) induced by OA (Hernroth et al., 2016). HSPs will be a valuable biomarker as HSP genes and proteins are present in all organisms, yet caution should be applied when ascribing results to bacterial outcomes, as the expression of HSPs differs based on species, within the same species different tissues show different expression levels, sex and age plays a role in expression and the method of detection can also influence the results (de Jong et al., 2008).

3.4.1.4 Haematological and immunological parameters

Haematological parameters provide insight into the health and physiology of an organism (Kroon *et al.*, 2017). In particular haemocytes play a vital role in the innate immune system, by acting as defensive cells through tissue infiltration, aggregation, encapsulation, cytotoxic reactions, and phagocytosis of foreign particles (Rolton and Ragg, 2020). Haemocytes of mussels can kill *Vibrio* spp. through phagocytosis, production of highly reactive molecules (like ROS), as well as a number of antimicrobial peptides (AMPs), hydrolytic enzymes (Destoumieux-Garzón et al., 2020) and other immunological parameters that will be discussed below and presented in Table 3.4.

Total haemocyte count (THC): THC, is likely the most popular measure of haemocyte immune responses when investigating the effect of pathogens on mussels (Dalzochio et al., 2016). Several studies have shown that THC is affected by *Vibrio* spp. infections, showing patterns of increased and decreased THC responses. However, it should be kept in mind, that THC results are variable depending on the nature of injected bacteria, bivalve species, and the handling of the sample. For example, quantitative differences were reported when injecting *Mytilus galloprovincialis* with either living or heat-killed *V. splendidus* LGP32 and *V. anguillarium* (Ciacci et al., 2009, Costa et al., 2009, Parisi et al., 2019). In *P. canaliculus*, increased THC was seen following 6 hours of a *Vibrio* sp. infection (Nguyen et al., 2019c) and

3 days after exposure to *Photobacterium swingsii* infection (Azizan et al., 2023a), but decreased with combined *P. swingsii* infection and temperature stress (Azizan et al., 2023b). In a study by Laith et al. (2021) on *P. viridis*, the THC at the beginning of the experiment was higher than at other time points, suggesting that the challenged group did not regain natural immunity after 120 hours of infection with *V. alginolyticus*. A decrease in THC was seen in *M. edulis* exposed to manganese (Mn) and when inoculated with the bacterium *V. parahaemolyticus*, likely contributing to the impaired haematopoiesis ability to combat the bacteria while further reinforcing the haemocytopenia (Oweson and Hernroth, 2009). The monitoring of THC in infected mussels requires large sample numbers and rapid procedures when establishing biomarker values. As a result, the measurement of haemolymph cells (haemocytes), can serve as a good biomarker as it is relatively easy performed from the posterior adductor muscle (Eggermont et al., 2020), and more importantly, haemolymph sampling is non-destructive and can be performed on the same individual to increase experimental and statistical flexibility (Ford, 1986).

Haemocyte characteristics: Haemocytes populations (granular, semi-granular, or agranular cells) also play an important role in bivalve susceptibility to pathogens (Destoumieux-Garzón et al., 2020). This was seen in *M*. *galloprovincialis*, where granular and semi-granular cells, harboured phagocytic activity, produced ROS and nitric oxide (NO) after being challenged with *V. splendidus* LGP32 and *V. anguillarum* (Ciacci et al., 2009). Furthermore, that study provided evidence that haemocyte populations decrease after *Vibrio* spp. Infection, but restore after a recovery period (Ciacci et al., 2009). Other studies provide evidence that *V. splendidus* LGP32 and *V. anguillarium* infections cause the loss of haemocyte cell adhesion in mussels (Ciacci et al., 2010, Tanguy et al., 2013a), and also influence haemocyte motility (Sendra et al., 2020). Caution should be applied when aiming to use haemocytes as biomarkers, as haemocytes are involved in a wide range of physiological functions that may not be related to defence (e.g., metabolite transport, digestion, shell growth and repair, and repair of damaged tissue), therefore making it less specific immunomarkers (e.g., total count, viability, phagocytosis, oxidative activity, lysosomal content, antimicrobial peptides, expression of immune-related genes) for *Vibrio* spp. responses (Dolar et al., 2020).

Pathogen-associated molecular patterns (PAMPs): Haemocytes can also recognise pattern recognition receptors (PRRs), which are proteins capable of recognising molecules frequently found in pathogens (the so-called pathogen-associated molecular patterns (PAMPs)) to activate intracellular signalling pathways to finally trigger the synthesis of antimicrobial effectors. Bacterial challenges carried out with different *Vibrio* strains (*V. alginolyticus*, *V. anguillarum* and *V. splendidus*) revealed distinct patterns of gene expression

for most of the immune genes tested in *Bathymodiolus azoricus* and *M. galloprovincialis* (Martins et al., 2014). From that study, differences were found in two immune recognition genes (galectins and peptidoglycan recognition proteins (PGRPs), supporting different recognition mechanisms and capacity to counteract bacterial challenges in the mussels (Martins et al., 2014). In addition, toll-like receptors (TLRs) can also recognise PAMPs and initiate corresponding signalling transduction pathways via intermediators to activate a wide range of downstream immune factors, which eliminate invading pathogens (Saco et al., 2020). Several TLR genes that recognise *V. anguillarum* and *V. parahaemolyticus*, were identified and up-regulated in *Hyriopsis cumingii* (Zhang et al., 2017, Ren et al., 2013) and *M. coruscus* (Xu et al., 2018). While *PAMPs*' specificity, sensitivity, and need for standardization can limit their use as biomarkers, they have the potential for personalized medicine, vaccine development, and disease surveillance, among other areas of interest include novel *PAMPs*, *PAMPs* in non-infectious diseases, and the microbiome's influence on *PAMPs* and disease.

***Antimicrobial peptides*:** Mussels are also rich in antimicrobial peptides (AMPs), such as defensin, mytilin, mytichitin-CB, myticusin-1, myticin and mytimycin, which play a key role in immune defence processes (Rosani et al., 2011, Qin et al., 2014, Liao et al., 2013, Tanguy et al., 2018, Bouallegui, 2019, Van Hung et al., 2019, Sendra et al., 2020). A majority of the AMPs identified from *Mytillus* spp. are cysteine-rich subgroups, except for the recently identified linear/α-helical family (Leoni et al., 2017, Bouallegui, 2019). Although AMPs have been discovered mainly in *Mytillus* spp., the information on AMP in *Perna* spp. remains largely unknown. As AMPs exhibit antibacterial activity against *Vibrio* spp., including *V. splendidus*, these peptides appear to have an important role in the immune defence of mussels against bacterial infection. In addition, However, further research is needed to fully understand the role of AMPs in the immune defence against *Vibrio* spp. and other bacterial pathogens in *Perna* spp.

Phenoloxidase: Besides the AMP gene expression, another critical component of the immune system of bivalves, namely phenoloxidase activity (PO), has been examined in *Vibrio* sp. challenges (Luna-Acosta et al., 2017). An increase in PO detected in haemolymph, following *V. anguillarium*, *V. harveyi*, *V. coralliilyticus* infections has been seen in *Mytillus edulis*, *Perna viridis*, and *Hyriopsis cumingii* (Ren et al., 2013, Puspita and Hutabarat, 2015, Van Hung et al., 2019). Typically, PO is a by-product of a complex cascade of reactions that includes melanization, wound healing, phagocytosis, and pathogen killing (Gerdol et al., 2018), making PO a good indicator of mussels pathogen defence mechanisms (Muznebin et al., 2022a) and potentially a good biomarker for *Vibrio* spp. infections.

Lysosomal membrane stabilisation (LMS): As a biomarker, lysosomal responses can be used as a sensitive indicator of bivalve cellular stress in conjunction with environmental perturbation and pathogen infection (Moore et al., 2006). Based on transmission electron microscopy (TEM) analyses of *M. edulis*, haemocytes infected with *V. tapetis* showed rapid ultracellular damage and lysosomal fusion, with morphological changes at the plasma membrane and cytoplasmic levels. However, no *Vibrio* internalisation was observed in that study, indicating no intracellular degradation of bacteria (Balbi et al., 2019). Haemocyte lysosomal membrane stability (LMS) was evaluated as a marker of cellular stress in mussels induced by bacterial challenges (Balbi et al., 2019). Measured in both small and large granulocytes of *M. galloprovincialis,* cellular stress was confirmed following *V. cholerae* infection (Canesi et al., 2005). Furthermore, the measurement of LMS has been shown to be effective for comparing the cellular stress induced *in vivo* by heat-killed Gram (+) and Gram (−) bacteria, including *V. anguillarum* and *V. splendidus* (Ciacci et al., 2009). In a study by Canesi et al. (2010), it was demonstrated that both *V. splendidus* and *V. anguillarum* caused a decrease in the level of LMS in the digestive gland of *M. galloprovincialis* at all time points post-infection. Furthermore, a reduction in *Mytillus* LMS following infection with *V.splendidus* LGP32 and *V. anguillarum* have been shown to be mediated by activation of different immune signalling pathways, including p38 MAPK, PKC, PI-3 kinase (Ciacci et al., 2010). The results obtained *in vitro* indicate that in *Mytilus* haemocytes, live *V. aestuarianus* 01/032 does not affect LMS, but live *V. splendidus* LGP32 severely affects LMS, with effects stronger than those previously recorded with the heat-killed strain (Canesi et al., 2002a, Ciacci et al., 2010). The results of those studies suggest that lysosomal membrane destabilisation varies greatly depending on the bacterial species and the use of live or heat-killed injected bacteria (Canesi et al., 2006), necessitating detailed reporting when projecting lysosomal membrane results if this is a desired biomarker for *Vibrio* spp. infection in mussels.

Apoptotic markers: The physiological and irreversible process of programmed cell death characterised by the fragmentation of DNA (Kroon et al., 2017) is implicated in many processes, such as tissue and organ development, homeostasis, and immune defence (Sokolova, 2009, Gerdol et al., 2018). From the literature investigated, all studies reported, in Table 3.4, showed an increase in apoptosis in five different mussel species exposed to *V. splendidus* LGP32, *V. anguillarum*, *V. aestuarianus* 01/032 and other *Vibrio* species in field and laboratory studies (Tanguy et al., 2018, Parisi et al., 2019, Auguste et al., 2020, Lattos et al., 2021a). As such, apoptosis may be an appropriate biomarker, but further species-specific work is required to determine its suitability to assess mussel health. The proliferating cell nuclear antigen (PCNA), caspases (e.g., CASP8, CASP3, CASP7 and CASP6) and other components, such as the fas-associated death domain (FADD) are commonly used

biomarkers of apoptosis, as they involved in DNA replication and in the activation and implementation of the apoptotic programme. These apoptotic markers were reported significantly upregulated in *M. edulis*, *M. galloproviancialis*, *B. azoricus* and *Pinna nobillis* following infection with *V. splendidus* LGP32, *V. anguillarum* and other *Vibrio* species (Tanguy et al., 2013b, Parisi et al., 2019, Lattos et al., 2021a). In contrast, a study on haemocyte apoptosis (Caspase 3/7) in *P. canaliculus* revealed infection with *Vibrio* sp. DO1 did not result in a change in the proportion of haemocytes producing reactive oxygen species with temperature or injection treatments, suggesting that this bacterium did not trigger pro-oxidant or antioxidant response or induce apoptosis in mussels (Ericson et al., 2022). The research implies that as a sensitive biomarker tool to assess cellular stress in marine invertebrates, particularly mussels, haemocyte apoptosis is useful, but further assessment will require method standardization, establishing baseline data, identifying species-specific responses, and integrating other biomarkers.

Autophagic markers: Molecular autophagy has also been used in molluscan cells to identify cell injury and to remove unnecessary or dysfunctional components caused by a variety of environmental stressors and *Vibrio* sp. infections (Balbi et al., 2018). The autophagic process is a key regulator of innate immunity as it helps clear pathogens and regulates inflammation (Balbi et al., 2018). Effects of *V. tapetis* on autophagosome formation was studied in *M. galloproviancialis* haemocytes using TEM (Balbi et al., 2018). Upon infection with *V. tapetis*, large autophagosomes were rapidly developed, characterised by a double membrane, with heterogeneous contents. Electrophoresis and western blotting were performed by the same researchers in haemocytes incubated with *V. tapetis* to see if this bacterium affects autophagic marker expression. The results showed in mussel haemocytes, *V. tapetis* induced a significant increased (light chain 3) LC3-II expression, decreased levels of phosphorylated mammalian target of rapamycin (mTor) and SQSTM1/p62 (sequestosome 1). In addition, *V. splendidus* LGP32 induced PI3K, Akt, and mTOR pathways in *M. galloprovincialis* haemocytes from a complex network linked to the JAK-STAT signalling pathway that participates in immune response and the regulation of cell proliferation, autophagy, and apoptosis (Rey-Campos et al., 2019a). In *Pinna nobillis* specimens infected with *Vibrio* bacteria and additionally infected with *Haplosporadian* parasite, autophagic markers (ubiquitin, LC3, and SQSTM1/p62) were examined (Lattos et al., 2021b). The results showed higher levels of ubiquitin expression, LC3 was not detected through western blot, and the SQSTM1/p62 exhibited a tissue specific pattern of expression, indicating lower levels and therefore increased autophagy in individuals additionally infected with the *Haplosporidian* parasite.

Immunological biomarkers related to illness can be effectively measured using omics tools, such as transcriptomics, proteomics, and metabolomics (Haddad *et al.*, 2018). Transcriptional biomarkers linked to mussel health identified a significant impact of a wide range of vibrios (e.g. *V. splendidus*, *V. parahemolyticus*, *V. alginolyticus*, *V. alginnolyficus*, *V anguillarum*) in response to stress, redox balance, metabolism, apoptotic processes, and immunity (Venier et al., 2011, Tanguy et al., 2018, Martins et al., 2014, Moreira et al., 2015, Dong et al., 2015, Rey-Campos et al., 2019a, Rey-Campos et al., 2019b, Dong et al., 2017, Lori et al., 2020, Saco et al., 2020, Chen et al., 2021a, Yang et al., 2021, Romero et al., 2022). Likewise, mussel proteomics biomarkers have allowed the detection of a network of protein changes due to oxidative stress and disturbances in energy metabolism when challenged with *Micrococcus luteus* and *V. anguillarum* (Ji et al., 2013, Wu et al., 2013). Interestingly, protein biomarkers have been used to distinguish exposure and effect of *Vibrio* spp. infections between mussel species from very distinct natural habitats (Martins et al., 2014). As shown by Martins et al. (2014) *Vibrio* spp. (*V. alginolyticus*, *V. anguillarum* or *V. splendidus)* infections on *B. azoricus* resulted in alternations in protein sequences involved in metabolic and energy production processes along with nutritional demands, while infections in *M. galloprovincialis* putative protein functions related to structural and cellular integrity and signalling functions. Overall, the application of proteomic studies to detect changes in protein biomarkers in mussel species exposed to *Vibrio* could help identify those protein components involved in host-pathogen interactions that are more sensitive to detect the stress response to studied *Vibrio*. However, the application of this protein biomarker needs substantial development to provide clear connections between other environmental stressors, exposure, changes in peptides abundances, and species-specificity.

3.4.2 Biomarkers of effect

Biomarkers of effect relate to measurable alterations within tissues or body fluids of an organism, often linked to possible health impairment or disease (Dalzochio et al., 2016). For mussels infected with *Vibrio* spp., this most often links to histopathology indices due to pathogen infection (Table 3.5). Histopathological biomarkers enable the assessment of organismal health status (Stentiford et al., 2005, Bignell et al., 2008), while also providing information relating to physiological changes in reproduction and metabolic status and evidence on pathogens (Bignell et al., 2008) and associated tissue changes due to bacterial contamination (Stentiford et al., 2005, Bignell et al., 2008). While histopathological methods of monitoring body health are extremely efficient, they only provide a morphological picture of alterations, which is discernible only at the final stages of pathology and cannot always define physiological disruption (Kumeiko et al., 2018). Additionally, they remain limited biomarkers

based on only a few species of mollusc and lack sufficient detail for effective quantitative analyses. Often, semi-quantitative approaches are the preferred option, though they require well-trained researchers and extensive background knowledge (Costa et al., 2013). In this respect, histopathology may be used as a tool for providing supporting information for measures (need to combined with other biomarkers) that aim to assess historical exposure to, or effects of pathogens (Bignell et al., 2008). Histopathology of a range of mussel tissues following *V. splendidus, Vibrio* harveyi, *V. coralliilyticus*, *V. tubiashii*, *V. mediterranei*, and *V. hispanicu*s, *V. alginolyticus*, *V. splendidus* ME9 and *V. anguillarum* NB10 exposure has been shown to result in an increase in histological alterations including tissue necrosis, haemocyte infiltrations and bacterial/parasitic colonisation when mussels are exposed to *Vibrio* spp., both in the laboratory and in the field. These effects were seen in *M. edulis* (Ben Cheikh et al., 2017, Wang et al., 2021b), *M. galloproviancialis* (Parisi et al., 2019, Battistini et al., 2020), *P. viridis* (Laith et al., 2021)*, P. canaliculus* (Kesarcodi-Watson et al., 2009a, Azizan et al., 2023a) and *Pinna nobilis (Künili et al., 2021).* Interestingly, all species at adult stage showed abnormalities to haemocytes and tissues (e.g. digestive gland, mantle and gills) in response to *Vibrio* spp. infection, while detachment of cilia cells and velum was uniquely described in mussel larvae (Kesarcodi-Watson et al., 2009a, Ruiz et al., 2013, Ben Cheikh et al., 2017, Parisi et al., 2019, Battistini et al., 2020, Künili et al., 2021, Laith et al., 2021, Wang et al., 2021b, Azizan et al., 2023a). Exposures with *Vibrio* spp. concentrations of 10^{-6} CFU/mL resulted in the vacuolisation, necrosis, and tissue separations in hepatopancreas and digestive glands, sloughing of tubule epithelial cells, karyomegaly and hyperplasia (Laith et al., 2021). To complement histopathology findings, immunohistochemistry (IHC) can be used as an additional biomarker tool to examine the expression and localization of immune-related proteins within the tissues of infected mussels that are known to be involved in mussels' immune response to vibrio infections. For example, researchers may use antibodies specific to proteins, such as the water channel proteins aquaporins and the Na+/K+ ATPase biomarker of osmoregulatory processes and the proliferating cell nuclear antigen, PCNA, and Caspase-3 as biomarkers of apoptosis to detect their expression within the tissues (Parisi et al., 2019). Using IHC, Ben Cheikh et al. (2017) monitored the haemocytes exposed to *V. splendidus*-related strains by immunolabeling bacterial-like cells with full-length green fluorescent proteins (GFPs). The prominent changes in tissue structure seen in most studies investigated, suggest that histopathological and IHC markers are suitable biomarkers for providing insight into the effect of *Vibrio* spp. on mussel tissue and cell health. Taken together, descriptive data obtained from histology and IHC assessment provide additional information to support other biomarkers in integrated data monitoring programs.

3.4.3 Biomarkers of susceptibility

Susceptibility biomarkers describe an organism's inherent or acquired capacity, based on genetic factors and alterations in receptors, to respond to exposure following contact with pathogens. These types of biomarkers have been mostly investigated as bivalve immunomarkers and is still evolving with application to mussels. It has been shown that mussel susceptibility to specific *Vibrio* spp. (e.g., *Splendidus* clade, oyster pathogen) was determined by host physiology (Charles et al., 2020b). Also possible links between mussel and oyster susceptibility to *Vibrio* spp infection and their microbiome profile was made based on16S rRNA gene-based analysis (Vezzulli et al., 2018). Moreover, studies have found the genus *Vibrio* (potential pathogenic species such as *V. aestuarianus*) constitute a greater proportion of the microbiota in *Crassostrea gigas* compared to *M. galloproviancialis*, suggesting that oysters may offer better environments (i.e., host species intrinsic factors) for these bacteria to thrive in, than mussels. Positively, studies have suggested that shellfish innate immune memory or "immune priming" may help shellfish respond to pathogen infections (Rey-Campos et al., 2019a). Therefore, exposure to non-lethal doses of pathogens could provide molluscs with the ability to launch stronger immune responses during later phases of infection, potentially protecting mussels against *Vibrio* spp. infections. As shown in the mussel, *M. galloprovincialis*, priming with *V. splendidus* altered immune responses and modulated the number of differentially expressed genes that enabled the mussel to cope with infections (Rey-Campos et al., 2019a). Interestingly, successes in developing resistance to virus ostreid herpesvirus-1 (OsHV-1) are now being applied for resistance to *Vibrio* bacteria in oyster *C. gigas*, which have been implicated in summer mortality (de Lorgeril et al., 2018, Zhai et al., 2021). This work has demonstrated that *Vibrio* spp. resistance can be enhanced by genetic improvement and now can be translated to mussel.

Table 3.1 Biotransformation linked biomarkers detected in mussels infected with *Vibrio* spp.

Mussel species	**Sample**	**Methods**	***Vibrio* species**	**GSH/GSSH**	**GST**	**Total GSH**	**Reference**
Mytllus galloproviancialis	Digestive gland	Real time q-PCR; Bioassay	*V. splendidus LGP32 and V. anguillarum (ATCC19264)*		+		Canesi et al. (2010)
	Hepatopancreas	Proteomics-2DE; ^{1}H NMR metabolomics	*Micrococcus luteus and V. anguillarum*		+		Wu et al. (2013)
	Gills	Proteomics; ^{1}H NMR metabolomics	*Micrococcus luteus and V. anguillarum*		+		Ji et al. (2013)
Mytilus coruscus	Mantle, muscle, gills, haemocyte, gonad, hepatopancreas	Cloning; Real time qRT-PCR	*V. alginolyticus and V. harveyi*		+		Liu et al. (2016)
Elliptio complanata	Haemolymph	Bioassays	*V. anguillarum*		+		François et al. (2015)
Perna canaliculus	Haemolymph, gill, hepatopancreas	GCMS-metabolomics	*V. sp. DO1 (V. coralliilyticus / neptunius-like isolate)*	+		+	Nguyen et al. (2019c)
	Haemolymph	GCMS-metabolomics	*V.sp. DO1 (V. coralliilyticus / neptunius-like isolate)*	+		+	Nguyen et al. (2018c)

Abbreviations: GSH/GSSH: reduced glutathione/oxidised glutathione ratio; GST: glutathione s-transferase, GCMS: Gas chromatography–mass spectrometry; qRT-PCR: quantitative real time polymerase chain reaction; 2DE: Two-dimensional electrophoresis gel; ^{1}H NMR: Proton NMR spectroscopy; + Increased.

Table 3.2 Oxidative stress biomarkers detected in mussels infected with *Vibrio spp.*

Mussel species	**Sample**	**Methods**	***Vibrio* species**	**SOD**	**CAT**	**GPO_X**	**LPO_X**	**ROS**	**Defensin**	**Lysozyme**	**MT**	**Reference**
Mytillus edulis	Haemolymph	Bioassay	*V. tubiashii*	+								Parry and Pipe (2004)
	Haemolymph	Microsphere-based multiplex branched DNA assay; flow cytometry; bioassay	*V. splendidus*	+				+	-	+		Tanguy et al. (2013a)
	Haemolymph, gills	Proteomics	*V. parahaemolyticus, V. tubiashii, V. splendidus, V. alginolyticus;*	+/-								Hernroth et al. (2016)
Mytillus galloproviancialis	Haemolymph	Real time q-PCR	*V. splendidus LGP32, Vibrio anguillarum*					+		+/-		Li et al. (2008)
	Haemolymph	Flow cytometry; morphological analysis; bioassays	Fluorescein-labelled *V. alginolyticus*					+				García-García et al. (2008)
	Haemolymph	Real time q-PCR; bioassay	*V. anguillarum*					+		+		Costa et al. (2009)
	Haemolymph	Bioassays; flow cytometry	*V.splendidus LGP32, V. anguillarum*							+		Ciacci et al. (2009)
	Digestive gland	Real time q-PCR; Bioassays	*V. splendidus LGP32 and V. anguillarum (ATCC19264)*		+						+	Canesi et al. (2010)
	Haemolymph	Bioassay; Real time qRT-PCR	*V. splendidus LGP32, Vibrio anguillarum*					+/-				Ciacci et al. (2010)
	Haemolymph	Transcriptomics (microarray)	*V. splendidus*	+								Venier et al. (2011)

	Haemolymph	Real time q-PCR; Bioassay	*V. anguillarum*					+		+		Wang et al. (2013a)
	Digestive gland, gills	Real time qRT-PCR	*V. harveyi*	+								Liu et al. (2014c)
	Hepatopancreas	Real time qRT-PCR	*V. harveyi*	+								Liu et al. (2014b)
	Haemolymph	Bioassays; electrophoresis; Western blotting	*V. parahaemolyticus Conero, V. alginolyticus 1513, V. vulnificus 509*					+		+		Ciacci et al. (2017)
	Haemocytes, gills, mantle	Real time q-PCR	*V. splendidus LGP32*	+	+			+				Sendra et al. (2020)
Mytilus coruscus	Adductor muscle, haemocytes, mantle, gills, gonad, hepatopancreas	Cloning; Real time qRT-PCR	*V. parahemolyticus*	+/-								Wu et al. (2017)
	Adductor muscle, haemocytes, mantle, gills, gonad, hepatopancreas	Cloning; Real time qRT-PCR	*V. parahemolyticus*								+	Ge et al. (2020)
Perna canaliculus	Haemolymph	Flow cytometry	*Vibrio sp. DO1 (V. coralliilyticus / neptunius-like isolate)*					+				Nguyen et al. (2018c)
	Haemolymph	Flow cytometry	*Vibrio sp. DO1 (V. coralliilyticus / neptunius-like isolate)*					+				Ericson et al. (2022)
	Haemolymph	Bioassays	*Photobacterium swingsii*				+					Azizan et al. (2023b)
Perna viridis	Haemolymph	Bioassays	*V. alginolyticus*					+				Laith et al. (2021)
Pinna nobilis	Digestive gland, gills	Bioassays	*V. harveyi and Vibrio sp.*	+	+	+						Künili et al. (2021)

Elliptio complanata	Haemolymph	Bioassays	*V. anguillarum*				+					François et al. (2015)
Bathymodiolus azoricus	Digestive gland, gills	Gene expressions-qPCR; Protein sequence analyses-HPLC-ESI-MS/MS	*V. alginolyticus, V.anguillarum and V. splendidus*			+						Martins et al. (2014)
Gigantidas platifrons (or Bathymodiolus platifrons)	Gills	Transcriptomics -qRT PCR	*V. alginolyticus*	+								Chen et al. (2021a)

Abbreviations: THC: total haemocyte count; Lab: laboratory; SOD: Superoxidase mutase; CAT: Catalase; GPOX : glutathione peroxidases; LPOX: lipid peroxidation; GST-π : glutathione transferase pi class; ROS: reactive oxygen species; Zn: Zinc; Mn: manganese; Cu 2+:Copper; Pb2 :Lead; GCMS: Gas chromatography–mass spectrometry; PCR: polymerase chain reaction q-PCR: quantitative polymerase chain reaction; RT-PCR: quantitative real time polymerase chain reaction; HPLC-Esi-MS/MS: high-performance liquid chromatography/electrospray ionization tandem mass spectrometry 2DE: Two dimensional electrophoresis gel; 1H NMR: Proton NMR spectroscopy; + increase; - inhibition; = no significant increase; +/- mixed response

Table 3.3 Metabolite and protein biomarkers detected in mussels infected with *Vibrio* spp.

Mussel Species	**Sample**	**Method**	***Vibrio* species**	**Metabolites**	**Proteins**	**Reference**
Mytillus galloproviancialis	Haemolymph	qPCR	*V. splendidus LGP32, V. anguillarum*		+HSP70	Cellura et al. (2007)
	Haemolymph	qPCR	Heat-killed *V. splendidus LGP32, V. anguillarum*		+HSP70	Cellura et al. (2006)
	Gills	Proteomics; ^{1}H NMR metabolomics	*V. anguillarum*	Amino acids, betaine, ATP	Procollagen-proline dioxygenase, protein disulphide isomerase, nucleoside diphosphate kinases, electron transfer flavoprotein	Ji et al. (2013)

	Hepatopancreas	Proteomics- 2-D gel electrophoresis and ^{1}H NMR metabolomics	*V. anguillarum*	Glutamine, succinate, aspartate, glucose, ATP, homarine and tyrosine	Arginine kinase and small heat shock protein	Wu et al. (2013)
	Digestive gland, gills	^{1}H NMR metabolomics	*V. harveyi*	Hypotaurine, homarine glycine, dimethylglycine, taurine, betaine, aspartate, glucose, and ATP	Cu/Zn-SOD, HSP90, defensin and lysozyme	Liu et al. (2014b)
	Hepatopancreas	^{1}H NMR metabolomics and Real time qRT-PCR	*V. harveyi*	Valine, leucine, isoleucine, threonine, alanine, arginine and tyrosine, AMP, ATP, choline, phospho-choline, glycero-3-phosphocholine, taurine and betaine	Cu/Zn-SOD, HSP90, lysozyme and defensin	(Liu et al., 2014c)
	Haemolymph	Real time qRT-PCR-Gene expression, HPLC-ESI-MS/MS - protein assay	*V. alginolyticus, V. anguillarum, V. splendidus*		+HSP70	Martins et al. (2017a)
	Haemocytes	Confocal laser scanning microscopy; Bioassays; TEM; electrophoresis and western blotting	*V. tapetis*		Total protein content	Balbi et al. (2018)
Mytillus edulis	Haemolymph	Functional assay, Illumina sequencing and transcriptome analysis, Microsphere-based multiplex branched DNA assay	*V. splendidus LGP32*		Various HSPs in transcriptome	Tanguy et al. (2018)

Mytilus coruscus	Muscle, haemocytes, mantle, gills, gonad, hepatopancreas	Cloning; Real time qRT-PCR	*V. parahemolyticus*		+HSP90	Liu et al. (2016)
	Haemolymph	Transcriptomics; qRT-PCR	*V. alginolyticus*		+HSP70	Dong et al. (2017)
Bathymodiolus azoricus	Digestive gland, gills	Gene expressions-qPCR; protein sequence analyses-HPLC-ESI-MS/MS	*V. alginolyticus, V. anguillarum, V. splendidus*		+HSP70	Martins et al. (2014)
	Gills, digestive gland, mantle	qPCR	*V. diabolicus*		+HSP70	Martins et al. (2015)
Perna canaliculus	Haemolymph	GCMS-metabolomics	*Vibrio sp. DO1 (V. coralliilyticus/neptunius-like isolate)*	Succinic acid, fumaric acid, malic acid, itaconic acid, gamma-aminobutyric acid, tryptophan, and aspartic acid		Nguyen et al. (2018c)
	Haemolymph	GCMS-metabolomics	*Vibrio sp. DO1 (V. coralliilyticus/neptunius-like isolate)*	Malonic acid, fumaric acid, lactic acid, itaconic acid, glutamic acid, glycine, hydroxyproline, glutamic acid, pyroglutamic acid, S-adenosylmethionine, methionine, serine, valine, leucine, isoleucine creatinine and linoleic acid		Nguyen et al. (2018b)
	Mantle, gill, muscle, hepatopancreas	Targeted GCMS-metabolomics	*Vibrio sp. DO1 (V. coralliilyticus/neptunius-like isolate)*	Kreb Cycle metabolites		Nguyen and Alfaro (2019)

	Haemolymph	GCMS-metabolomics	*Vibrio sp. DO1 (V. coralliilyticus/neptunius-like isolate)*	Various metabolites linked to branched chain amino acid pathway, glutathione pathway, urea cycle metabolites and oxidative stress metabolites		Ericson et al. (2022)
	Haemolymph	GCMS-metabolomics	*V. mediterranei*	Various metabolites, largely amino acids		Azizan et al. (2023c)
	Haemolymph	GCMS-metabolomics	*V. mediterranei* and *P. swingsii*	Fatty and amino acids		Azizan et al. (2023c)

Abbreviations: AMP: adenosine monophosphate; ATP: adenosine triphosphate; AMP: Lab: laboratory; SOD: Superoxidase mutase; ROS: reactive oxygen species; Cu 2+:Copper; GCMS: Gas chromatography–mass spectrometry; PCR: polymerase chain reaction q-PCR: quantitative polymerase chain reaction; RT-PCR: quantitative real time polymerase chain reaction; HPLC-Esi-MS/MS: high-performance liquid chromatography/electrospray ionization tandem mass spectrometry 2DE: Two dimensional electrophoresis gel; 1H NMR: Proton NMR spectroscopy; + increase; - inhibition; = no significant increase; +/- mixed response.

Table 3.4 Haematological and immunological biomarkers detected in mussels infected with *Vibrio* spp.

Mussel species	**Sample**	**Methods**	***Vibrio* species**	**THC**	**Haemocyte char.**	**PAMPs**	**AMP**	**PO**	**Lysosomal membrane stabilisation**	**Autophagy / Apoptosis**	**Other**	**Reference**
Mytillus edulis	Haemolymph	Bioassay	*V. tubiashii*	+								Parry and Pipe (2004)

	Haemolymph, digestive glands	Bioassay	*V. parahaemolyticus*	-							Bactericidal response	Oweson and Hernroth (2009)
	Haemolymph	Bioassay; flow cytometry; multiplex DNA assay	*V. splendidus LGP32-GFP; V. splendidus LGP32 Δvsm; V. splendidus 7SHRW*		+		+				+Oxidative burst, weak and delayed haemocyte response	Tanguy et al. (2013a)
	Larvae	Real time qRT-PCR; Real time PCR	*V. coralliilyticus*		+		+	+				Van Hung et al. (2019)
	Gills	Bioassays; proteomics	*V. parahemolyticus CCUG 43363, V. tubiashii ATCC 19109, V. splendidus CCUG 20273, V. alginoyticus CCUG 16315 T*				+				+Myticin	Hernroth et al. (2016)
	Larvae	Bioassays	*V. splendidus (EU358783), V. coralliilyticus / neptunius-like isolate (EU358784)*					+				De Rijcke et al. (2016)
	Haemolymph	Functional assay, Illumina sequencing and transcriptome analysis, Microsphere-based multiplex branched DNA assay	*V. splendidus LGP32*			+	+			+	Sequences encoding for galectins, fibrinogen-related proteins, MyD88, lysosomal hydrolases and protease inhibitors	Tanguy et al. (2018)

	Haemolymph, whole animals	Bioassay; histological and immune-histochemical analyses	*GFP-V. splendidus*	+	+							Ben Cheikh et al. (2017)
Mytillus edulis chilensis	Gills	qPCR-	*V. anguillarum*			+	+				+C-type Lectin and Mytilin B, -Myticin A and PGRP	Castillo et al. (2017)
Mytillus galloproviancialis	Haemolymph	Bioassays; flow cytometry	*V.splendidus LGP32, V. anguillarum*	+	+/-				+		In-vitro bactericidal assay	Ciacci et al. (2009)
	Haemolymph	Cytology, electrophoresis; Western blotting; bioassay	*V.splendidus LGP32 V. anguillarum*	+	+				+		+Phosphorylation of mitogen-activated protein kinases (MAPKs), + Phosphorylation of PKC-like proteins	Ciacci et al. (2010)
	Haemolymph	Pyrosequencing	*V. splendidus*				+				+Mytilin, myticin, and mytimycin	Rosani et al. (2011)
	Haemolymph	Real time qRT-PCR- Gene expression, HPLC-ESI-MS/MS -protein assay	*V. alginolyticus, V. anguillarum, V. splendidus*			+	+				Aggrecan, LBP; LBP-BPI; MyD88; LITAF; NF-κB	Martins et al. (2014)
	Haemolymph	Clearance measurements; biochemical assays	Heat-killed *V.splendidus LGP32,V. anguillarum (ATCC 19264)*	+	+/-						Bacteria clearance; haemocyte sub-populations; THC variations; Function of haemocytes sub-populations	Parisi et al. (2008)

	Haemolymph, adductor muscle	Clearance measurement; Western blotting	*V. splendius* LGP32							+		Parisi et al. (2019)
	Haemolymph	Bioassays; electrophoresis; Western blotting	*V. parahaemolyticus Conero,V. alginolyticus 1513, V. vulnificus 509*				+			+	O^{2-} and NO production, -p38 MAP Kinase and apoptotic processes.	(Ciacci et al. 2017)
	Haemocytes, gills, mantle	Real time q-PCR; flow cytometry	*V. splendidus LGP32*		-		+			+	Irg1 expression; antibacterial activity of dimethyl itaconate	Sendra et al. (2020)
	Haemolymph	Transcriptomics	*V. splendidus*			+					+Mytibase clusters and singletons	Venier et al. (2011)
	Haemocytes	Confocal laser scanning microscopy; Bioassays; TEM; electrophoresis and western blotting	*V. tapetis*							+	+	-Cytochrome C activity; NO; + LC3-II, p-mTor and p62; haemocyte functional parameters,	Balbi et al. (2018)
	Haemolymph	Transcriptomics	*V. splendidus*		+		+					+ Haemocyte structures	Rey-Campos et al. (2019b)
	Haemolymph	Transcriptomics; qPCR	*V. splendidius* LGP32		+/-		+			+		+ Myticin C; + granulocytes; - hyalinocytes	Rey-Campos et al. (2019a)
	Haemolymph	Bioassays; TEM	*V. coralliilyticus ATCC BAA-450, V. coralliilyticus TAV24*						+				Balbi et al. (2019)

	Gills	Bioassays; transcriptomics	*V. splendidus*				+			+	+ Immune response activation pathways	Saco et al. (2020)
	Mantle, gills	Microbiological analyses; MALDI-TOF analysis	*Vibrio splendidus clade, V. aestuarianus, and V. harveyi*							+		Battistini et al. (2020)
Mytillus coruscus	Haemocytes, digestive gland, mantle, posterior adductor muscle, gills, foot muscle, gonad	Liquid chromatography peptide sequence analysis	*V. harveyi*			+	+				+Myticusin-1	Liao et al. (2013)
	Haemocytes, digestive gland, mantle, posterior adductor muscle, gills, foot muscle, gonad	Bioassays; liquid chromatography	*V. parahaemolyticus (CGMCC1.1616), V. harveyi (CGMCC1.1601)*			+	+				Antimicrobial peptide with chitin-biding domain (mytichitin-CB)	Qin et al. (2014)
	Haemolymph	Transcriptomics; qRT-PCR	*V. alginolyticus*			+	+			+	+1270 and -265 genes; C1q, Rac1, Hepc2, IRF-2, RIG-I, CYP450, CP-I and CAT	Dong et al. (2017)
	Muscle, mantle, gill, gonad hepatopancreas, haemocytes	RT-PCR; qRT-PCR	*V. parahaemolyticus*			+					Expression patterns of McTLR-a mRNA	Xu et al. (2018)

	Haemolymph, gills, gonads	Transcriptomic analysis	*V. parahemolyticus*	+		+	+				Validation of DEGs	He et al. (2022)
Bathymodiolus azoricus	Haemolymph	qRT-PCR; HPLC-ESI-MS/MS	*V. alginolyticus, V. anguillarum, V. splendidus*			+	+				Aggrecan; LBP; BPI LBP-BPI; MyD88; LITAF, NF-κB.	Martins et al. (2014)
	Gills, digestive gland, mantle	qPCR	*V. diabolicus*			+	+				+Carcinolectin, serpin-2, SRCR, IRGs, RTK, TLR2, NF-κB, ferritin genes\	Martins et al. (2015)
	Gills	qPCR; FISH	*V. diabolicus*				+			+	+Haemolysin	Barros et al. (2016)
	Gills	qPCR	*V. diabolicus*				+			+	Caspases 8 and 9, BAX, and members of the Bcl-2 family	Martins et al. (2017)
Gigantidas platifrons (or Bathymodiolus platifrons)	Gills	Transcriptomics	*V. alginolyticus*			+	+				+ Expression of other immune effectors	Chen et al. (2021)
Perna viridis	Haemolymph	Bioassays	*V. harveyi*					+				Puspita and Hutabarat (2015)
	Haemolymph, hepatopancreas	Bioassays	*V. alginolyticus*	+							Lysozyme activity	Laith et al. (2021)
	Haemocytes, digestive gland, muscle, gills, mantle	Transcriptomics; qRT-PCR	*V. parahaemolyticus*								Mytilin-like AMPs in pernalin 1, pernalin 2, pernalin 3, and pernalin 4	Zeng et al. (2022)

Perna canaliculus	Gills, Hepatopancreas, haemolymph	Metabolomics; flow-cytometry	*Vibrio* sp. *DO1 (V. coralliilyticus / neptunius-like isolate)*	+						+		Nguyen et al. (2019b)
	Haemolymph	Metabolomics; flow-cytometry	*Vibrio* sp. *DO1 (V. coralliilyticus* / *neptunius-like isolate)*	-						+		Ericson et al. (2022)
	Haemolymph	Flow cytometry	*Photobacterium swingsii*	-							+Bacterial clearance	Azizan et al 2023
	Haemolymph	Flow cytometry	*Photobacterium swingsii*	+							-Bacterial clearance	Azizan et al 2023
Pinna nobilis	Digestive gland, gills	Bioassays	*V. harveyi and Vibrio sp.*							+/-		Künili et al. (2021)
	Digestive tissue	Bioassays; PCR	*Vibrio mediterranei*	+	+							Prado et al. (2020)
Hyriopsis cumingii	Haemolymph, hepatopancreas, gills, mantle	qRT-PCR	*V. anguillarum*			+		+			+HcToll1	Ren et al. (2013)
	Haemolymph, hepatopancreas, gills, mantle	qRT-PCR; bacterial clearance assays	*V. parahaemolyticus*			+					+HcToll3	Zhang et al. (2017)

Abbreviations: THC: total haemocyte count; Lab: laboratory; SOD: Superoxidase mutase; CAT: Catalase; DEG: differentially expressed genes; GPOX : glutathione peroxidases; LPOX: lipid peroxidation; Zn: Zinc; Mn: manganese; Cu $^{2+}$:Copper; Pb^{2+} :Lead; BCL: B-cell lymphoma; BAX: BCL-2-associated X protein); C1q: complement C1q protein; Rac1: ras-related C3 botulinum toxin substrate 1; Hepc2: hepcidin 2; IRF-2: interferon regulatory factor 2; RIG-I: retinoic acid-inducible gene I; CYP450: cytochrome P450; CP-I: cathepsin I and CAT: catalase; HcToll: Toll-like receptor; LC3-II: microtubule-associated protein light chain 3; LBP: Lipopolysaccharide Binding Protein; LBP-BPI: Bactericidal/Permeability-Increasing Protein; LITAF: Lipopolysaccharide (LPS)-induced Tumor necrosis factor-alpha TNF-α factor Jun-like; p62: sequestosome-1; McTLR-a mRNA: messenger RNA transcript of the Toll-like receptor; MyD88: Myeloid Differentiation primary response gene-88; NF-κB: Nuclear-Factor kappa B; MAPKs: mitogen-activated protein kinases; p-mTOR : phosphorylated mammalian target of rapamycin; Protein Kinase C: PK C-like proteins; PGRPs: Peptidoglycan recognition proteins; Serpin-2 : serine protease inhibitor; SRCR: Scavenger receptor cysteine-rich; IRGs: Immunity-Related GTPases; RTKs: Receptor Tyrosine Kinases; TLR2: Toll-like receptor 2;

GCMS: Gas chromatography–mass spectrometry; PCR: polymerase chain reaction; q-PCR: quantitative polymerase chain reaction; RT-PCR: quantitative real time polymerase chain reaction; HPLC-Esi-MS/MS: high-performance liquid chromatography/electrospray ionization tandem mass spectrometry 2DE: Two dimensional electrophoresis gel; 1H NMR: Proton NMR spectroscopy; + increase; - inhibition; = no significant increase; +/- mixed response.

Table 3.5 Histological and immunohistochemical biomarkers detected in mussels infected with *Vibrio* spp.

Mussel species	**Sample**	**Methods**	***Vibrio* species**	**Histology**	**Immuno-histochemistry**	**Other**	**Reference**
Mytillus edulis	Haemolymph, whole animals	Histological; immune-histochemical (IHC) analyses	*GFP-V. splendidus*	+	+	Tissue necrosis, haemocyte infiltrations in digestive gland, mantle or gills, granulomas	Ben Cheikh et al. (2017)
	Larvae	H&E-stained histological analysis; TEM	*V. splendidus ME9, V. anguillarum*	+		Disorganized cilia in the stomach, Digestive organs necrosis, velum detachment	Wang et al. (2021)
Mytillus galloproviancialis	Haemolymph, adductor muscle	Histology; immunohistochemistry	*V. splendius* LGP32	+	+	Histology PAM: Adductor muscle lost cellular volume, and, in turn, large white spaces appeared both among fibres and among fascicles IHC of PAM: High number of immunopositive cells (the antibody directed against Na^+/K^+ ATPase, proliferating cell nuclear antigen, PCNA, and Caspase-3)	Parisi et al. (2019)
	Haemolymph	Bioassays; TEM	*V. coralliilyticus ATCC BAA-450, V. coralliilyticus TAV24*		+	Haemocytes showed rapid cell damage followed by lysosomal fusion events	Balbi et al. (2019)
Perna viridis	Haemolymph, hepatopancreas	Histology	*V. alginolyticus*	+		Haemocyte infiltration, normal B-cells, normal F-cells, haemocyte infiltration in connective tissue,	Laith et al. (2021)

						vacuolation of F cells, vacuolation of B-cells, tubular structure, hyperplasia, degeneration of lumen, necrotic foci, karyomegally, destruction of hepatopancreas tissue. necrotic connective tissue and bacterial colonization	
Perna canaliculus	Larvae	TCD bioassays; Koch postulate	*Vibrio sp. DO1 (V. coralliilyticus / neptunius-like isolate) and V. splendidus*	+		Detachment of cilia cells, deterioration of soft tissue	Kesarcodi-Watson et al. (2009a)
	Whole tissues	Flow cytometry, Histopathology, species-specific qPCR	*Photobacterium swingsii*	+		Haemocyte infiltration, haemocyte infiltration in connective tissue, digestive gland dilation, digestive gland epithelium atrophy, muscle degeneration and necrosis with haemocytic infiltration	Azizan et al 2023
Pinna nobilis	Digestive gland, gills	Bioassays	*V. harveyi and Vibrio sp.*	+	+	Haemocytic infiltrations of the connective tissue around the digestive gland and intestine, parasitic stages of *Haplosporadian pinnae* in the intestinal lumen, epithelial deformations and necros	Künili et al. (2021)
	Digestive gland, gills, mantle	Histopathology; immunohistochemistry; SDS-PAGE / immunoblot; Dot blot analysis	*Vibrios*	+		Heavy inflammation responses and heavy lesions in the connective tissue of the digestive gland	Lattos et al. (2021a)

Abbreviations: IHC: Immunohistochemical; PAM: posterior adductor muscle; PCNA: proliferating cell nuclear antigen; H&E: haematoxylin and eosin; TEM: transmission electron microscopy; TCD: thrombin clotting time; Na+/K+ ATPase: sodium-potassium pump; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; q-PCR: quantitative polymerase chain reaction.

3.5 CONSTRAINTS AND POTENTIAL OF BIOMARKER-BASED APPROACHES

Despite the advancements in measurable responses to assess the relative condition of mussels following *Vibrio* spp. infections, connections between biomarker responses and baseline conditions (indicative of good health) are still needed. There are three steps to establishing connections, according to Waller and Cope (2019). Identifying biomarkers or other measures that will provide the most informative information is the first step in developing health assessment tools for mussels. Next, determining characteristics of a 'healthy' or 'normal' individual (mussels) are needed, by characterising the baseline condition across multiple species, life stages, and geographic regions (Waller and Cope, 2019). The final step, would involve evaluating how health characteristics shift from baseline conditions as the result of disease, exposure to microbiological outbreaks, or other environmental stressors (Waller and Cope, 2019). These three steps would help with the tabulation of a database with relevant information of biomarkers of interest. Ideally, a biomarker would be transformed into a point-of-use test that mussel farmers, scientists and decision makers could effectively use in the field to monitor for *Vibrio* spp. Ultimately, the high-throughput platforms and laborious sample preparation methods, should be integrated into an affordable, hand-held tool that is easy to use and interpret by anyone (Pinu et al., 2019). Such devices are applied in other areas and can include lateral flow devices, dipstick approaches or electrochemical detection (Trivedi et al., 2017). However, before any of this becomes a reality, reference intervals describing the dispersion of test parameter values of animals exposed to a certain condition (or apparently healthy) are required, to generate accurate assessment tools and for the interpretation of results (Geffré et al., 2009). Once a suspected biomarker with associated conditions has been identified, validation is required before it can truly be used as a biomarker (Hey et al., 2019). There have been many factors that have hindered biomarker validation, including failure to disclose methodologies or replicate results, not having a clear plan for the biomarker's intended use, not understanding its form and function, not selecting adequate controls, mismatches with age, gender, tissue infection, etc. (Holland, 2016).

Several variables (i.e., age, season, sex etc) can also influence biomarker reference intervals, necessitating a detailed description of the organism under investigation (Gutierrez et al., 2020). As summarised above, many of the biomarkers exhibited variable responses due to the mussel species, the type of tissue being analysed, sampling location and time, diet, exposure to chemical contaminants, temperature stress, life history, gender and more (Fig. 3.2) (Moore et al., 2007). The natural ecosystem variations over time make it a substantial

challenge to determine disturbances at an ecological level. The presence of other biological contaminants, atmospheric deposition of chemical contaminants, and global climate change likely mean there are no truly undisturbed ecosystems (Oliver and Fisher, 1999, Hook et al., 2014). It is reasonable, however, for the investigated biomarker to be linked to adverse effects at the organism level (particularly effects biomarkers). Apart from biological variability, analytical variables can also influence the outcome of a biomarker. To ensure accurate and reproducible data between laboratories, it is necessary to create a standard protocol for sample collection, processing, and storage, as well as to verify the sensitivity and specificity of the biomarkers used (Moore et al., 2007). These variables are best confounded when collecting large populations of mussels representative of healthy and diseased populations (Moore et al., 2007, Hook et al., 2014, Brosset et al., 2021, Zare Jeddi et al., 2021). Therefore, better establishment of baseline concentrations of the population under investigation is essential (Oliver and Fisher, 1999, Brosset et al., 2021), while also considering repeated evaluates from the same populations and locations, as well as the investigation of the environmental factors that affect the mussel traits (Oliver and Fisher, 1999). In addition, the more data relating to the mussel and environment should be reported with the measured outcomes to search for similarities and changes among studies.

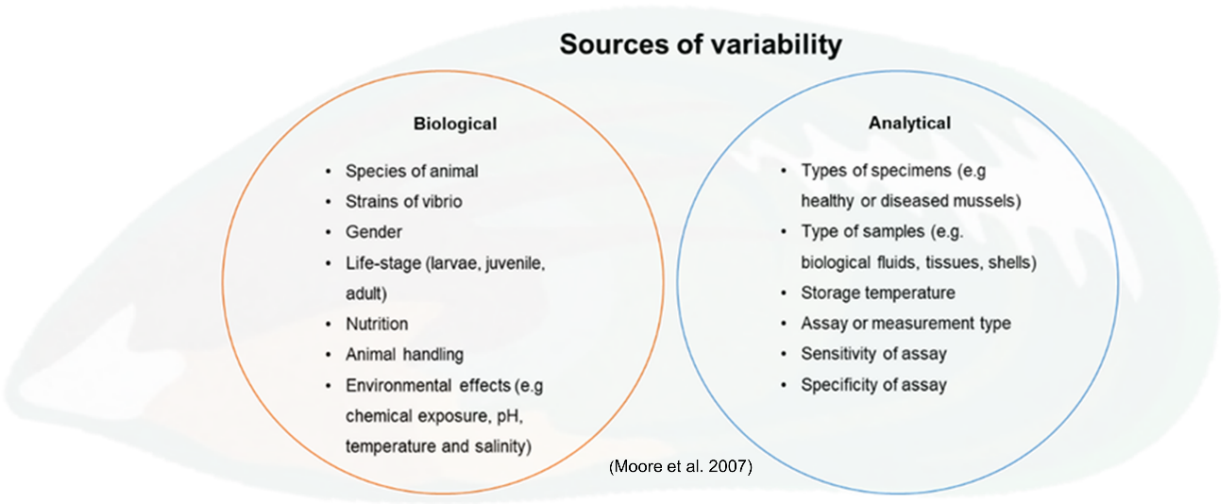

Figure 3.2. Potential sources of variation identified in mussel-vibrio studies for biomarker validation. Adapted from Moore et al. (2007).

A daunting challenge in the development of specific biomarkers for mussels-vibriosis comes to play when aiming to separate differences due to multiple microbial infections (Oliver and Fisher, 1999, Newton and Cope, 2006). For instance Dolar et al. (2020) reported that bacterial and viral infections had different immune responses in the crustacean *Porcellio scaber*. The

authors showed that animals with a viral infection had significantly higher total haemocyte counts and higher proportions of three types of haemocytes compared to animals with bacterial infection. Research in controlled environments and field testing are both necessary to develop stress-specific biomarkers (Waller and Cope, 2019). Additionally, the variability of physiology in mussels is typically attributed to the seasonal reproductive cycle of the species and changes in temperature and salinity of their habitats (Oliver and Fisher, 1999). The variability caused by different nutrition, climate change (i.e. ocean acidification), diseases, phenotypic plasticity as well as genetic stock, is less understood, but undoubtedly significant (Oliver and Fisher, 1999, Paillard et al., 2004, Moore et al., 2007, Gestal et al., 2008). Also, oysters collected at the same time of year from the same site were found to differ greatly among individuals, with coefficients of variation between 50 to 100% for immunomarker measurements, such as haemocyte numbers, rate of locomotion, and nitroblue tetrazolium reduction test (Oliver and Fisher, 1999). There is a need for studies such as this on mussels to correlate environmental factors, stressors, or diseases with biomarkers.

Compared to individual platforms alone, integrating data from various platforms could identify a biomarker panel with better sensitivity and specificity (Hook et al., 2014). There are a variety of multi-biomarkers that are commonly used together as a model for biomonitoring programme, including oxidative stress, pathological alteration, metabolites, and genes and proteins (as highlighted by this current manuscript). Several studies have shown a practical application of the multi-biomarkers in management plans to indicate the presence of pathogenic *Vibrio* spp. and their detrimental effects on mussels. Matozzo et al. (2018) used MANOVA analysis on an array of effect biomarker responses in haemocyte (total haemocyte count, haemocyte diameter and volume, lysozyme and lactate dehydrogenase activities in cell-free haemolymph, and micronuclei frequency) and in gills and digestive gland (cortisol-like steroids and lipid peroxidation levels) alongside pathological alterations and microbiological analyses, to correlate both exposure and effects of stressors. When integrated, these datasets have been successfully used to forecast stressful conditions, routinely measured, bimonthly, from four rearing sites in the Gulf of La Spezia. It has been suggested that the health status of farmed mussels (*Mytilus galloprovincialis*) deteriorates with time, resulting in them becoming susceptible to pathogenic bacteria, viruses, and protozoa (Matozzo et al., 2018). Shellfish management could be made more proactive by following this type of study, which can inform managers on population dynamics, provide early warning signals, and provide information about what is driving the changes. Several measures of oxidative stress (SOD, CAT, GPOx), pathological alteration, molecular and microbiological analysis were recently used by Künili et al. (2021) to evaluate the health status and mass mortality of *P. nobilis* populations. The authors demonstrated that mussels that encountered

oxidative stress and vibriosis due to coinfections of various *Vibrio* spp. Would impact the health of other populations. Thus, by applying initial screening of oxidative stress biomarkers for vibriosis it becomes possible to understand the baseline prevalence of *Vibrio* spp., which in effect helps to determine whether infectious agents can be transferred during field and hatchery operations and whether quarantine or disinfection procedures are necessary.

Diagnosis is another important area in mussel health management, that could potentially benefit from omics technologies. A rapid and accurate diagnosis of pathogens is crucial to the management of diseases and to the study of infections and immune responses in hosts. Most recently, it was observed that dual RNA-seq, in which transcriptional biomarkers in both the pathogen and the host were analysed simultaneously can provide direct insight into the host-pathogen interaction as opposed to traditional approaches (e.g., microarrays or reverse transcription PCR). While this biomarker technique is still in its infancy and has yet to be investigated in *Vibrio*-mussel interactions, it has the potential to define and refine host-related factors related to pathogen virulence determinants, thereby identifying biomarkers relevant to pathogen-specific illness and disease outcomes (Westermann et al., 2012, Nuss et al., 2017).

3.6 CONCLUSIONS

Multifactorial causes of mussel mortalities are threating this industry, one of which is highlighted by the family *Vibrionaceae*. Considering that mussels are usually eaten raw or undercooked, they represent an important means of transmitting *Vibrio* bacterial pathogens from animals to humans. Additionally, *Vibrio* spp. infections can weaken mussel condition making them more susceptible to a second stressor with mortality as outcome. However, before a worst-case scenario is experienced, it is our responsibility to support biosecurity management standards and build resilience. In addition, the physiological response of mussels to *Vibrio* spp. infection can improve farming and processing efficiency while also adding value in terms of market-desired traits. By implementing integrating research utilising genomics, transcriptomics, proteomics, metabolomics, immunology, and histopathological assessments, it becomes possible to characterise biomarkers across multiple levels of biological organisation and build on scientific and practical knowledge. This review highlighted various biomarkers that could be suitable for assessing Vibrionaceae in mussels. Ultimately, by measuring responses of mussels to *Vibrio* spp. infections quantitative data may be linked to biomarkers of exposure, effect, and susceptibility. Building on this concept of physiological biomarkers of mussel responses to Vibrionaceae infections through well reported research and validation steps, will be key in strengthen efforts towards *Vibrio* spp. management and

monitoring in mussels. Biobanking of baseline information on the response of mussels to *Vibrio* species infections will also be required to develop effective animal health strategies and policies and increase confidence in the basis for risk management interventions.

Chapter 4: Beyond relaxed: Magnesium chloride anaesthesia alters the circulatory metabolome of a marine mollusc (*Perna canaliculus*)

"The joy of discovery is certainly the liveliest that the mind of man can ever feel."
- Claude Bernard

Before addressing the biological questions, a best practice mussel handling procedure is established, this chapter aims to investigate the effect of the muscle relaxant magnesium chloride ($MgCl_2$) on the metabolic response of Greenshell™ mussels.

Publication from this chapter:

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Abstract

The New Zealand Green-lipped mussel industry is well-established providing vastly to aquaculture exports. To assess mussel health and reproduction status, visual examination of organs and/or collection of haemolymph is commonly applied. Anaesthetics, such as magnesium chloride ($MgCl_2$) can be utilized to prevent muscle contraction and keep shells open during sampling. The specific effects of muscle relaxing agents on baseline metabolism in invertebrates is unknown, but it is evident that molecular, cellular, and physiological parameters are altered with these chemical applications. To this end, metabolomics approaches can help elucidate the effects of relaxing agents for better assessment of their use as a research tool.

Adult, Green-lipped mussels were anaesthetized for 3 h in a $MgCl_2$ bath, whereafter haemolymph samples were collected and analysed via gas chromatography-mass spectrometry applying methyl chloroformate alkylation derivatization.

Anesthetized mussels were characterized as non-responsive to manual manipulation, with open valves, and limited siphoning function. Metabolite profiling revealed significant increases in the abundances of most metabolites with an array of metabolic activities affected, resulting in an energy imbalance driven by anaerobic metabolism with altered amino acids acting as neurotransmitters and osmolytes.

This research is the first to use metabolomics approaches to identify the metabolic consequences of this commonly used bivalve relaxing technique. Ultimately the use of $MgCl_2$ anesthetization as a sampling strategy should be carefully evaluated and managed when performing metabolomics-related research.

Key words

Green-lipped mussel; Anaesthesia; $MgCl_2$; GC-MS metabolomics; Physiology; Relaxant

4.1 INTRODUCTION

Green-lipped mussels, *Perna canaliculus*, are important cultured species for the New Zealand aquaculture industry (Symonds et al., 2019). Adequate health monitoring, metabolic conditioning, and reproduction maintenance often requires visual examination of mussel soft body parts and/or collection of tissues or biofluids. Mechanical prying (using a reversing plier) of mussel shells forces valves apart to achieve an opening for assessment/sampling. This

procedure can fracture the shell, damage the mantle, tear the adductor and exhaust the animal (Lellis et al., 2000). A routine method to assist sample collection is to first anesthetise animals. In aquaculture, anaesthetics are implemented to reduce research-related mortalities, to minimize handling stress, and to facilitate tissue biopsies (Butt et al., 2008, Suquet et al., 2010).

Bivalves can be anaesthetized using compounds such as clove oil, *tricaine methanesulfonate*, pentobarbital sodium, and magnesium chloride ($MgCl_2$) (Lellis et al., 2000). $MgCl_2$ is the most commonly applied relaxant for research and commercial applications. $MgCl_2$ is non-toxic at appropriate levels, cost effective, easily available and administered, and acts as an efficient relaxant with limited safety concerns for the operator (Acosta-Salmón et al., 2005, Arafa et al., 2007, Puchnick-legat et al., 2015). $MgCl_2$ aesthetic action is attributed to the magnesium ions (Mg^{2+}) which serve as a calcium channel blocker, preventing calcium ions (Ca^{2+}) from entering the cell. Consequently, intracellular Ca^{2+} concentration is kept below the threshold required to initiate release of acetylcholine, which next fails to transmit an action potential. Blocking of calcium channels forces the excitation-contraction coupling in the adductor muscle to fail, preventing muscle contraction and keeping the shells open (Namba et al., 1995).

There remain significant uncertainties about the molecular, cellular, and physiological effects of anaesthetics and muscle relaxing agents in invertebrates (Lewbart and Mosley, 2012, Armstrong et al., 2018). Metabolite profiles provide information about what is happening at a metabolic/physiological level. Metabolites are end products of gene expression and can define the phenotype of a cell or tissue under a specific condition, contributing knowledge about the functional state of cells (Patti et al., 2012, Tugizimana et al., 2018). Metabolite profiles are thus direct signatures of biochemical activity. Metabolic changes associated with anaesthetics (sedation/relaxants) have been assigned to disturbances in the homeostasis of intermediary carbohydrate metabolism, energy metabolism, and decreased metabolite concentrations due to slower metabolism (Ghini et al., 2015, Young et al., 2015).

Ideally, anaesthesia should keep physiological functions within normal ranges (Pugliese et al., 2016), as reported in juvenile mussels where the use of $MgCl_2$ showed no alterations in the intrafilamentary space of the gills before and after relaxation (Gui et al., 2016). Yet, the metabolic effects of $MgCl_2$ in mussels are not well-characterised. The current study is designed to determine if the relaxant action of $MgCl_2$ influences the metabolome of Green-lipped mussels. These findings are expected to shed light on the nature of the metabolic effect of $MgCl_2$ on mussels and will inform use thereof in future experiments.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

A total of 54 adult Green-lipped mussels (average wet weight of 45.74 ± 8.16 g and shell length of 93.60 ± 5.21 mm) were collected from Kaiaua, Thames, New Zealand (37°02′51.2″S 175°18′56.1″E) and transported in coolers to the Auckland University of Technology where they were kept in a recirculating seawater system for 24 h prior to the start of the experiment. Following acclimation, 50% of the animals were anesthetized in 5 L containers with aeration using 100 g/L (525 mM) $MgCl_2$•$6H_2O$ (Sigma- Aldrich, New Zealand, M9272) (Namba et al., 1995). After 3 h in the anaesthetic solution, mussels were assessed and classified as anesthetized if shell gaping was observed without shell closure after three successive pressures on their valves (Alipia et al., 2014). The remaining animals were sampled from the acclimation tank as non-anesthetized controls. Mussels were weighed to the nearest 0.01 g and the shell lengths were measured to the nearest 0.10 mm along the longest axis, using callipers. Additionally, mussel gender was assessed by observing the colour of the gonad, which is a cream (milky white) colour in males and a yellow (orange) colour in females, with 28 females and 26 males recorded (Fig. S1).

4.2.2 Sample collection, preparation, and analyses

Haemolymph was collected from the posterior adductor muscle upon opening of the shell by gently inserting a 23-gauge needle attached to a 1 mL sterile syringe. Immediately after withdrawal, 400 µL haemolymph was transferred to a 2 mL cryovial containing 20 µL 10 mM d_4-alanine (internal standard), flash frozen in liquid nitrogen, and stored at –80°C until metabolomics analysis (Nguyen et al., 2018a, Nguyen et al., 2018c). Quality control (QC) samples were prepared by pooling haemolymph from all the experimental groups, homogenising, and sub-aliquoting them; each pooled QC sample was processed as a biological sample.

Haemolymph samples were prepared for metabolomics analyses as previously described by Young et al. (2019). In brief, the samples were dried under vacuum and extracted twice with 500 µL of 50:50% followed by 80:20% methanol:water solutions. Samples were vortexed (1 min) and cold centrifuged (10,000 rpm; 10 min; 4º C) after both rounds of extractions. Extract supernatants were combined, dried in a SpeedVac concentrator (4 h; 0°C), and derivatized using methyl chloroformate (MCF) alkylation. The samples were re-suspended in 400 µL of 1 M sodium hydroxide and transferred to silanised borosilicate glass tubes containing 334 µL of methanol. 40 µL of MCF reagent was added and the mixture was vortexed for 30 sec. A second 40 µL aliquot of MCF reagent was added with subsequent vortexing (30 sec). 400 µL

of chloroform was added followed by vortexing for 10 sec, then 800 µL of 50 mM sodium bicarbonate was added followed by vortexing for 10 sec. Derivatized extracts were centrifuged (1174 g; 5 min; 6°C), the aqueous layer was discarded, and the residual water removed using sodium sulphate salt. To minimize potential technical biases, all samples were completely randomised prior to extractions and derivatizations being performed over three consecutive days using the same batches of reagents. Lastly, the MCF derivatives were transferred to vials and analysed via a gas chromatography-mass spectrometry (GC-MS) system coupled to an ISQ mass selective detector [EI] operated at 70 eV, which was fitted with a ZB-1701 GC capillary column (30 m × 250 µm id × 0.15 µm with 5 m stationary phase, according to settings provided by Smart et al. (2010).

Several derivatized quality control (QC) samples were employed to ensure reproducibility of GC-MS measurements (Young et al., 2019). These included chloroform solvent, non-derivatized n-alkanes (C10–C40), blank samples containing 20 µL of 10 mM d_4-alanine, amino acid mixtures (20 µL, 20 mM) and pooled samples of treated mussels (with and without treatment of magnesium chloride). These QC samples were injected every five samples and assessed at the beginning of the analytical run and at the intervals throughout the analysis to ensure the reproducibility of the GC-MS measurement. Together, QC samples made up more than 30% of all injections performed. All samples were injected in a random order.

4.2.3 Data processing and statistical analysis

Deconvolution of chromatographic data was performed using the Automated Mass Spectral Deconvolution and Identification System (AMDIS v2.66) software. Metabolites were identified using Chemstation software (Agilent Technologies) and customized R xcms-based scripts to interrogate an in-house library of MCF-derivatized compounds using the data processing workflow developed by Aggio et al. (2011). Records were quality assessed and manually checked for the presence of contaminants with aberrant records being removed. Metabolite peak intensity data were normalized against the internal standard to compensate for potential technical variation. Compound identifications were based on matches (≥ 70%) to both the MS spectrum of the derivatized metabolite and its respective chromatographic retention times and accurate mass. As such the identified compounds can be assigned a level 1 identification (Sumner et al., 2007b, Schrimpe-Rutledge et al., 2016). The systematical error was removed by random forest (SERRF) to normalize the QC samples, correct the data, and obtain the results.

Data pre-treatment steps were completed by using the SIMCA software (version 14.0, Umetrics, Umea, Sweden) and the webserver MetaboAnalyst 4.0 (www.metaboanalyst.ca)

(Chong et al. 2018). Missing value estimation was performed (1/5th minimum value of variable). Univariate analyses were performed on non-transformed data, whereas multivariate analyses were conducted after generalized log transformation and auto-scaling. Unsupervised principal components analysis (PCA) was used to identify natural groupings of all samples based on a correlation matrix of the variables. A heatmap of altered metabolites (students' t-tests; $p< 0.05$) combined with hierarchical cluster analysis (HCA) (Euclidian distance; Ward's method) was used to visualize differences in relative metabolite abundances and to construct dendrograms to assess similarity/dissimilarity among samples and among metabolites. False discovery rate (FDR) correction was used to reduce the risk of the false positive by adjusted p-value (<0.1) based on the Benjamini Hochberg method (Benjamini and Hochberg, 2000). Statistical analyses were performed using MetaboAnalyst 4.0 (Chong et al., 2018) . Differential correlation analyses (Young et al., 2019) on every pairwise metabolite-metabolite abundance was performed on anesthetized and non-anaesthetized groups of mussels separately (Pearson correlation, XLSTAT software package [Addinsoft, New York, USA]). Major differences (Δ r difference >0.5) in the correlation values between the datasets were sought to investigate changes in metabolic network structure.

4.3 RESULTS

A total of 121 metabolites were detected by GC-MS across all samples, of which 89 were reliably annotated. Multivariate PCA was first performed to reduce dimensionality and to provide an intuitive overview of inherent sample clustering based on the correlation matrix of all variables; gender did not appear to impart an influence on the haemolymph metabolome so was removed as a factor from subsequent analyses (Supplementary Figure S1). Separation between anesthetised and non-anesthetised mussels are clearly evidenced in the 2D score plot (Fig. 4.1A), indicating major differences in metabolite profiles between the treatment groups.

To further explore the correlation structure of the data, differential correlation analysis (Pearson) for the abundances of every pairwise metabolite was performed on mussels from each treatment group, and cross referenced against each other (Supplementary material). Through this approach, altered correlations induced by $MgCl_2$ sedation were sought. For example, creatinine positively correlated ($r = 0.79$) with glycine in non-anesthetized mussels, but this relationship was disrupted in $MgCl_2$ exposed mussels to form a negative association ($r = -0.10$) (Fig. 4.1B). Correlations between metabolites provide an alternative fingerprint of the biophysical system and are expected to reflect underlying biochemical networks. A total of 683 pairwise metabolite correlations were identified as being disturbed by $MgCl_2$ treatment,

further substantiating a considerable treatment effect but at a different metabolic mode of organisation.

Lastly, variable selections were made following conventional univariate statistical analyses. After 3 h of $MgCl_2$ incubation, 93 features in mussel haemolymph were identified as being significantly different (t-tests; FDR-corrected p<0.1) from non-anesthetized controls (Figure 4.1C; Table 1). Levels of 92 features were higher in $MgCl_2$-treated mussels, whereas 1 feature was lower. These results indicate an overall concentration of metabolites. The anatomy of mussels and the deposition of metabolic products into the haemolymph are summarized in Fig. 4.2B, which is evidenced by a general and marked increase in total metabolite abundances (sum of the peak heights) being detected in mussel haemolymph after $MgCl_2$ exposure (Fig 4.2C). The mechanism of $MgCl_2$ on synaptic terminals is illustrated in Fig 4.2A.

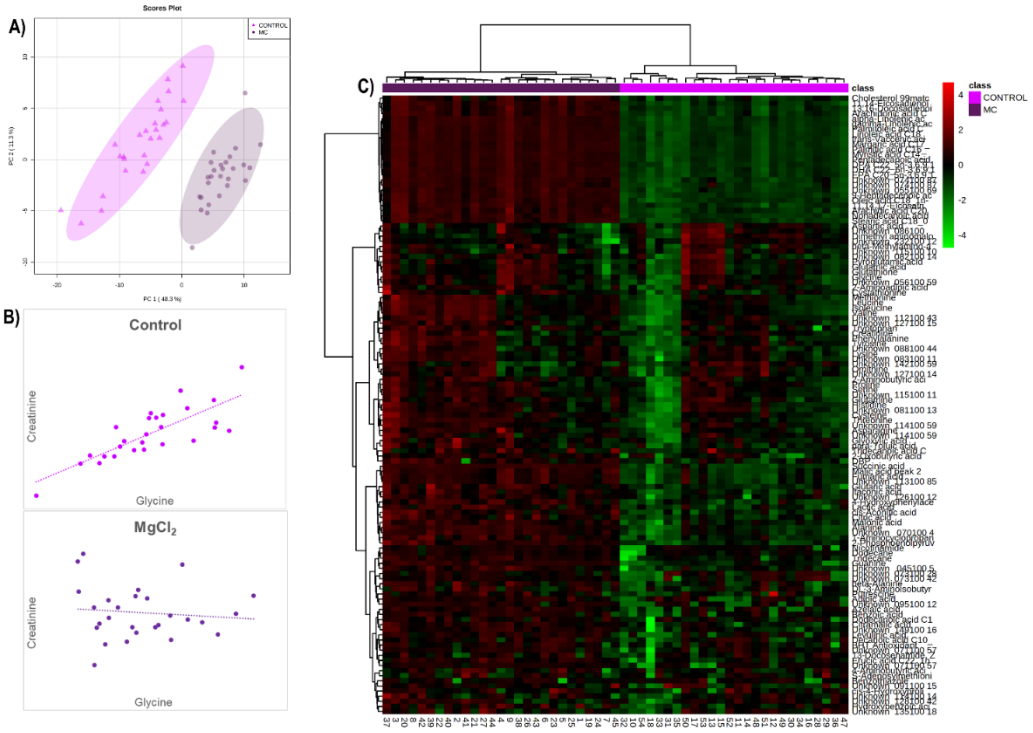

Figure 4.1. Effect of $MgCl_2$ anesthetisation on the mussel (*Perna canaliculus*) haemolymph metabolome. (**A**) PCA score plot showing the first two principal components and clear distinction of the sample groupings, (**B**) an example of a disrupted metabolite–metabolite correlation following $MgCl_2$ treatment, and (**C**) a heatmap of statistically altered (t-tests; FDR-corrected p<0.1) features with combined hierarchical cluster analyses of metabolites and samples based on autoscaled data (rows = features/metabolites; columns = samples/individual mussels per treatment; red–green colour scale = relatively high-to-low metabolite abundances).

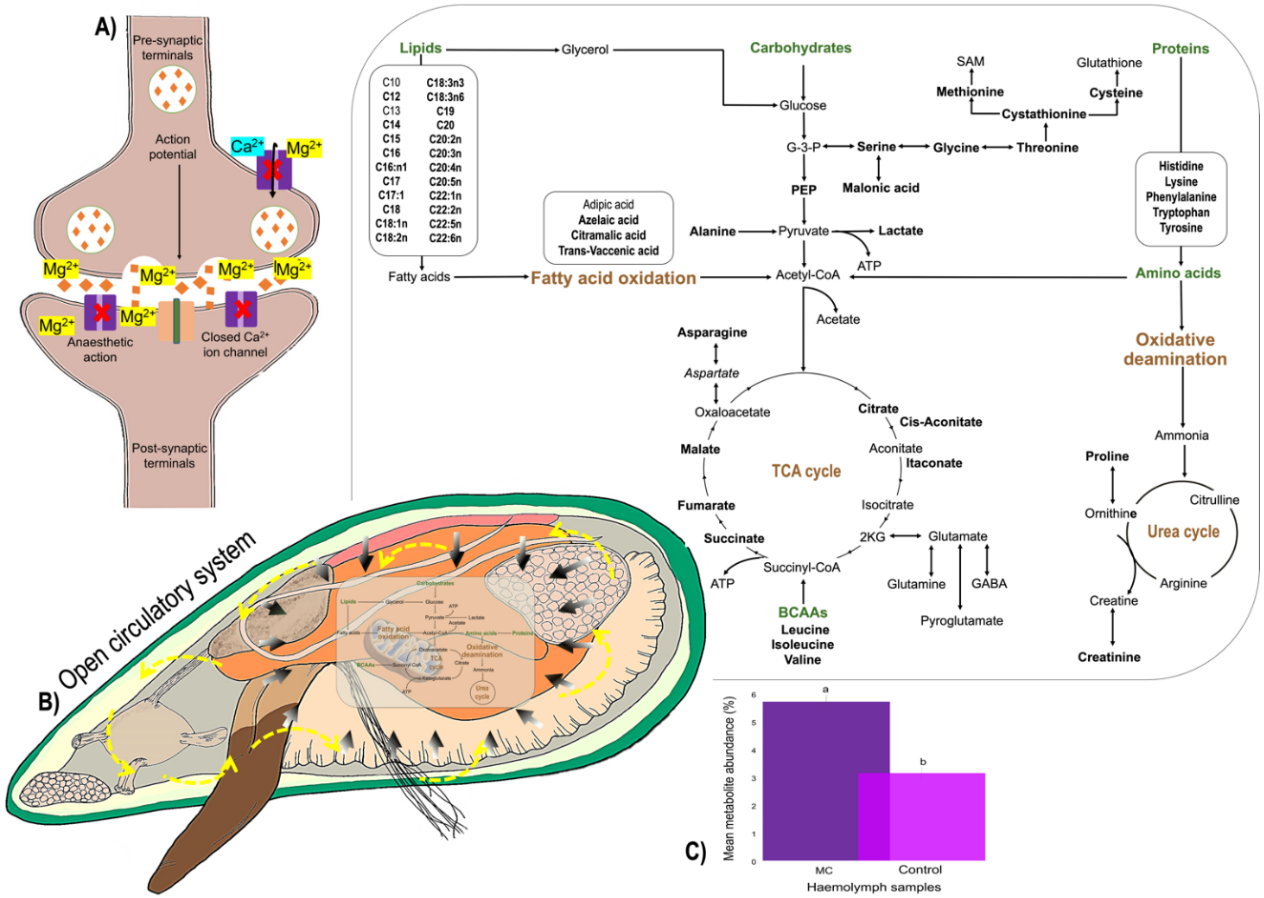

Figure 4.2. Schematic diagram highlighting: (**A**) mechanism of magnesium chloride ($MgCl_2$) anaesthesia on neurotransmission via blocking of calcium ions channels, (**B**) the open circulatory system (yellow arrows) in *Perna canaliculus* serving as an accumulation point for metabolic products, and influx of metabolites (black arrows) into the haemolymph during anaesthesia and (**C**) gross metabolite accumulation effect of $MgCl_2$ anaesthesia in haemolymph (mean sum of the peak heights [±SE]).

Table 4.1 Metabolites in mussels altered (t-tests; p<0.1; FDR<0.1) by $MgCl_2$ anaesthesia. Abbreviation: FC, Fold change; FDR, False discovery rate; DBP, Dibutyl phthalate; DHA, Docosahexaenoic acid; DL, Dextrorotatory and Levorotatory; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; MgCl2/ Control, $MgCl_2$- anesthetized mussels versus non-anesthetized control mussels.

n	Metabolite	FC[a]	log2(FC)	FDR	$MgCl_2$/ Control	n	Metabolite	FC[a]	log2(FC)	FDR	$MgCl_2$/ Control
1	1-Aminocyclopropane-1-carboxylic acid	2.04	1.03	1.72E-09	↑	62	Pentadecanoic acid C15_0	6.38	2.67	3.98E-19	↑
2	11,14,17-Eicosatrienoic acid	6.77	2.76	1.07E-12	↑	63	Phenylalanine	2.18	1.12	2.86E-06	↑
3	11,14-Eicosadienoic	4.84	2.28	8.91E-15	↑	64	Proline	1.41	0.50	1.08E-02	↑
4	13,16-Docosadienoic acid C22_2n-6,9c	5.86	2.55	5.87E-11	↑	65	Serine	1.50	0.58	2.30E-03	↑
5	2-Aminobutyric acid	1.44	0.52	1.90E-03	↑	66	Stearic acid C18_0	6.77	2.76	2.62E-15	↑
6	2-Oxobutyric acid	2.02	1.01	1.71E-02	↑	67	Succinic acid	27.91	4.80	5.57E-19	↑
7	2-Phosphoenolpyruvic acid	6.54	2.71	8.20E-07	↑	68	Threonine	2.31	1.21	1.05E-08	↑
8	4-Hydroxyphenylacetic acid	3.33	1.74	1.52E-08	↑	69	trans-Vaccenic acid	9.05	3.18	5.57E-19	↑
9	9-Heptadecenoic acid C17_1n-8t	6.43	2.68	2.02E-17	↑	70	Tridecane	2.05	1.04	1.75E-10	↑
10	Alanine	2.71	1.44	4.11E-15	↑	71	Tryptophan	2.01	1.01	2.68E-04	↑
11	alpha-Linolenic acid C18_3n-3,6,9c	14.25	3.83	8.07E-16	↑	72	Tyrosine	1.62	0.69	9.36E-04	↑
12	Arachidic acid C20_0	4.99	2.32	5.21E-10	↑	73	Unknown 045100 5989.0 7467.8	2.15	1.11	1.69E-07	↑
13	Arachidonic acid C20_4n-6,9,12,15c	5.19	2.37	4.76E-14	↑	74	Unknown 070100 4228.5 698.0	3.37	1.75	4.78E-12	↑
14	Asparagine	2.08	1.06	4.66E-04	↑	75	Unknown 055100 6977.2 7466.3	8.06	3.01	3.11E-15	↑
15	Azelaic acid	2.23	1.16	2.99E-03	↑	76	Unknown056100 5924.8 11510.8	1.53	0.62	2.81E-02	↑
16	Benzoic acid	2.75	1.46	5.73E-04	↑	77	Unknown 071100 5792.3 4375.3	1.87	0.90	6.55E-05	↑
17	beta-Alanine	1.98	0.98	4.60E-05	↑	78	Unknown 071100 5794.4 8572.9	1.55	0.63	1.77E-02	↑
18	BHT Antioxidant	1.65	0.72	1.33E-02	↑	79	Unknown 074100 8768.8 5533.1	5.40	2.43	5.81E-19	↑
19	Cholesterol	7.17	2.84	7.52E-07	↑	80	Unknown 074100 8769.0 4332.9	4.86	2.28	6.94E-16	↑
20	cis-Aconitic acid	2.49	1.32	1.91E-08	↑	81	Unknown08110013651.615242.5	1.54	0.62	1.93E-02	↑
21	Citramalic acid	2.03	1.02	5.55E-08	↑	82	Unknown 083100 11176.7 5656.9	2.03	1.02	5.07E-05	↑
22	Citric acid	1.94	0.96	8.84E-06	↑	83	Unknown 113100 8548.1 5921.2	2.95	1.56	1.07E-08	↑
23	Creatinine	1.29	0.37	5.17E-02	↑	84	Unknown 114100 5915.3 8214.4	1.60	0.68	2.07E-03	↑
24	Cystathionine	2.45	1.29	1.17E-02	↑	85	Unknown 126100 12746.7 5933.2	2.45	1.29	1.16E-06	↑
25	Cysteine	1.74	0.80	7.69E-05	↑	86	Unknown 149100 16732.8 5717.4	2.07	1.05	7.34E-09	↑
26	Dibutyl phthalate	1.80	0.85	6.78E-06	↑	87	Unknown 088100 4430.3 5919.6	2.57	1.36	3.78E-03	↑
27	DHA C22_6n-3,6,9,12,15,18c	8.68	3.12	1.09E-17	↑	88	Unknown 095100 12631.0 3911.2	2.44	1.28	4.11E-04	↑
28	Dimethyl aminomalonic acid	0.65	-0.61	3.44E-02	↓	89	Unknown 112100 4360.3 11549.2	1.61	0.69	1.62E-04	↑
29	DL-3-Aminoisobutyric acid	2.13	1.09	7.97E-03	↑	90	Unknown 114100 5961.6 14633.7	1.44	0.52	3.78E-03	↑
30	Dodecane	1.89	0.92	4.48E-06	↑	91	Unknown 115100 11895.4 8669.3	1.72	0.78	1.42E-04	↑
31	Dodecanoic acid C12_0	1.61	0.68	2.07E-03	↑	92	Unknown 127100 15949.8 5948.8	1.45	0.54	7.56E-04	↑
32	DPA C22_5n-3,6,9,12,15c	13.99	3.81	3.50E-13	↑	93	Valine	2.15	1.11	1.84E-08	↑
33	EPA C20_5n-3,6,9,12,15c	8.90	3.15	4.66E-19	↑	94	13-Docosenamide, Z	1.04	0.06	8.33E-01	

34	Erucic acid C22_1n-9c	1.94	0.96	7.71E-07	↑	95	2-Aminoadipic acid	1.52	0.60	8.00E-02	
35	Fumaric acid	3.13	1.65	5.02E-09	↑	96	4-Aminobutyric acid	1.32	0.40	1.08E-01	
36	gamma-Linolenic acid C18_3n-6,9,12c	12.26	3.62	4.11E-15	↑	97	Adipic acid	1.39	0.48	3.19E-01	
37	Glutamine	1.35	0.43	4.10E-02	↑	98	Aspartic acid	0.89	-0.17	3.95E-01	
38	Glutaric acid	3.14	1.65	6.78E-06	↑	99	Benzothiazole	1.05	0.07	7.33E-01	
39	Glycine	1.41	0.50	5.10E-04	↑	100	beta-Methylamino-alanine	1.24	0.31	3.46E-01	
40	Glyoxylic acid	1.32	0.40	2.65E-03	↑	101	cis-4-Hydroxyproline	1.29	0.36	1.72E-01	
41	Guanine	34.45	5.11	2.03E-05	↑	102	Decanoic acid C10_0	1.47	0.56	0.10489	
42	Histidine	1.57	0.65	2.53E-03	↑	103	Glutamic acid	1.31	0.39	1.60E-01	
43	Hydroxybenzoic acid	1.63	0.70	5.59E-04	↑	104	Glutathione	1.21	0.28	3.19E-01	
44	Isoleucine	2.16	1.11	2.59E-08	↑	105	Ornithine	1.29	0.37	2.14E-01	
45	Itaconic acid	2.09	1.06	1.61E-06	↑	106	Putrescine	1.13	0.18	7.25E-01	
46	Lactic acid	3.85	1.95	8.20E-07	↑	107	Pyroglutamic acid	1.27	0.34	1.82E-01	
47	Leucine	1.83	0.87	1.53E-06	↑	108	S-Adenosylmethionine	1.13	0.17	6.63E-01	
48	Levulinic acid	2.27	1.18	3.98E-04	↑	109	Tridecanoic acid C13_0	1.82	0.86	3.11E-01	
49	Linoleic acid C18_2n-6,9c	8.19	3.03	3.32E-18	↑	110	Unknown07310028183.314759.4	0.55	-0.86	2.48E-01	
50	Lysine	1.66	0.73	1.54E-04	↑	111	Unknown07310042977.435544.6	0.60	-0.73	3.47E-01	
51	Malic acid	5.07	2.34	6.21E-10	↑	112	Unknown08210014256.811042.6	1.09	0.13	7.65E-01	
52	Malonic acid	2.37	1.24	5.21E-10	↑	113	Unknown 086100 5956.6 12839.3	0.79	-0.34	5.11E-01	
53	Margaric acid C17_0	7.92	2.99	7.91E-17	↑	114	Unknown 091100 15035.5 6511.2	1.13	0.17	4.96E-01	
54	Methionine	1.67	0.74	1.33E-04	↑	115	Unknown11410014731.911527.2	1.44	0.53	1.08E-01	
55	Myristic acid C14_0	7.09	2.83	7.41E-19	↑	116	Unknown 115100 10070.4 5660.1	1.07	0.09	7.25E-01	
56	Nicotinamide	2.03	1.02	1.66E-05	↑	117	Unknown12710014334.910132.5	1.05	0.07	8.01E-01	
57	Nonadecanoic acid C19_0	7.07	2.82	3.67E-14	↑	118	Unknown 128100 4211.8 820.77	1.20	0.26	1.36E-01	
58	Oleic acid C18_1n-9c	5.82	2.54	6.56E-16	↑	119	Unknown 135100 18028.0 7719.9	1.15	0.20	3.47E-01	
59	Palmitic acid C16_0	7.17	2.84	8.51E-20	↑	120	Unknown 142100 5919.9 14117.0	0.79	-0.34	2.48E-01	
60	Palmitoleic acid C16_1n-7c	9.31	3.22	4.72E-16	↑	121	Unknown23210012872.015651.0	0.65	-0.62	1.03E-01	
61	para-Toluic acid	1.43	0.51	1.89E-04	↑						

4.4 DISCUSSION AND CONCLUSIONS

Here, we provide new insights into the metabolic response of a marine mollusc (*P. canaliculus*) following exposure to $MgCl_2$. Our findings clearly demonstrate that $MgCl_2$ substantially alters the haemolymph metabolome in mussels, indicating major physiological dysregulation. $MgCl_2$ relaxes the adductor muscle through its action in the neuronal and abdominal ganglion. It is suggested that $MgCl_2$ produces an axonal blockade at the sodium channel in the membrane of the presynaptic terminals, causing a reduction in the release of excitatory neurotransmitters (i.e., acetylcholine and GABA) (Namba et al., 1995, Winlow et al., 2018). Ultimately, the anaesthetic agent causes a perturbation in the synaptic transmission and leads to failure of the extraction-contraction coupling in the adductor muscle which results in mussels opening their valves. Mussels have an open circulatory system and all organs are surrounded by haemolymph (McMahon and Bogan, 2001). $MgCl_2$ relaxes the adductor muscle and appears to concentrate metabolic substrates within the haemolymph as demonstrated by the increased abundance of most haemolymph metabolites within the anesthetized mussels. The fact that the majority of haemolymph metabolites were affected by anaesthetic treatment provides clear evidence of a major physiological response. Interestingly, the metabolic results may also suggest anaerobic activity which can be ascribed to reduced respiration during sedation, triggering a hypoxic response (Dunphy et al., 2015, Nguyen et al., 2018a, Nguyen et al., 2018c, Powell et al., 2017)

Findings from the current study supports reduced energy sources, activation of anaerobic energy metabolism under aerobic conditions, and elevated energy expenditure as previously noted in response to anaesthetics (De Zwaan et al., 1982). The first evidence to confirm the change in energy state in the current study is elevated lactic acid. In the absence of oxygen the accumulation of nicotinamide adenine dinucleotide (NADH) is countered by the lactic acid pathway which converts pyruvate to lactic acid (by means of lactate dehydrogenase and coenzymes) simultaneously oxidizing NADH to NAD^+, allowing anaerobic glycolysis to function (Venter et al., 2018a, Venter et al., 2018b). This has been reported in the marine mussel, *Perna viridis*, exposed to copper and cadmium; and in the freshwater mussel, *Unio tumidus,* exposed to pharmaceuticals (Wu and Wang, 2011, Falfushynska et al., 2014). On the other hand, opine production presents an alternative to the well-established lactate pathway for anaerobic energy production (Grieshaber et al., 1994). Instead of pyruvate being reduced to lactate-by-lactate dehydrogenase (LDH), marine invertebrates utilize opine dehydrogenases (ODHs) to

catalyse the reductive coupling of pyruvate with amino acids. This process contributes to maintaining the NADH/NAD+ balance. A well-studied example of this pathway comes from the king scallop *Pecten maximus* (*Pm*OcDH) (Van Thoai et al., 1969), and recently, an enzyme from the Mediterranean mussel *Mytilus galloprovincialis* (*Mg*OcDH) was also identified (Vázquez-Dorado et al., 2011).

Anaerobic glycolysis together with limited oxidative phosphorylation in the anaesthetized mussels, resulted in increased tricarboxylic acid (TCA) cycle intermediates (citric acid, glutaric acid, fumaric acid, itaconic acid, cis-aconitic acid, malic acid, succinic acid and its isomer, citramalic acid) which serves as a second indication of an altering energy status of anesthetised mussels. One vital metabolite for invertebrates during anoxic scenarios is succinic acid, which is the end-product of the glucose/aspartate-succinate pathway. In essence, this pathway involves the reversal of the second half of the TCA cycle, as a mean to use accumulating reduced equivalents for the synthesis of nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD), ensuring equivalents for adenosine triphosphate (ATP) production during anoxia (Harcet et al., 2013). In the current study, the abundant succinic acid (foldchange >225) supports the use of this pathway for anaerobic energy production, as reported in anoxic blue mussels, *Mytillus edulis* (Rivera-Ingraham et al., 2013). The cellular implications for the use of $MgCl_2$ may also indicate inhibition of the enzymes such as succinate dehydrogenase (SDH), causing elevation of succinic acid. The accumulation of succinic acid in the haemolymph of anesthetized mussels, suggesting that this metabolite could serve as an anaerobic substrate for amino acid (i.e. alanine) production in the cytosol as well as for the mitochondrial generation of succinate (Pisarenko et al., 1990).

Additional anaerobic pathways activated in the anesthetized mussels to counter the energy and redox (NADH: NAD^+) imbalance involves amino acids, which serves as substrates or intermediates of anaerobic energy metabolism (Zurburg and De Zwaan, 1981)(Zurburg and De Zwaan 1981). In particular, alanine (generally produced when glucogenic amino acids are transaminated from pyruvate) was elevated in the current study, and plays an important role in the buffering of H^+ ions, regulation of intracellular osmotic pressure, and serves as a substrate for opine production in molluscs (Venter et al., 2018a, Venter et al., 2018b). Other amino acids detected as increased in the anesthetized mussels include: leucine, isoleucine, glycine, cysteine, serine, tyrosine, tryptophan, alanine isomers (phenylalanine, beta-alanine), which serve as gluconeogenic substrates during anaesthesia. This supports the fact that protein synthesis is typically reduced during hypoxia (Haider et al., 2020). The amino acids, methionine and cystine,

detected in this study are commonly associated with oxidative stress. Prolonged increases in these metabolites have been related to oxidative injury to mitochondrial proteins and lipids in scallops (Ivanina and Sokolova, 2016). However, the exact impact of 3-hour $MgCl_2$ incubation on oxidative activity in *P. canaliculus* remains to be evaluated. Further linking to neurological influences due to $MgCl_2$ exposure is the reduced amino malonic acid and derivative, dimethyl amino malonic acid. This metabolite is believed to inhibit neuronal communication (Xu et al., 2020b), thereby reducing stress in mussels.

During hypoxic exposure, ammonium excretion is limited, as seen in the blue mussel, *Mytilus edulis* (Haider et al., 2020). A similar action can be linked to an anaerobic state of *P. canaliculus* following $MgCl_2$ incubation. Metabolic pathways responsible for nitrogenous waste (urea cycle and purine metabolism) were affected in this group with increased creatinine and decreased aspartic acid, supporting the suppression of urea production, and resulting in accumulation of urea cycle and associated intermediates. Increased guanine linked to the purine nucleotide cycle also attest to the fact that purines are degraded at a slower rate in the anesthetized group limiting ammonia production (Weihrauch and Allen, 2018). Changes as these have previously been recorded in unionid mussels (Leonard et al., 2014), supporting nucleic base changes in *P. canaliculus.*

A number of metabolite–metabolite correlations were found to be affected by $MgCl_2$ sedation. Although difficult to interpret in cases where functional associations between metabolites are not currently known, these results do indicate severe metabolic perturbations in the underlying structure of biochemical networks. Elucidating the origin of metabolite correlations is anticipated to provide insight into biochemical processes and their regulation. As an exemplary highlight resulting from this analysis, correlations between glycine and creatinine were found to be substantially affected. Glycine is a precursor in the biosynthesis of creatine, via guanidinoacetate and S-adenosylmethionine intermediates. Creatine is catabolized to the excretion product creatinine by the phosphotransferase enzyme phosphocreatine, which is utilized by invertebrates for rapid ATP production (Ellington, 1989). Glycine also has a prominent role as an osmolyte in molluscs (Young et al., 2019) and serves as a precursor in strombine production, via condensation with pyruvate and an opine specific dehydrogenase which is important for anaerobic energy production in invertebrates (Alfaro et al., 2019a). This disruption in glycine–creatinine correlation signals a significant shift in basal metabolism, in the anesthetized mussels, supporting the other metabolomics findings indicative of changes in energy metabolism.

Apart from a phosphocreatine energy producing route, fatty acid oxidation results in the production of acetate enabling limited energy production via beta-oxidation during anaerobiosis (De Zwaan et al., 1982). Results from the current study suggest that homeostasis of synthesis and degradation of lipids and their derivatives were affected by $MgCl_2$–induced hypoxia. Increases in linoleic acid and docosahexaenoic acid (DHA) may attest to the presence of stress as they play important roles as cell membrane constituents, with the potential to activate cell surviving cascades in response to hypoxia, as seen in *Apostichopus japonicus* (Li et al., 2019a). In addition, elevated concentrations of fatty acids (saturated, monounsaturated, polyunsaturated) support the functioning of membrane-bound proteins (enzymes, ion channels and receptors) assisting with mussel survival by reducing the stimulation of the sympathetic nervous system during anesthetization (Havel and Goldfien, 1959, Cooperman, 1970, Zhou, 2012).

The overall metabolic response of *P. canaliculus* due to $MgCl_2$ incubation shows a higher metabolite abundance, with energy producing pathways attesting to a shift towards anaerobic energy production. The use of $MgCl_2$ was deemed an effective relaxant for use on the Sydney rock oyster when evaluating immunological parameters, with effects of the relaxant lasting no longer than 96 h (Butt et al., 2008). From the current metabolomics-based study, $MgCl_2$ causes major changes in metabolite profiles of mussel haemolymph samples. Thus, $MgCl_2$ (and anaesthesia in general) should be used with extra caution to assist easier sampling of haemolymph from mussels since potential bias may be artificially induced into the metabolome signature. Building from this research it would be useful to determine if the effect of $MgCl_2$ masks specific metabolite signature changes induced by other treatments (e.g., thermal stress and pathogen infections) leading to an influence on the biological interpretability of the data.

Chapter 5: Pathogenicity and virulence of bacterial strains associated with summer mortality in marine mussels (*Perna canaliculus*)

"The sea, the great unifier, is man's only hope. Now, as never before, the old phrase has a literal meaning: we are all in the same boat."
- Jacques Yves Cousteau

Since the use of magnesium chloride ($MgCl_2$) as a relaxant agent is discontinued for the remaining experimental chapters in this thesis, this chapter aims to identify and characterise isolated bacterial pathogens belonging to the *Vibrionaceae* family and test the survivability of these isolates on mussels through an injection challenge test.

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Abstract

The occurrence of pathogenic bacteria has emerged as a plausible key component of summer mortalities in mussels. In the current research, four bacterial isolates retrieved from moribund Greenshell™ mussels, *Perna canaliculus,* from a previous summer mortality event, were tentatively identified as *Vibrio* and *Photobacterium* species using morpho-biochemical characterisation and MALDI-TOF MS and confirmed as *V. celticus*, *P. swingsii*, *P. rosenbergii and P. proteolyticum* using whole genome sequencing. These isolates were utilised in a laboratory challenge where mussels were injected with cell concentrations ranging from 10^5 to 10^9 CFU/mussel. Of the investigated isolates, *P. swingsii* induced the highest mortality. Additionally, results from quantitative polymerase chain reaction analysis, focusing on known virulence genes were detected in all isolates grown under laboratory conditions. *P. rosenbergii* and *P. swingsii* showed the highest expression levels of these virulence determinants. These results indicate that *Photobacterium* spp. could be a significant pathogen of *P. canaliculus*, with possible importance during summer mortality events. By implementing screening methods to detect and monitor *Photobacterium* concentrations in farmed mussel populations, a better understanding of the host-pathogen relationship can be obtained, aiding the development of a resilient industry in a changing environment.

Keywords

Genotyping; mussels; MALDI-TOF-MS; pathogenicity; phenotyping; *Photobacterium*; summer mortality; *Vibrionaceae.*

5.1 INTRODUCTION

The New Zealand (NZ) aquaculture industry has developed into a specialised and high-quality food production sector in the last 50 years, with Greenshell™ mussels (*Perna canaliculus*) as the dominant aquaculture species (Heasman et al., 2020). Compared to other cultured shellfish species worldwide, *P. canaliculus* has experienced relatively few health issues with occasional pathogens and parasites detected in farmed populations (Castinel et al., 2019). However, mass mortalities of mussels were recorded in the North Island of New Zealand during the austral summer of 2011-2012 and Marlborough Sounds over the summer of 2012–2013 (Mackenzie, 2014, Macara, 2018, Anderson et al., 2019). More recently, in the summer of 2018, elevated seawater temperatures (up to 25°C) and significant mussel mortalities were reported in farms across Marlborough and Coromandel regions (Neilson, 2019, Peart, 2019, Webb and Duncan, 2019). Summer mortality is loosely defined as unexplained mortality events resulting from many potential causes related to the host, pathogen, or environment (Webb and Duncan, 2019). Climate change projections for NZ estimate that seawater

temperatures will rise by 1–3°C over the next 80 years; likely compounding further incidences of summer mortality in response to thermal stress and increasing prevalence of pathogens (Heasman *et al.*, 2020). It is believed that the combination of elevated temperatures with pathogen burdens which appear to proliferate during the summer months may lead to physiological 'tipping points' in mussels resulting in death (Li et al., 2020). For example, Li et al. (2020) found metabolic perturbations and cytoskeleton protein structural changes in gill tissues in Greenshell™ mussels from a summer mortality event. *P. canaliculus* has also been shown to experience increased production of intracellular reactive oxygen species (ROS) induced by *Vibrio* sp. DO1 infection (Nguyen and Alfaro, 2020b) and vibriosis associated with elevated water temperatures (Newton and Webb, 2019), suggesting that a range of pathogens could take advantage of immunocompromised mussels under certain stressful conditions.

Previous studies reported a combination of *Vibrio*-*Photobacterium* species along with other marine bacteria as normal inhabitants of seawater, marine sediments, seaweeds and animals (Urbanczyk et al., 2011, Labella et al., 2017). They can interact with aquatic species as non-specific commensals, parasites, and saprophytes (Urbanczyk et al., 2011). These microorganisms appear to cause disease under changing environmental conditions (e.g., rising ocean temperature and salinity) by taking advantage of the host's stressed condition (Matanza and Osorio, 2018). For example, *Photobacterium damselae* subspecies [subsp. *damselae* (*Pdd*) and subsp. *piscicida* (*Pdp*)] have received considerable attention as emerging temperature-dependent pathogens for many aquatic organisms, including fish, molluscs, and crustaceans (Matanza and Osorio, 2018). Yet, little is known about the molecular mechanisms implemented by other species such as *P. swingsii and P. rosenbergii*, even though these bacteria have been shown to be responsible for disease in corals (*Pachyseris speciosa, Merulina ampliata*, *Barabattoia amicorum),* larvae of the giant clam (*Tridacna gigas*) and wild blue mussels (*Mytilus edulis*) (Sutton and Garrick, 1993, Thompson et al., 2005b, Eggermont et al., 2017). For example, *Photobacterium* species were found in haemolymph and tail fans of wild lobsters (*Jasus edwardsii*) captured on the east coast of the North Island of New Zealand. Half of the lobsters had tail fan necrosis (TFN), indicating that these bacteria might play an important role in shellfish health (Zha et al., 2018). Furthermore, *Vibrio splendidus* and *V. coralliilyticus/neptunius*-like isolates have been associated with batch losses of *P. canaliculus* larvae during hatchery culture (Kesarcodi-Watson et al., 2009a), and experiments using a *V. coralliilyticus/neptunius*-like isolate suggest pathogenicity in adult *P. canaliculus* (Nguyen et al., 2018b, Nguyen et al., 2018c, Nguyen et al., 2019b). Although some of these bacterial strains are known to affect numerous aquatic species worldwide, no study has yet characterised pathogenicity and virulence of bacteria specifically associated with summer mortality in *P. canaliculus*.

Identification and characterisation of bacteria at the species level can be a challenging task since some taxa nested within specific clades have high degrees of both genetic and phenotypic similarities. Bacterial typing systems detect differences in phenotypic or genotypic characteristics of strains, and based on their resolution power, can be used to distinguish genera, species, or strains (van Belkum et al., 2007, Li et al., 2009b). Techniques for bacterial strain typing include light or electron microscopy, physiological tests (e.g., growth temperature, pH value, salt concentration and oxygen requirements), biochemical profiling (Donelli et al., 2013, Travers et al., 2014), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Moussa et al., 2021), polymerase chain reaction (PCR)-based methods, and other sequence-based techniques, including multi-locus sequence typing (MLST) and whole-genome sequencing (Amaral et al., 2014, Culot et al., 2021). A multi-technique approach enables detailed information on bacterial strains with high accuracy of identification from the outbreak environments.

Considering the status of *P. canaliculus* as an important aquaculture species and associated bacterial incursion, the aim of this study was to characterise bacterial strains associated with a Greenshell™ mussel summer mortality event and assess their pathogenicity. The specific objectives were to: 1) characterise the bacterial strains using phenotypic and genotypic approaches, 2) determine the pathogenicity of the bacterial strains on Greenshell™ mussels through laboratory challenge experiments, and 3) quantify expressions of known virulence factor genes within the bacterial strains. Results from this investigation will aid in determining the importance of pathogenic bacteria on increased mortality in *P. canaliculus* during summer months.

5.2 MATERIALS AND METHODS

The integration of phenotyping (cell morphology, physiological and biochemical profiling), protein profiling via MALDI-TOF MS, and genotyping methods (whole genome sequencing and qPCR analysis of known virulence genes related to motility and chemotaxis, extracellular polysaccharide production, biofilm formation, heat-shock protein, cytotoxin and production of lytic enzymes) were implemented to identify and characterise bacterial strains isolated from tissues of farmed mussels during a mortality event (Fig. 5.1).

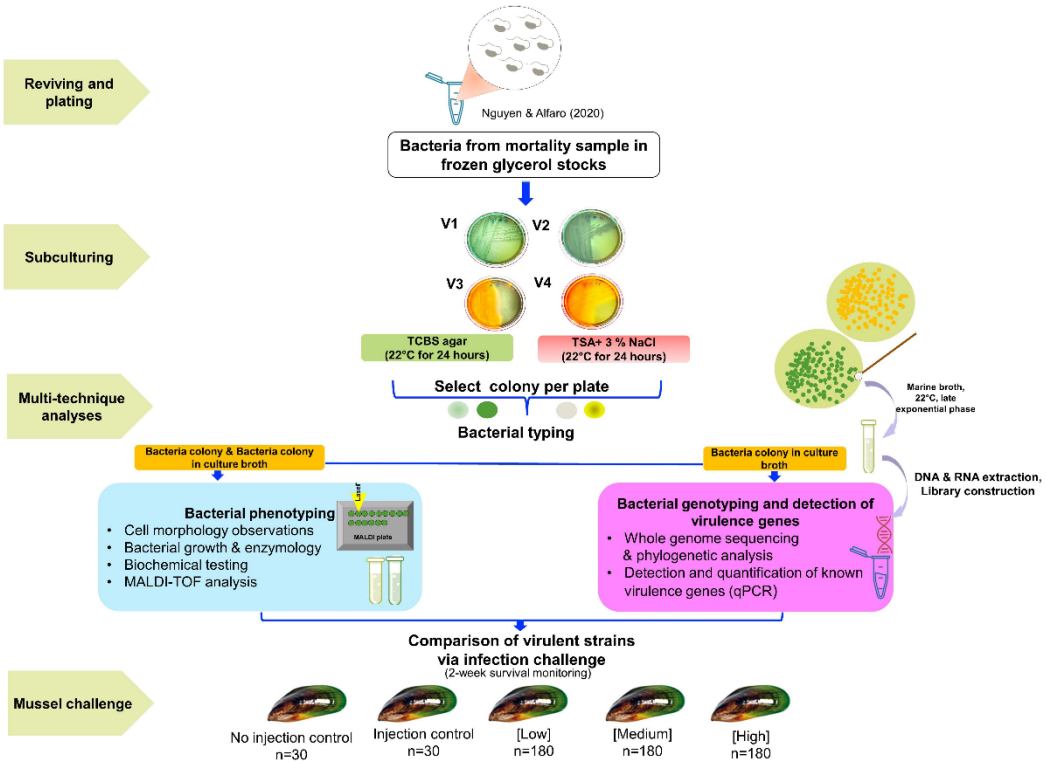

Figure 5.1. Experimental approach including phenotypic and genotypic bacterial isolate characterisation followed by a three-week controlled laboratory mussel infection challenge.

5.2.1 Bacteria isolation from farmed mussel populations

Greenshell™ mussels (average ± SD wet weight of 46 ± 8 g and shell length of 94 ± 5 mm) were collected from a mussel farm in Kaiaua, Thames, New Zealand (37°02′51.2″S 175°18′56.1″E) in the austral summer of 2017-2018, during a mass mortality event (Nguyen and Alfaro, 2020b). Bacteria were isolated from moribund mussels, by plating 100 µL of haemolymph in dilution on thiosulfate-citrate-bile salts sucrose (TCBS) agar (Fort Richard Laboratories, New Zealand) and incubating at 22°C for 24 h. Individual colonies of different morphologies and sizes were selected and suspended in 500 µL sterile sea water, whereafter aliquots of 50 µL were transferred to new TCBS plates (repeated twice). Isolates with *Vibrionaceae* morphology characteristics (1–3 µm, motile rods, sometimes curved, observed under bright field microscope) (Buller 2004) and the isolates were frozen at –80°C in marine broth supplemented with 50% glycerol (v/v) (Buller, 2013, Nguyen et al., 2018b).

5.2.2 Strain and culture conditions

A total of four selected predominant bacterial colonies across samples were inoculated on TCBS agar and grown overnight at 22°C. A single colony was picked from each strain and individually inoculated into 10 mL of fresh marine broth 2216 medium (Difco, USA) and grown

for 24 h at 22°C with constant agitation. Cell density was determined at 600 nm using a spectrophotometer. Growth rates of the isolates were calculated as described by Hall et al. (2014). Freshly grown cultures (OD_{600} = 1) were diluted in fresh marine broth. Aliquots (250 µL) were loaded into a 96-well plate in triplicate. Absorbance was measured every 30 min until stationary phase with a multi-mode microplate reader (FLUOstar® Omega, Germany) at 22°C. Data were analysed with the Omega software, as described previously (Hall et al., 2014).

5.2.3 Bacterial phenotyping

5.2.3.1 Cell morphology

Isolates were streaked onto pre-poured TCBS (Fort Richard Laboratories, New Zealand), trypticase soy agar (TSA) with 3% salt (Fort Richard Laboratories, New Zealand) and McConkey agar plates (Fort Richard Laboratories, New Zealand) and incubated at 22°C for 24 h. Morphological characteristics, including colony shape, colour, size, edge, elevation (colony surface shape) and texture were noted along with gram staining and motility.

5.2.3.2 Physiological and biochemical profiling

Phenotypic profiles were further evaluated with biochemical, thermal, and halophilic analyses using a similar system to that described by Le Roux et al. (2004) Isolates were grown overnight on TCBS and tested using 35 API-based biochemical tests (API 20E and API 20NE, BioMérieux, France), NaCl tolerance (growth in 6.5% brain heart infusion and sheep blood agar) and temperature tolerance (growth on TSA +3% salt at 4, 22, 37, and 41°C). For API testing, the isolates were prepared in 3% saline solution instead of 0.85% as per manufacturer's instructions. API results were compared against APIWeb™ identification system (Table S1), profiles published by Le Roux et al. (2004) and Buller (2004b).

5.2.3.3 MALDI – TOF analysis

Single colonies were obtained from 24 h old TSA + 3% salt cultures and analysed using MALDI – TOF following the simple and the formic acid protocol (Bruker MALDI Biotyper, Bruker Daltonik, Germany). Spectral profiles were analysed automatically by the instrument's software (flexAnalysis version 3.4, Bruker Daltonics, Inc.)

5.2.4 Bacterial genotyping

5.2.4.1 DNA extraction and sequencing

A DNA Mini Kit (Qiagen, Germany) was used for bacterial deoxyribonucleic acid (DNA) extractions in accordance with the manufacturer's protocols. DNA was extracted from fresh marine broth cultures grown overnight at 22°C. Concentrations of extracted DNA were assessed using the dsDNA High Sensitivity (HS) Assay kit on a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Oregon, USA). Quality of DNA was determined by NanoDrop spectrophotometer (Thermo Fisher Scientific, Delaware, USA). After extraction, DNA libraries were prepared from the normalized PCR amplification products (1 ng total of DNA extract) using the Nextera XT DNA Library Preparation kit (Illumina, California, USA) following the manufacturer's protocol. The concentration of the purified libraries was assessed by using the Qubit dsDNA HS Assay kit, and quality by the 4200 Tapestation HS D5000 kit, (Agilent Technologies, Waldbronn, Germany). Each DNA library was then normalised and pooled to an equimolar concentration of 4 nM. Following denaturation using 0.2 N NaOH, the pooled libraries were diluted further to 20 pM and spiked with 1% of PhiX following Illumina's recommendation. Sequencing was performed on the spiked libraries on an Illumina MiSeq using the MiSeq reagent Kit v3 (600 cycles) (Illumina, California, USA), with paired-end sequencing of 2x 300bp.

5.2.4.2 Bioinformatic analyses

Reads were assessed via FastQC v. 0.11.9 (Andrews and others, 2010) which discovered trace amounts of reads still containing adapter sequences, which were then trimmed via AdapterRemoval v. 2.3.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The paired-end sequencing reads were assembled using SPAdes v. 3.14.1 (Schubert et al., 2016) with the "isolate" setting for bacterial isolate genome assembly. Quality and completeness of the assemblies were assessed via QUAST v. 5.0.2 (Gurevich et al., 2013) and BUSCO v. 5.0.0 (Simão *et al.*, 2015). Selected conserved genes (*recA*, *mreB*, *rpo*, *gyr*, *fur*) identified by BUSCO (from the bacteria_odb10 lineage) were used for phylogenetic analysis. RAxML v. 8.2.12 (Stamatakis, 2014) was used to analyse the concatenated nucleotide gene alignment (supermatrix) for a partitioned bootstrap (1000) phylogenetic inference.

5.2.5 Bacterial infection challenge on mussels

Adult *Perna canaliculus* (n=650) were obtained from Kaiaua Marine Farms Ltd. (Firth of Thames, New Zealand) and transported to the aquaculture facility at the Auckland University

of Technology. Animals were labelled, weighed (54 ± 9 g; average ± SD) and measured (90 ± 7 mm; average ± SD) upon arrival, then housed for a 7-day acclimation period in a recirculating seawater system (16°C; 35 ppt; pH 8.2). Baseline bacterial colony counts were collected from a subsample of animals to determine the number of *Vibrio* spp. present. A 100 µL aliquot of pure haemolymph was diluted (up to 10^3) and spread onto a TCBS agar using the spread plate technique. All plates were incubated at 22°C for 24 h before being quantified to obtain CFU/mL of *Vibrio* in the haemolymph.

Prior to the infection challenge experiment, the four bacterial isolates characterised above (V1-4) were revived and suspended in sterile fresh marine broth to obtain a suspension with an OD_{600} of 1. Then, the cultures were quantified and low (10^5 colony forming units (CFU)/mL), medium (10^7 CFU/mL) and high (10^9 CFU/mL) concentrations were prepared to use in the mussel challenge experiment. Three bacterial concentrations were tested: two consistent with the loads of *Vibrio* spp. in the environment and infected mussel tissues (10^5 and 10^7CFU/ml) and one higher (~10^9 CFU/ml).

Each bacterial isolate was injected as a 100 µL stock into the posterior adductor muscle of 30 individual mussels per group (un-injected, injected with marine broth, injected with infectious stocks of low, medium, and high doses). After injection, each animal was placed into its own 2 L container with seawater and air supply. This was repeated for all four bacterial isolates, injecting low, medium, and high infection concentrations sequentially at fixed intervals of approximately 1 hour per isolate (540 infected mussels in total). Additionally, 30 non-injected control mussels and 30 marine broth injected control mussels were included in the challenge experiment. Altogether, 600 mussels were monitored daily for two weeks. Mussels were not fed during the experiment. Each day, mussels were checked individually to record survivability by performing the British Standard Squeeze method, which classified a mussel as dead if the animal was unable to adduct valves following 10 rapid squeezes (Dunphy et al., 2015). If the mussel shells stayed apart following 10 squeezes, the animal was classified as dead and removed from the tank system and recorded. Next, the sex of the animal was determined by assessing the colour of the gonads (male = white, female = orange). Observations on spawning occurrences and water quality parameters (nitrite, nitrate, ammonia, dissolved oxygen, oxidation reduction potential and pH) were checked before daily water exchanges of 50% volume.

5.2.6 Detection of known virulence genes

5.2.6.1 RNA extractions, cDNA synthesis and quantification

After the bacterial isolates were grown at 22°C until late exponential phase in marine broth, 500 µL of the bacterial samples were pipetted from the culture tube and placed in a 1.5 mL RNase/DNase sterile microcentrifuge tube, flash frozen in liquid nitrogen, and maintained at –80°C until subsequent molecular analyses. Total RNA was isolated from replicate bacterial samples with TRIzol Reagent (Thermo Fisher, USA), in accordance with the manufacturer's instructions. Purified RNA was reconstituted in RNase-free water and any contaminating DNA was removed by treating with Turbo DNase (Ambion, USA) following the manufacturer's recommendations. The extracted total RNA pellet was re-suspended in nuclease-free water (MilliporeSigma, USA) and cleaned using a TURBO DNase Free TM Kit (Invitrogen, New Zealand). The quality and quantity of the supernatant was then determined using a Qubit 2.0 fluorometer (Life Technologies, USA) and Qubit RNA BR Assay Kit (Life technologies, USA). A total of 500 ng of RNA was synthesised into cDNA using OneStep RT-PCR Kit (Qiagen, Germany) and stored at -20 °C for later analysis.

Specific primers for nine target sequences were designed using the Primer 3.0 software (https://bioinfo.ut.ee/primer3-0.4.0/) (Table S1). The *elf* (Adeyinka *et al.*, 2019) and *fur* (Coutard *et al.*, 2007) genes, which are considered to be housekeeping genes, were used as a control in the qPCR. Target sequences were designed based on the consensus of sequences that are deposited in GenBank. Nine target sequences were chosen, all of which are known to be virulence determinants of several pathogenic *Vibrionaceae* bacteria:

- Heat-shock protein 60 (Hsp60) – The *hsp60* gene is produced by many marine bacteria to survive under adverse conditions and cross-protects against other stresses (Wong et al., 2002).This stress protein acts as chaperones in restoring the normal functions of inactivated proteins (Gophna and Ron, 2003).
- Zinc-metalloprotease (*zm*) - The *zm* gene is present in pathogenic strains of family *Vibrionaceae*, but also in non-pathogenic strains. The protein homologue of this gene is a key virulence factor of many *Vibrio* pathogens of fish, shellfish, coral and humans, acting to digest mucin and other connective tissue components, such as collagen IV and fibronectin (Sussman et al., 2009).
- Single-zinc metalloprotease (*vcpA*) – This gene has been identified as a key toxin in the disease process for *V. coralliilyticus* pathogenesis (de O Santos et al., 2011). It might play a regulatory role on protein expression/secretion (chitinase *chi* gene, hemolysin/cytolysin *vthA* gene, aminopeptidases *amp* gene, hemolysin related gene *hcp* gene, rugosity and *b*iofilm structure *m*odulators C *rbmC* gene) (Travers et al., 2015).

- Toxin R (*toxR*) - The *toxR* gene is a virulence regulator in *Vibrionaceae* bacteria, including *V. cholerae*, *V. harveyi*, *V. parahemolyticus* and *Photobacterium profundum* (Zhang et al., 2018). This membrane-localized regulatory protein plays an essential role in modulating bacterial persistence and virulence (Ruwandeepika et al., 2011).
- Outer membrane protein U (OmpU) – The *ompU* gene may act as adhesins to host tissue and mediate resistance to cationic antimicrobial peptides and proteins, which has been linked to virulence phenotype (Duperthuy et al., 2010).
- Chitinase (*chi*) – This gene appears to serve as a virulence factor for many bacterial pathogens, including *V. harveyi* clades and *V. cholerae* during the infection process through sugar-binding properties that promote adhesion to surface of host cells (Frederiksen et al., 2013).
- Mannose-sensitive hemagglutinin pillus adhesins (*mshA*) – This gene is known to mediate attachment of bacterial-host cell adherence and subsequent pathogenesis in many bacteria, including the *V. cholerae* (O'Boyle et al., 2013).
- Lipase (*lip*) – This gene has been reported to have a potentially pathological role in many *Vibrionaceae* bacteria, such as *V. harveyi* and *V. vulnificus*. Lipase may constitute virulence factors by affecting several immune system functions through long-chain unsaturated fatty acids generated by lipolytic activities (Su et al., 2004).
- Palatin-like-Phospholipase (*plp*) - This lipolytic gene is an essential virulence factor of many marine bacteria, such as *Photobacterium demselae* and *V. vulnificus*, which secretes a phospholipase by a type II secretion system. It can disrupt the phospholipid membrane and thus lead to host cell lysis, contributing to systemic infection (Wan et al., 2019a).

5.2.6.2 Quantitative polymerase chain reaction (qPCR)

Quantitative PCR (qPCR) assay was prepared by a slight modification of the method described by Delorme et al. (2020b). The 20 µL reactions were performed using SYBR® Green PCR Master Mix (Applied Biosystems™, USA) and 2 µL of 10 ng/µL of DNA template. A standard curve of amplified PCR product was included in every run along with two non-template controls. All target genes were tested in triplicate along with controls in a LightCycler 480 thermal cycler (Roche, Switzerland), programmed with the SYBR® Green I detection system as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 55°C or 59°C for 1 min. *ELF* and *FUR* were used as an endogenous reference to normalize the expression levels between samples. The relative mRNA expression fold change for all target genes was determined by the delta-delta Ct (2- ddCT) method.

5.2.7 Statistical analyses

Relative expression levels of all nine mRNA genes were transformed using $y = x^{-0.2}$ as described by Delorme et al. (2020b). Data and statistical tests were computed with Statistical Package for Social Science (SPSS) software (v. 17) and XLSTAT software package (Addinsoft, New York, USA). After a significant ANOVA result, significant differences in relative expression levels of all nine mRNA genes between different bacterial isolates were determined using Tukey's HSD (honestly significant difference) as a post hoc test with α = 0.05. All graphs were plotted using Graph Pad Prism Software version 5.01 (Dotmatics, USA). Using MetaboAnalyst v 4.0 software (Chong et al., 2019), a heatmap of virulence gene expressions combined with hierarchical cluster analyses (HCA) (Euclidian distance; Ward's method) was used to visualize differences in relative gene expression abundances and provide dendrograms to assess similarity/dissimilarity among bacterial isolates and among virulence genes expressions. In addition, a heatmap was constructed to illustrate the correlation analyses among expression of virulence genes graphically, using Pearson correlation as the distance metric.

Cumulative mortality data were plotted as scatterplots using Displayr v1.2.10044 software available at www.Displayr.com (Displayr Australia Pty Ltd), and LOESS smoothers (span α = 0.75) with 95% confidence intervals applied. Survival was analysed using a Kaplan-Meier analysis from the binary data (survived or died) of all mussels and treatments via XLSTAT software package. To assess the significant differences (α = 0.05) in survival probability curves among treatments, tests based on Chi-squared distribution (Log rank test) were performed. Chi-squared tests (α = 0.05) were used to test the association between mortality data and potential covariates of sex and spawning. Projection to latent structures-regression (PLS-R) was used to explore utility of the virulence gene data to predict pathogenicity. First, based on the survival data, the bacterial strains were ranked from lowest to highest pathogenicity (scale 1–4). A PLS-R model was constructed with latent X-variables (i.e., the virulence gene expressions) as predictors and pathogenicity as the Y- response (i.e., pathogenicity rank) using the model equation:

$$Y = 4.96299849720592 - 1.67953652052005 * hsp60 + 8.35392542231148E - 02 * zm - 1.04285186469871 * vcpA + 1.38880688465376 * toxR + 1.77912344877999 * ompU + 0.461104422216688 * mshA + 0.130124905491064 * chi - 0.135915416459983 * lip - 3.88849921754515 * plp$$

This model was validated using a 5-fold cross-validation to assess model performance via its R^2 and Q^2 values.

5.3 RESULTS

5.3.1 Phenotyping characterisation

5.3.1.1 Morphology and growth characteristics

Translucent, and creamy grey colonies were observed on TSA 3% salt plates. Based on colonies forming on TCBS agar, isolates were divided into two groups, sucrose fermenters (yellow colonies) and non-sucrose fermenters (green colonies). Colonies showed *Vibrionaceae* colony characteristics, including circular colonies, yellow or green in colour and bacterial colony diameters ranging from 2–5 mm in size. All the isolates were motile. Additionally, the strains were gram-negative with round shapes differing in length. The growth curves for four bacterial isolates, except V1, displayed a diauxic lag phase pattern characterised by two distinct exponential phases (Figure S1). The diauxic lag phase was particularly noted for the three strains tested V2, V3 and V4. V1 showed a monophasic growth pattern.

5.3.1.2 Biochemical profiles

Isolated bacteria (V1-4) showed successful growth on TCBS, TSA 3%, in Brain Heart Infusion broth with 6.5% NaCl and blood agar plates at 22°C. No growth was seen on McConkey agar plates or at 4 or 41°C. The biochemical tests which had any discriminatory results are summarised in Table 2 and Table S2.

5.3.2 MALDI-TOF MS

One isolate (V4) gave a genus identification and a probable species identification, when compared with the MALDI-TOF Biotyper database v 2021. V4 was identified as *Vibrio gigantis* (1.91–2.12 score) with *Vibrio pomeroyi* (1.84–2.00) as the next closest match. For the remaining isolates (V1-V3), the MS measurement resulted in an unreliable identification; with the scores lower than 1.8, being 1.45. The identities of all strains of V1–V4 were confirmed by whole genome sequencing.

5.3.3 Genotyping characterisation

5.3.3.1 Strain identification and phylogeny

Phylogenetic relationships of isolated bacteria were based on selected conserved genes (*recA*, *mreB*, *rpo*, *gyr*, *fur*) of whole genome sequencing data of *Vibrionaceae* (Fig. 5.2).

Isolates corresponded to two main genera of marine bacteria: *Vibrio* (1 isolate) and *Photobacterium* (3 isolates*)*. V4 - *Vibrio celticus* isolated in this study is a species known to belong to the *Splendidus* clade of the genus *Vibrio*. Three isolated strains related to the *Photobacterium* clade were identified as V1 - *Photobacterium swingsii*, V2 - *Photobacterium rosenbergii* and V3 - *Photobacterium proteolyticum*, respectively. There was 99% similarity of the nucleotide sequence identities among the isolates and their associated known strains when compared with the conserved genes with sequence length for *recA* (1047 bp), *mreB* (1044 bp), *rpo* (1047 bp), *gyr* (2418 bp) and *fur* (447 bp).

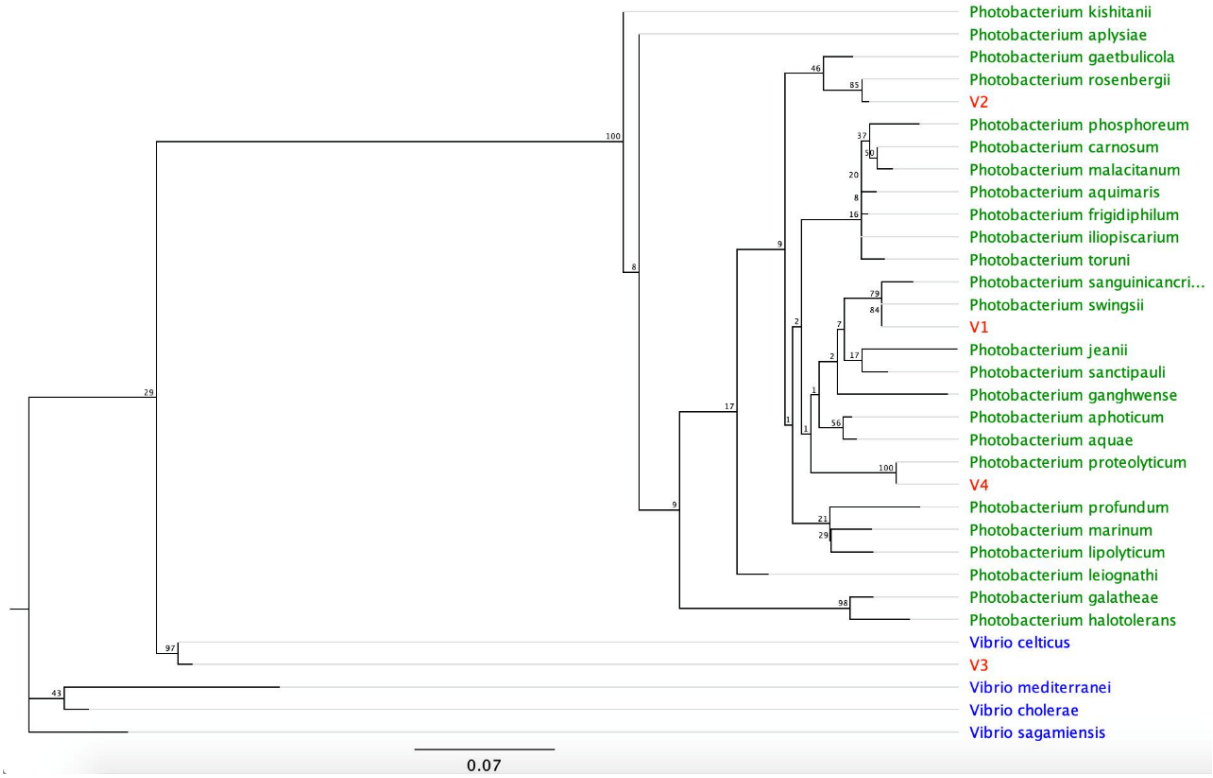

Figure 5.2. Phylogenetic analysis of *Vibrionaceae* bacteria based on selected conserved genes (*recA*, *mreB*, *rpo*, *gyr*, *fur*, *hsp60*). The evolutionary history was inferred using the maximum likelihood method, based on the general time reversible model. The scale bar shows the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown at the branch node.

5.3.4 Laboratory challenge experiments

5.3.4.1 Mussel survival

Mussel survival was monitored for two weeks, and Kaplan-Meier survival curves were plotted from these data (Fig. 5.3). Isolated V1, identified as *Photobacterium swingsii,* resulted in the highest mussel mortality rate with 73.3% in medium infected groups, and 72.5% in highly infected groups at the end of experiment. Equality of the survival curves across treatment levels for each bacterial strain were tested (Table 5.1). Additionally, mortality for mussels

injected with high concentrations of V2 *(Photobacterium rosenbergii*) and V3 (*Photobacterium proteolyticum)* showed statistical differences from the injected-challenge control (Table 5.1). Mussels injected with V4 (*Vibrio celtius)* showed the highest mussel survival rate (i.e., 74.7%), indicating that V4 was avirulent, having the smallest effect on mussel survival.

Table 5.1 Summary of survival analysis: mean fold change, fold change percentage and p-values via log-rank test of equality across four treatments. p-value < 0.05, highlighted in bold, indicate there was a statistically significant difference between survival curves, which means mortality in these treatments are higher than the injected-challenge control. V4 not displayed as all comparisons were non-significant.

		Injected-Challenge Control vs Treatment		
Strain	**Doses**	**Fold change (FC)**	**FC%**	**p-value**
V1 (*Photobacterium swingsii*)	L	0.60	60	1.000
	M	1.45	145.16	<0.001
	H	1.12	111.85	<0.001
V2 *(Photobacterium rosenbergii*),	L	1.65	165.16	0.501
	M	0.26	25.78	0.092
	H	7	700	0.021
V3 (*Photobacterium proteolyticum)*	L	1.72	172.26	0.245
	M	0.36	36.33	0.153
	H	4.77	477.29	0.021

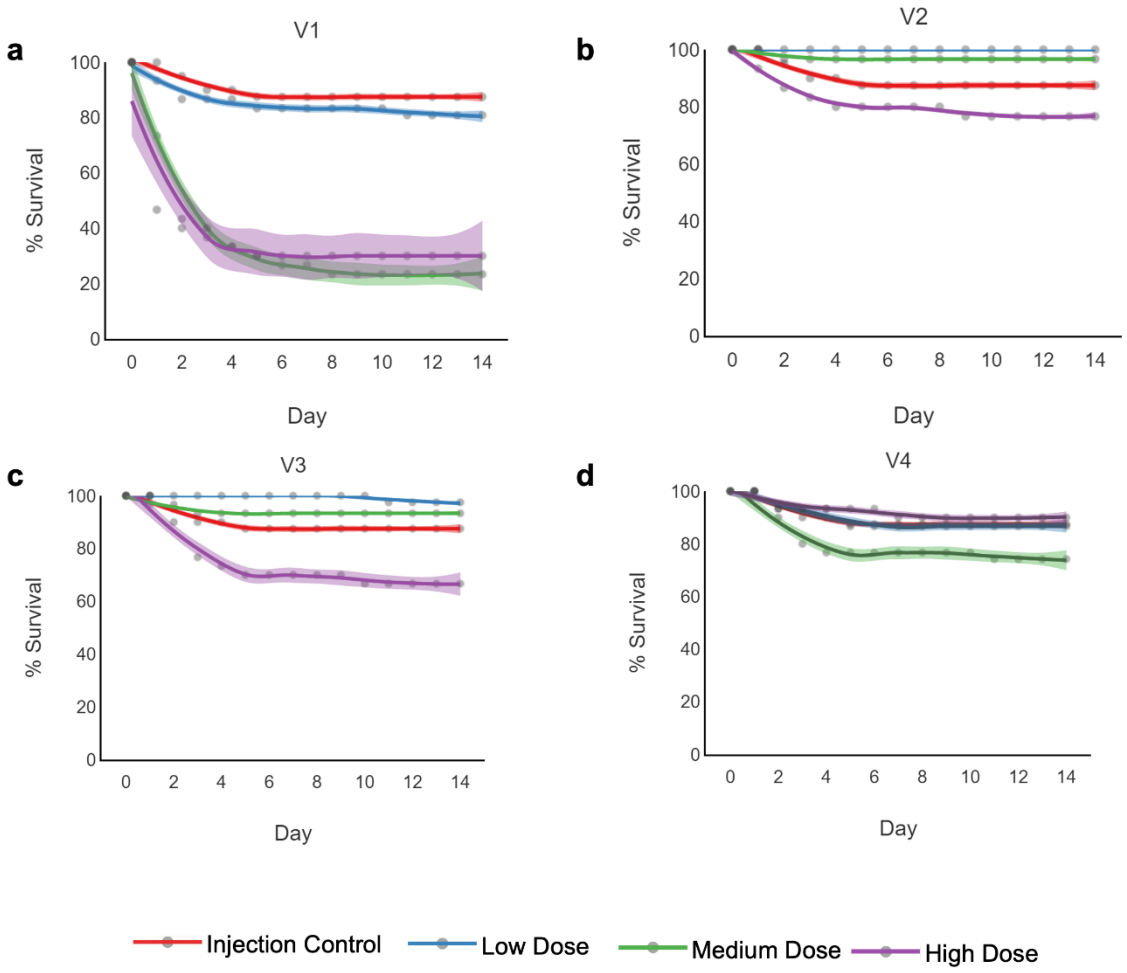

Figure 5.3. Survival plots of *P. canaliculus* after injection with V1-4 (corresponding to subfigures A-D). *Photobacterium swingsii* at high (purple), medium (green) and low (blue) doses along with the injected-challenge control (red) doses, with 33 tanks/replicates containing 1 animal per tank per treatment group (refer to online version for colour interpretation).

At the end of the experiment, the relationship between the dosage and mortality was not linear across the treatment groups with four bacterial species. Additionally, a Chi-square test showed that spawning and the probability of mortality after infection across all strains were likely to be dependant. However, susceptibility to the bacterial injections did not appear to be associated with mussel sex (Table S3).

5.3.5 Detection of virulence genes

Genes known to be virulence determinants in the most frequently reported species of *Vibrio* (e.g., *V. cholerae*, *V. harveyi*, *V. coralliilyticus*, *V. vulnifucus* and *V. splendidus*) were detected in all the isolates used in this study. These nine virulence genes were expressed in all isolates at different expression levels (Fig. 5.4), with low to relatively low intra-strain variation (Table

S5). The *hsp60* gene encoding heat shock protein was found predominantly in in isolates V2 and V1 and was expressed in all isolates at different levels, with a 63-fold difference in expression levels between the isolate showing the highest expression (V2) and the one showing the lowest expression (V4) (Fig. 5.4). Additional genes which were amplified in four isolates were *zm* and *vcpA*. V2 showed the highest expression of *zm* gene compared with the other isolates and showed a significant difference, with 2.3-fold difference between the highest (V2) and the lowest (V3) values. The highest expression level of *vcpA* gene was observed in V2 followed by V3 and V1, and this gene showed significant difference between the highest and the lowest value in different isolates (Fig. 5.4).

Highest expression of *toxR* gene was found in V2, with a significant ($p < 0.05$) 2.2-fold difference between the highest and the lowest values (Fig. 5.4). *ompU* gene expression showed the highest values in V3 with highly significant difference ($p < 0.001$) when compared with the isolate V4. There was 2.7-fold difference in expression levels between the isolate showing the highest expression and the one showing the lowest. The other lytic enzymes known as Chitinase (*chi*) produced by *Vibrio* spp. and belonging to the *Harveyi* clade was chosen. Expression of *chi* gene was substantially different ($p < 0.001$) between V2 and V4 isolates. Expressions of *mshA* gene were similar ($p > 0.05$) across isolates (Fig. 5.4). Isolate V2 showed the highest expression of the *lip* gene, whereas isolate V4 had the lowest expression (Fig. 5.4). When comparing both V2 and V4, the expression level of the *lip* gene showed in Fig. 5.4 was statistically significant ($p < 0.05$). In this study, the *plp* gene expression showed a lower variation in the different isolates (Table S5), although the difference in expression was highly significant ($p < 0.0001$) between the isolates V2 and V4, as well as between isolates V1 and V4.

The results in Fig. 5.4j demonstrate that HCA heatmap classified each of the four groups of bacterial strains into three distinct clusters. V1, and V3 combine to form their own cluster while V2 and V4 form their own separate cluster. V2 displayed higher expression of the corresponding genes while V4 showed relatively low expression of these genes. Correlation analysis revealed that the expression of the *hsp60* gene was significantly positively correlated ($r = 0.626$; $p = 0.0004$) with the expression of the *vcpA* metalloprotease gene, virulence regulator gene *toxR* ($r = 0.702$; $p = 0.01$), *ompU* ($r = 0.557$; $p = 0.02$), and the *plp* ($r = 0.855$; $p = 1.15E-05$) genes, but was not significantly correlated with the expression of the *zm, chi*, *mshA* and *lip* genes (Fig. 5.4k). The expression of the lipase gene showed a highly significant correlation ($r = 0.890$; $p = 0.0001$) with the expression of *chi* gene. Finally, *vcpA* metalloprotease gene expression showed a significant correlation (*ompU*: $r = 0.878$, $p = 0.0001$; *plp*: $r = 0.820$, $p = 0.001$) with the expression of the *ompU* and *plp* genes.

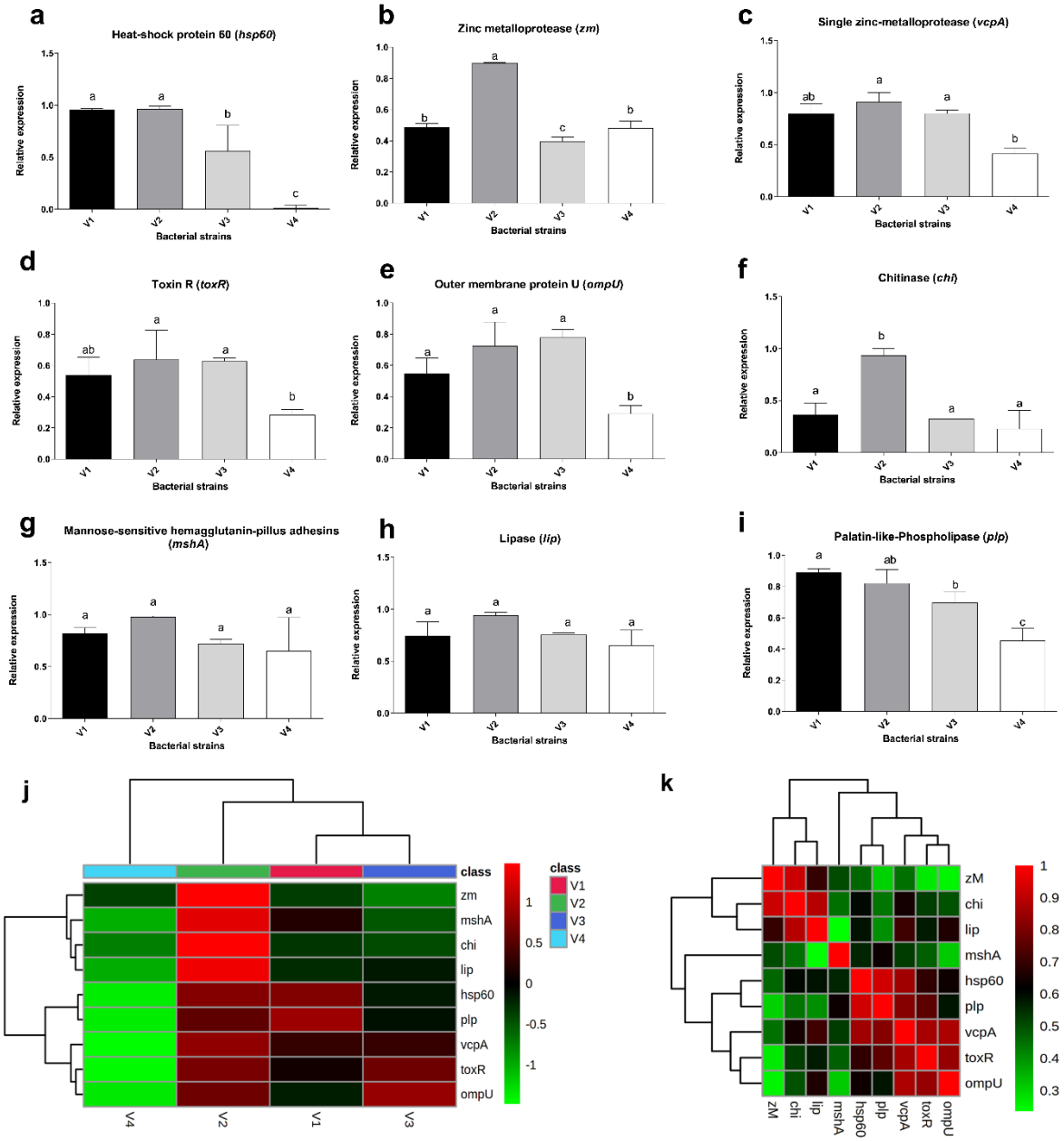

Figure 5.4. (**a**–**i)** Comparative analysis of the nine virulence-associated genes in four bacterial isolates using qPCR. The gene is represented as relative gene expression**.** Bars represent the means and standard errors of three biological replicates. (**j**) Hierarchical cluster analysis heatmap (HCA heatmap) of the bacterial strains based on their virulence gene expression based on one-way ANOVA (rows/columns = genes/isolates, red–green colour scale = relatively high-to-low relative gene expressions). The red colour indicates highly expressed genes, while the green colour indicates low expressed genes. **(k)** A correlation heatmap with combined hierarchal cluster analyses of the virulence genes based on Pearson correlation (rows/columns = genes, red–green colour scale = relatively high-to-low relative gene expressions).

In order to estimate which of the virulence gene expressions are strong predictors of bacterial pathogenicity, PLS-R was performed (Fig. 5.5). The ability of the PLS-R model (Fig. 5.5a) to predict pathogen rank was good (5-fold CV; two component model; R^2 = 0.86; Q^2 = 0.50)

Three features comprising *hsp60*, *plp* and *vcpA* genes contributed most strongly (VIP > 1.0) towards predictivity of the model (Figure 5.5b).

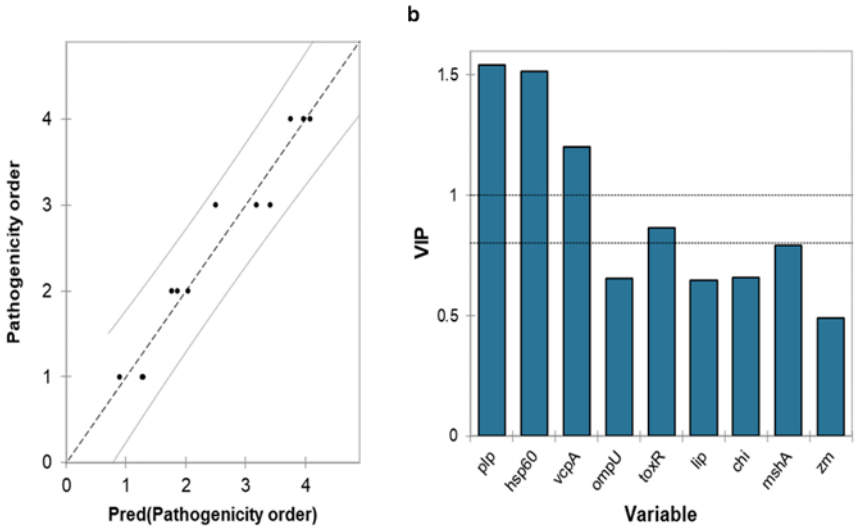

Figure 5.5. PLS Regression models for predicting pathogenicity performance based on virulence gene expressions. a) Predicted vs observed pathogenicity with trend line b) Variable Importance in the Projection (VIP) based on PLS-R model.

5.4 DISCUSSION

Aligning with current research focusing on bacterial implications in aquatic disease outbreaks during summer months, these results revealed four bacterial isolates recovered from moribund mussels belonged to the family *Vibrionaceae*. These bacterial isolates had the characteristics of the genus *Vibrio* and *Photobacterium*, matching the morphology profile of gram-negative, straight or comma-shaped rod bacteria, with polar flagella, as previously described in a wide range of diseased and healthy aquatic animals (Buller, 2004a, Dworkin et al., 2006, Sawabe et al., 2013, Romalde et al., 2014). Application of phenotypic, MALDI-TOF, and whole-genome sequencing in the present study allowed identification of these four bacteria isolates as V1: *Photobacterium swingsii*; V2: *Photobacterium rosenbergii;* V3: *Photobacterium proteolyticum;* and V4: *Vibrio celticus*. *Vibrio* and *Photobacterium* species have been well described in the literature, including basic biology (Boyd, 2007, Thompson et al., 2009, Moi et al., 2017). taxonomical classifications (Thompson et al., 2009, Labella et al., 2017), animal diseases (Prayitno and Latchford, 1995, Abdel-Aziz et al., 2013, Le Roux et al., 2015), ecology and distributions (Thompson et al., 2005a, Burtseva et al., 2020) the microbiome (Rubiolo et al., 2019, Wei et al., 2019), physiology and metabolism (Proctor and Gunsalus, 2000, Buijs et al., 2020) and their repertoires of virulence factors (Johnson, 2013, Moi et al., 2017). More specifically, the involvement of *Vibrio* bacteria belonging to the *Harveyi* clade (King et al., 2018), *Splendidus* clade (Kesarcodi-Watson et al., 2009b), *V. mediterranei* (Andree et al.,

2021, Prado et al., 2020b) and *V. coralliilyticus* (Kesarcodi-Watson et al., 2009a) have been reported in association with mussel mortality. Reports of mussel mortalities associated with *Photobacterium* species are scarce (Eggermont et al., 2017), but equally important when considering biosecurity threats and management strategies. Acquisition of baseline data on bacteria isolated from wild mussel populations following a mortality event, is the first step towards establishing disease control measures and monitoring initiatives for this commercially important bivalve.

One of the bacterial isolates tested (V1: *Photobacterium swingsii*) caused *P. canaliculus* mortalities of 73.3% in medium infected groups, and 72.5% in highly infected groups (by the end of the challenge). None of the mussels died in the non-injected control. However, mortalities that occurred in injected-challenge control (5 from 30 mussels) within 2-5 days post-infection, may be attributed to the procedure of injection in the adductor muscle, which caused disruption of tissues and variability (Gay et al., 2004). Ultimately, higher mortalities were recorded for bacteria-injected mussels than injected-control mussels. In addition to V1, mortalities were also recorded in *P. canaliculus* following infection with high doses of V2 *(Photobacterium rosenbergii*) and V3 (*Photobacterium proteolyticum)*. These bacterial strains have been previously linked to vibriosis in oysters (Gomez-Gil et al., 2011), octopus' lesions (Fichi et al., 2015) and coral bleaching (Thompson et al., 2005b) supporting pathogenicity of these bacteria as seen in the current study. *P. swingsii* was first characterised from diseased Pacific oyster (*Crassostrea gigas*) and wild spider crab (*Maja brachydactyla*) isolates (Gomez-Gil et al., 2011) and has since been reported in blue mussels (Eggermont et al., 2017), abalone (Jiang et al., 2013) and octopus (Fichi et al., 2015) .

This is the first known study to date that has identified pathogenic outcomes for *P. canaliculus* infected with *P. swingsii*. The high mortality rates of mussels exposed to *P. swingsii* in our challenge-experiment suggests that it is likely to be a causal factor in mortality. The present study indicates that in all isolates, mortality rates further increased within the 14-days observation period after exposure to the higher pathogen dose (compared to the minimum infection dose), although the high dose applied (10^9 CFU/ml) may not reflect natural infection. Prior to the challenge experiment, only a few living bacteria, including *Vibrio* colonies, were found in the haemolymph of unchallenged mussels (at a dilution of 10^0). In the current study mussel, mortality due to the presence of other pathogens, can be excluded as only the studied strains were identified in mussel haemolymph samples analysed by Sanger sequencing post-challenge (Table S4). In the present trial, no prophylactic measure was employed to depurate *P. canaliculus* prior to challenge experiment, since antibiotics were not guaranteed to eradicate *Vibrio* species (Andree et al., 2021, Prado et al., 2020b) and its applicability for the

in vivo experiments can also compromise the health of the target organism (Marques et al., 2006). Our study has also demonstrated that mortality was significantly higher in mussels which had spawned following stimulation by bacterial injection (see Supplementary material in excel file), indicating that they were more susceptible to mortality caused by bacterial invasion. After stimulation by bacterial injection, mussels produced a range of effectors, such as antimicrobial peptides and bacteriolytic enzymes, which are involved in the mussel immune defence (Gerdol and Venier, 2015). However, the ability to recover immune function after simulated bacterial challenge can be impacted by the physiological stress of spawning (Li et al., 2012). This suggests that after exhausting the basal levels of antimicrobial effectors to bacterial injection, spawned mussels have insufficient metabolic resources to initiate the expression of new proteins and peptides to continue fighting infection, which led to mortality (Li et al., 2012). Moreover, *P. canaliculus* used in the present trial were not fed during the infection trial, and this may have contributed to their susceptibility towards bacterial infection. Future efforts on animal models and infection protocols are certainly needed to clearly identify the pathogens of molluscs and their effects.

Our study showed that all nine virulence genes examined, *hsp60*, *zm*, *vcpA*, *toxR*, *ompU*, *mshA*, *chi*, *lip,* and *plp* were expressed in all bacterial isolates investigated. These virulence genes were associated with various functions (i.e., attachment, colonisation, biofilm formation, cell lysis, and defence against host-produced antimicrobial peptides), all contributing to potential pathogen function in this host (Johnson, 2013). Niu et al. (2013) indicated that according to the ecological and evolutionary view of bacterial pathogenomics, pathogenic, symbiotic, and commensal bacteria often share their habitats with bacteriophages and each other, which promote these microorganisms to adopt similar strategies and molecular systems to interact with their hosts. Although our data showed V2 (*P. rosenbergii*) exhibited maximum expression levels (i.e., seven of the tested virulence genes), the presence of a large number of virulence genes does not guarantee production of active virulence enzymes/proteins involved in pathogenicity (Li et al., 2011). The mRNA transcript levels may be upregulated in V2 (*P. rosenbergii*), but a bank of already present virulence genes in V1 (*P. swingsii*) might actually be activated upon contact with the host and lead to programmed cell death, which supports the scenario of pathogenicity of this bacterium in the mussel challenge test, as well as its potential contribution in summer mortalities Our results indicate that using robust PLS-regression models to predict pathogenicity potential just from the virulence gene expression of bacteria can be effective (Fig. 5). Three virulence genes, *hsp60*, *plp* and *vcpA* showed high levels of expression in bacterium with high pathogenicity rank, indicating these virulence genes might be important for bacterial pathogenesis. The Hsp60, also known as GroEL, is described as the most abundant protein synthesised by most pathogenic bacteria and is

involved in the pathogenesis of several infectious disease with increasing evidence that this stress protein is involved in host-cell adhesion and invasion (Garduño et al., 1998, Hennequin et al., 2001, Oliver et al., 2020) as well as in modulating the host immune response (Oliver et al., 2020). Furthermore, these results suggest that the *plp* gene encoding palatin-like phospholipase contributes to the pathogenicity of V1 (*P. swingsii*) via its invasive activity. This agrees with other research indicating that *Photobacterium* strains can produce phospholipase which is a thermostable extracellular cytotoxin that can degrade fish erythrocytes (Moi et al., 2017). The metalloprotease *vcpA* gene is known to have a role in the virulence of *Vibrio coralliilyticus, V. cholerae, V. splendidus, and V. tubiashii* towards corals and molluscs (Sussman et al., 2009, Munn, 2015). As seen in numerous analyses, it was shown to facilitate bacterial invasion and the infection (Galvis et al., 2021). The virulence of these isolated bacteria is unlikely to be exclusively attributed to the nine selected genes that we assessed, and additional virulence genes/factors (even critical virulence genes from other species) probably also contribute to their pathogenicity.

Following from this study, there are more areas of research that can be explored to provide further understanding and management of pathogenic bacteria which potentially cause summer mortality. The use of alternative bacterial growth media can help to detect other species of bacteria not investigated in this study. From the TCBS agar medium used in this study, it was determined that V1 (*P. swingsii*) can cause higher mortalities in our mussel challenge experiment, future studies via the use of 16S rRNA gene sequences and a designated genus specific quantitative polymerase chain reaction (qPCR) are needed to verify its prevalence and abundance in summer mortality samples across mussel populations. Generally, in nature, hosts are usually infected by several bacterial strains with different pathogenic genotypes, which often leads to increased virulence (Van Baalen and Sabelis, 1995, Susi et al., 2015, Andree et al., 2021). Future work will need to incorporate coinfection trials, to determine this potential synergistic effect among the bacterial isolates investigated. Previous observations have shown that the pathogenicity of bacteria from *Vibrio-Photobacterium* sp. is multifactorial and complex and may involve the products of several different genes acting either alone or in concert (Croci et al., 2001, Ruwandeepika et al., 2011). We hypothesise that pathogenicity of the tested isolates is likely due to synergistic effects conferred by the coexistence of several virulence genes, requiring further investigation on the interconnections between regulatory mechanisms that affect virulence gene expressions. Despite the usefulness of the virulence gene expression derived from the *in vitro* grown bacteria investigated so far, further work will be needed to look at virulence factor production *in vivo* during bacterial infections within the intact host to provide a more complete picture of bacterial pathogenesis. Finally, further investigation will be needed to elucidate the complex

relationships among water temperatures, thermal stress effects on host physiology and immunity, regulation of virulence in bacterial pathogens, and their roles in mussel summer mortality events (Lam et al., 2014).

5.5 CONCLUSIONS

Our findings reveal that bacteria isolated during a summer mortality event in *P. canaliculus* are pathogenic towards mussels and have potential to cause significant deleterious effects. By means of a laboratory infection challenge and profiling of virulence gene expressions, we conclude that *Photobacterium* species, particularly *P. swingsii* could be important pathogens and emergent disease in Greenshell™ mussels during periods of environmental stress. However, to create a definite link to summer mortalities, prevalence alone is not the only indicator of the role this bacterium plays in mussel health. Additional studies are needed to ascertain if there are consistent bacteria in both healthy and diseased populations at the mortality hotspots which would also enable collection of intrinsic farm factors (e.g., frequency of disease between and within farms) and management factors. Other intrinsic and extrinsic factors, including physiological and/or genetic state of the host, environmental influences, and the presence of various opportunistic infectious agents contributing to disease risk and mortality must be identified and quantified. *In vivo* laboratory experiments, extensive field data and novel mathematical models that link gradients of different abiotic factors and different bacterial infection intensity to identify a threshold of disease resistance in mussels must be developed to infer the cause of host mortality. As summer mortalities of *P. canaliculus* are being experienced on commercial farms, ongoing research focusing on temporospatial *Photobacterium* ecology and methods to assess the pathogenic potential of a strain are pivotal in areas where Greenshell™ mussel aquaculture is prominent. This information is essential to understand how bacterial pathogens cause episodes of mortality during the warmer summer months and to predict their impacts on future marine-based food production and security.

Chapter 6: Quantification of *Photobacterium swingsii* and characterisation of disease progression in the New Zealand Greenshell™ mussel, *Perna canaliculus*

"The ocean is a silent teacher, whispering its lessons to those who are willing to listen. It holds the secrets of life, evolution, and the interconnectedness of all living things."
- Rachel Carson

Since the potential pathogen was identified for the first time from moribund mussels in the previous chapter, this chapter aims to evaluate the association between *Photobacterium* and Greenshell™ mussels over time through laboratory-challenged mussels.

This chapter has been submitted for publication:

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Abstract

Greenshell™ mussels (*Perna canaliculus*) are endemic to New Zealand and support the largest aquaculture industry in the country. *Photobacterium swingsii* was isolated and identified from moribund Greenshell™ mussels following a mass mortality event. In this study, a challenge experiment was used to characterise, detect, and quantify *P. swingsii* in adult *P. canaliculus* following pathogen exposure via injection into the adductor muscle. Mock treatments were included to control for the effects of the injection. Survival of control and infected mussels were monitored. Sampling of haemolymph, for bacterial colony counts and haemocyte flow cytometry analyses; histology sections for histopathological assessments and tissues (adductor muscle, gill, digestive gland) for quantitative polymerase chain reaction (PCR) analyses, were conducted at 12, 24, 48 hours post-challenge (hpc). The most profound effects of bacterial injection on mussels were seen at 48 hpc where mussel mortality, haemocyte counts and haemolymph colony forming were the highest. The quantification of *P. swingsii* via targeted PCR showed highest levels of bacterial DNA at 12 hpc in the adductor muscle, gill, and digestive gland. Histopathological observations suggested a non-specific inflammatory response in all mussels associated with a general stress response. This study highlights the physiological effects of *P. swingsii* infection in Greenshell™ mussels and provides histopathological insight into the tissue injury caused by the action of injection into the adductor muscle. The multi-technique methods used in this study can be applied for use in early surveillance programs of bacterial infection on mussel farms.

Keywords

Bacterial quantification; Greenshell™ mussels; Flow cytometry; Haemocytes; Histopathology; *Perna canaliculus*; *Photobacterium*; Pathogenesis; qPCR.

6.1 INTRODUCTION

Greenshell™ mussels are the most important aquaculture species in New Zealand, typically sold as a variety of food products worldwide (Miller et al., 2023). Aquaculture development and increasing disease occurrences, have prompted interest in studies on aquatic diseases which are likely to keep growing in the face of future threats from climate change, invasive species and pollution (Lane et al., 2022). Even though the Greenshell™ mussel industry has experienced relatively few disease issues, the presence of pathogens and parasites continue to be reported (Castinel et al., 2019). *Vibrio* spp. such as *Vibrio splendidus*, a *Vibrio coralliilyticus/neptunis*-like isolate (Kesarcodi-Watson et al., 2009a), *Vibrio mediterranei*

(Azizan et al., 2023c) and *Photobacterium swingsii* (Azizan et al., 2022) have been largely found in aquatic environments housing Greenshell™ mussels.

Photobacterium is one of the eight genera in the Family Vibrionaceae (order "Vibrionales", class Gammaproteobacteria) and is the largest genus after *Vibrio* (Labella et al., 2017). Several species in this genus, including *P. rosenbergii, P. swingsii, P. jeanii, P. sanctipauli,, and P. damselae,* have been shown to cause pathologies in animal hosts, as reviewed by Labella et al. (2017). Of particular interest for the current research are *P. swingsii* infections that have previously been detected in diseased Pacific oysters (*Crassostrea gigas*), wild spider crabs (*Maja brachydactyla*) (Gomez-Gil et al., 2011), blue mussels (Eggermont et al., 2017), abalone (Jiang et al., 2013) and octopus (Fichi et al., 2015). Additionally, mussels (*P. canaliculus*) injected with *P. swingsii* showed high mortality, along with expression of virulence genes (*hsp60*, *zm*, *vcpA*, *toxR*, *ompU*, *mshA*, *chi*, *lip*, and *plp*), suggesting pathogenesis of this bacterium to Greenshell™ mussels (Azizan et al., 2022). Despite advances that have been made, the mechanisms of bacterial pathogenesis of *P. swingsii* in Greenshell™ mussels are poorly understood.

Monitoring programs use a variety of diagnostic techniques aims to assess the condition and health status of farmed and wild mussel populations (Webb and Duncan, 2019). The most used methods for disease testing include bacterial culture, PCR, enzyme-linked immunosorbent assay (ELISA), histopathology, fluorescent *in situ* hybridisation (FISH) and immunohistochemistry, all with advantages and disadvantages (Lane et al 2022). Histology can be used in pathogen studies to help characterise the route of colonisation of organisms as it may allow for the evaluation of the presence, distribution and impact of an organism at defined time points in the host (Wang et al., 2021a). The use of qPCR allows for discrimination of congeneric pathogens (Piesz et al., 2022, Ríos-Castro et al., 2022), and is useful to monitor the progress of an infection (Mackay et al., 2002). By monitoring pathogen infections in a host over an extended period, researchers can better understand the pathogenesis of the bacterial infection and develop effective strategies for mitigating its impact (Burge et al., 2016a, DeCandia et al., 2018). For example, in the larvae of *M. edulis*, various *Vibrio* spp. and *Photobacterium* spp. isolates caused mortality after three days of infection, and genomic analyses identified virulence-related genes in the bacterial isolates responsible for the highest mortalities (Eggermont et al., 2017). In *P. canaliculus*, the effect of co-infection (using *P. swingsii* and *Vibrio mediterranei*) displayed varying responses overtime, with 100% mortality observed in juvenile mussels after 48h, while adults showed the same results after 72h (Azizan et al., 2023c). The effect of sampling time was clearly seen in a study on *M. galloprovincialis* challenged with *V. splendidus*. Herein , two peaks of bacterial colonies were detected at 1h

and 6h post challenge, demonstrating the recruitment of haemocytes and implementation of phagocytosis overtime (Parisi et al., 2019). Ultimately, strategies implemented by bacterial pathogens to avoid host defence mechanisms vary amongst species and can be complicated by different phases of infection (i.e., host-pathogen interactions or host immune response) (Labreuche et al., 2006).

The overall aim of this study was to evaluate the association between *Photobacterium* and Greenshell™ mussels' overtime. *P. swingsii* DNA load and distribution in digestive gland, adductor muscle and gill tissues were evaluated over a 48-hour period post-challenge using qPCR and histopathology assessments. The mussel immune response was evaluated by haemolymph response (i.e., total haemocyte count and haemocyte viability) over the same period. We envision that these data will aid further studies into the pathological significance of *P. swingsii* and potential disease control alternatives.

6.2 MATERIALS AND METHODS

6.2.1 Animal husbandry, bacterial exposure, and sampling

Adult *Perna canaliculus* (n=300) were obtained from Kaiaua Marine Farms Ltd. (Firth of Thames, New Zealand) and transported to the aquaculture facility at the Auckland University of Technology, Auckland, New Zealand. Upon arrival, animals were labelled, weighed (mean ± SE, 53.8 ± 7.9 g) and measured along the length (90.5 ± 5.5 mm) and then housed for a 7-day acclimation period in a recirculating seawater system (16°C; 35 ppt; pH 8.2).

Photobacterium swingsii was isolated from Greenshell™ mussels during a 2018 mortality event (Nguyen and Alfaro, 2020b), and were grown to a pure culture on thiosulfate-citrate-bile salts sucrose (TCBS) agar plates, then cultured in marine broth (Difco™) and incubated at 22°C for 24h, as previously described by Azizan et al. (2022). For the present study, the bacterium was harvested, washed, and suspended in marine broth (Difco™) medium at a concentration of 10^7 colony forming units (CFU)/mL for injection treatments. To control for the effects of the handling and the act of injection, a second group of *P. swingsii* served as a heat-killed control group, where the inoculum of 10^7 CFU/mL was boiled at 100°C for 10 min, before storage at 4°C for 6 days. Inactivation of the bacteria was confirmed by culturing broth on TCBS agar plates for 6 days at 22°C (Ciacci et al., 2009). No growth was observed over that period. Additional experimental groups included a group of untreated mussels (no injection control) and a group of mussels injected with marine broth (injection control).

A total of 75 mussels were allocated to each group (Fig. 6.1). At the onset of the experiment (time 0), 100 µL of either marine broth (G2), heat-killed *P. swingsii* suspended in marine broth (G3), or live *P. swingsii* suspended in marine broth (G4) (10^7 CFU/mL) were injected into the posterior adductor muscle of each individual mussel using a 25-gauge 1 mL syringe. After injection, all mussels including the no injection controls (G1) were placed into individual 2-L tanks with seawater and air supply. Mussel survival was monitored for 4 days, using the British Standard Squeeze method which classified a mussel as dead if the animal was unable to adduct valves following 10 rapid squeezes (Dunphy et al., 2015). Dead mussels were removed from the system and sexed accordingly. Daily water exchanges (50% of total volume) were performed along with water quality assessment of four saltwater parameters, namely, pH, ammonia, nitrite, and nitrate using a Marine Saltwater Master Test Kit (API Marine) (Azizan et al., 2022). The survival rate was calculated as the survival probability at any particular time (S*t*) (Goel et al., 2010), determined by the equation:

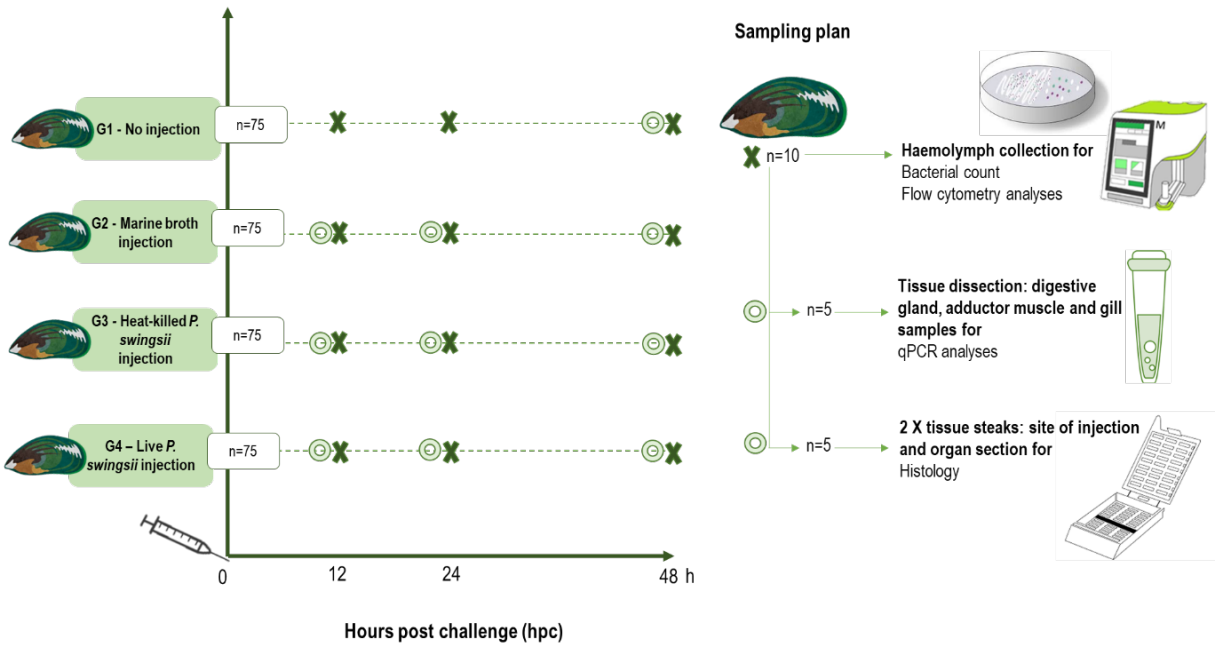


Figure 6.1. Experimental setup: Greenshell™ mussels were assigned four groups, including G1 = no injection control, G2 = control injected with marine broth, G3 = control injected with heat-killed bacteria, and G4 = treatment injected with live bacteria. Mussels were sampled after 12-, 24- and 48-hours post-challenge. At each sampling point, haemolymph was collected for flow cytometry and bacterial quantification and tissue samples for qPCR and sections for histological assessments (note, tissue samples for no injection control group were only collected at 48 hpc). Abbreviations: X = a total of 10 mussels per group were sampled, except for the no injection control, which was only tissue sampled at the end (48 hpc); O = a total of 5 individual mussels were used for histological sample collection, while a second set of 5 mussels were used for tissue collection for qPCR testing.

After 12 hpc (time 1), 24 hpc (time 2) and 48 hpc (time 3), a total of 10 mussels per group were sampled (apart from the no injection control which was only tissue sampled at the end –

48h). Mussels were opened to collect a haemolymph sample from the adductor muscle (Ericson et al., 2023c). For bacterial plating, a 10 µL aliquot of pure haemolymph was spread onto a TCBS agar plate using the spread plate technique (Demann and Wegner, 2019). All plates were incubated at 22°C for 24 – 48h before being counted to obtain CFU/mL of Vibrionaceae load in the haemolymph (Azizan et al., 2022). Following haemolymph collection, 5 individual mussels were used for histological sample collection, while a second set of 5 mussels were used for tissue collection for qPCR testing.

For flow cytometric analyses, a working haemolymph stock was first prepared with 50 µL of haemolymph diluted equally with the same volume of autoclaved filtered seawater. A total of 20 µL of the working haemolymph stock was added to 380 µL of Muse® Count & Viability Kit (200X, MCH100104; Luminex). The sample was vortexed, incubated at 18°C for 5 min before analysis using a Muse Cell Analyzer (Ericson et al., 2022).

For histological assessments, mussels (n=50) were shucked and placed in 4% formalin in seawater for 48 hours, whereafter the animals were cut into 2 histology sections, the first containing the adductor muscle (point of injection) tissue and the second section containing all the major organs (gill, mantle, digestive gland, gonad, connective tissue, and foot). Once cut and placed in histology cassettes, standard histological processing followed (Muznebin et al., 2022b).

For qPCR testing, mussels from each treatment were shucked and the digestive gland, adductor muscle and gill tissues were removed. Tissue samples of about 3x3 mm in size were placed into separate microcentrifuge tubes containing 200 µL RNAlater® and kept in the fridge for an hour to permeate the solution into tissue before storing in a -80°C freezer until qPCR analyses.

6.2.2 TaqMan qPCR for *Photobacterium swingsii*

DNA extraction

Tissues (adductor muscle, gill, digestive gland) from 50 mussels were extracted using the simple workflow of the MagMAX™ Core Nucleic Acid Purification Kit (Applied Biosystems™) executed on an automated KingFisher™ Flex system. Prior to extraction, tissues were homogenised in phosphate buffered saline (PBS) using 1.4 mm diameter ceramic beads in a MagNA Lyser (Roche Diagnostics, Basel, Switzerland), 6500 rpm, 30 s. No injection control, marine broth injection control and bacteria-infected mussel (killed *P. swingsii* and live *P. swingsii*) samples were then tested with a PCR specific to the *P. swingsii* 16S rRNA gene.

qPCR design and procedure

Primers and hybridising probe were designed using the Geneious Prime software (Dotmatics) to target the *P. swingsii* 16S rRNA genes. Geneious Prime and Primer BLAST were used to assess *in-silico* the specificity of the assay. PCR mix comprised of 12.5 µL SsoAdvanced™ Universal Probes Supermix (Bio-Rad), 8 µL nuclease free water, 1 µL (400 nM) of each primer, *P. swingsii* – 229F- 5'GGTCCATTTTCTGGCATGGC3' and *P. swingsii* – 324R- 5'TTGTGGCGACGATACACCAA3'; 0.5 µL of the probe (200nM)**,** *P. swingsii*-282P FAM-AGTTGACGGCGTACTCCTTG-BHQ1; and 2 µL of DNA template. The reaction was run on a CFX96 (Bio-Rad) thermocycler with the following conditions: 1 cycle of 95°C for 3', followed by 40 cycles of 95°C for 30'' and 60°C for 30''. Results were analysed using CFX Manager™ software.

Each qPCR included a set of standards prepared using *P. swingsii* genomic DNA (dilutions from known CFU concentrations) and no template controls. A PCR run was considered valid when the standards were detected, and the no template controls were not detected. All samples from valid runs showing a *Cq* before the 40 cycles cut-off were considered positive. Quantification of *P. swingsii* in samples was calculated by the software based on the standard curve.

6.2.3 Statistical analysis

A two-way ANOVA followed by a Bonferroni post-hoc test was used to analyse experimental data (bacterial load, total haemocyte counts, cell viability and qPCR), considering the effect of time and treatment. PCR quantitative data was log-transformed to deal with the wide range of results. For the log transformation, the lowest copy number result for any sample was added to all results to remove the impossibility of transformation for values = 0. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed in GraphPad Prism® version 9 (San Diego, CA).

6.3 RESULTS

6.3.1 Mussel survival

During the 72-hour monitoring period, mussel survival remained 100% for the no injection control group (G1), the marine broth injection group (G2), and the heat-killed bacterial injection group (G3). Within the group injected with live *P. swingsii* (G4), mortalities started to occur 24 hpc, with a 63.5% survival rate seen at the end of three days. Statistically, G4 differed from

the other experimental groups (log-rank test, $p < 0.001$; Fig. 6.2, as denoted by lowercase letters).

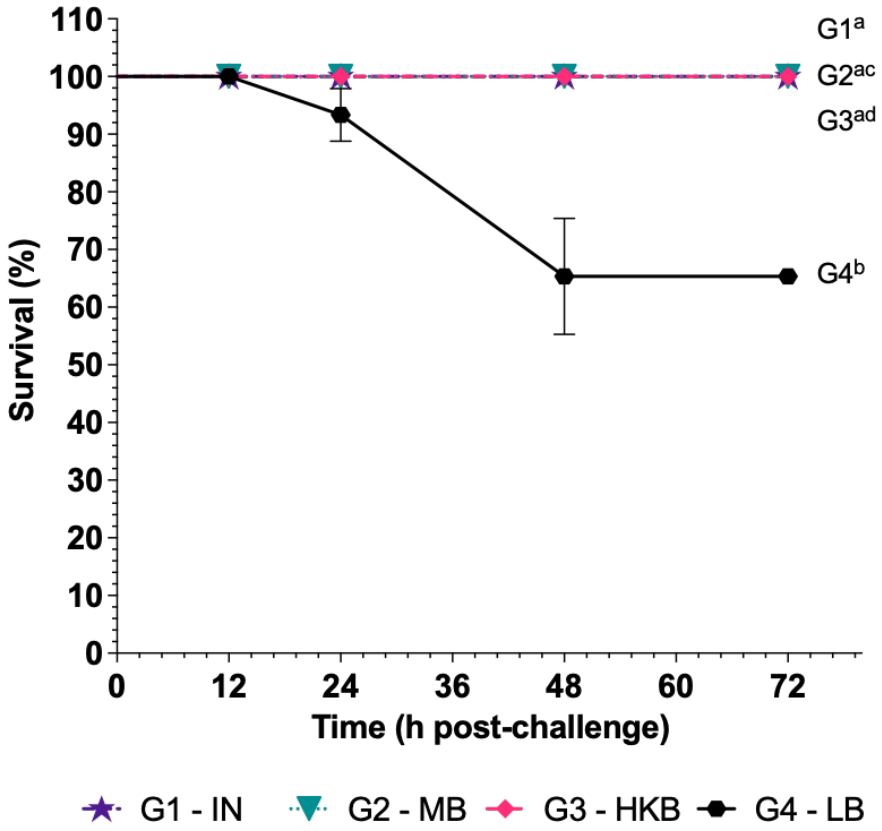


Figure 6.2. Survival curves for Greenshell™ mussel, following no injection (G1 – IN ★), injection with marine broth (G2 – MB ▼), injection with heat-killed bacteria (G3 – HKB ◆) and injection with live *P. swingsii* bacteria (G4 – LB ⬣), with 75 mussels were allocated to each group. Significant differences of log-rank (Mantel-Cox) test are indicated by different lowercase letters next to each survival curve when comparing the live bacteria injection group to the other groups ($p < 0.001$).

6.3.2 Bacterial quantification

The number of bacterial colony forming units (CFU) counted with characteristics of Vibrionaceae, within samples of mussel haemolymph within treatments (G1-G4) at 12, 24 and 48 hpc are reported in Fig. 6.3. Amongst treatment groups, at all sampling timepoints, haemolymph from mussels injected with live *P. swingsii* (G4) showed the largest number of CFU. Within G4, after 12 hpc, 43 CFU were detected. At 24 hpc, this value was two-fold higher with 109 CFU detected, and at 48 hpc the largest number of bacterial counts, of 273 CFU were detected. These changes were statistically significant when comparing the bacterial concentrations within G4 between timepoints 12 hpc and 48 hpc ($p < 0.001$) and between 24 hpc and 48 hpc ($p < 0.001$). Moreover, there was an interaction effect among treatment groups and sampling timepoint (Timepoint*Treatment, $p < 0.001$, 2-way ANOVA).

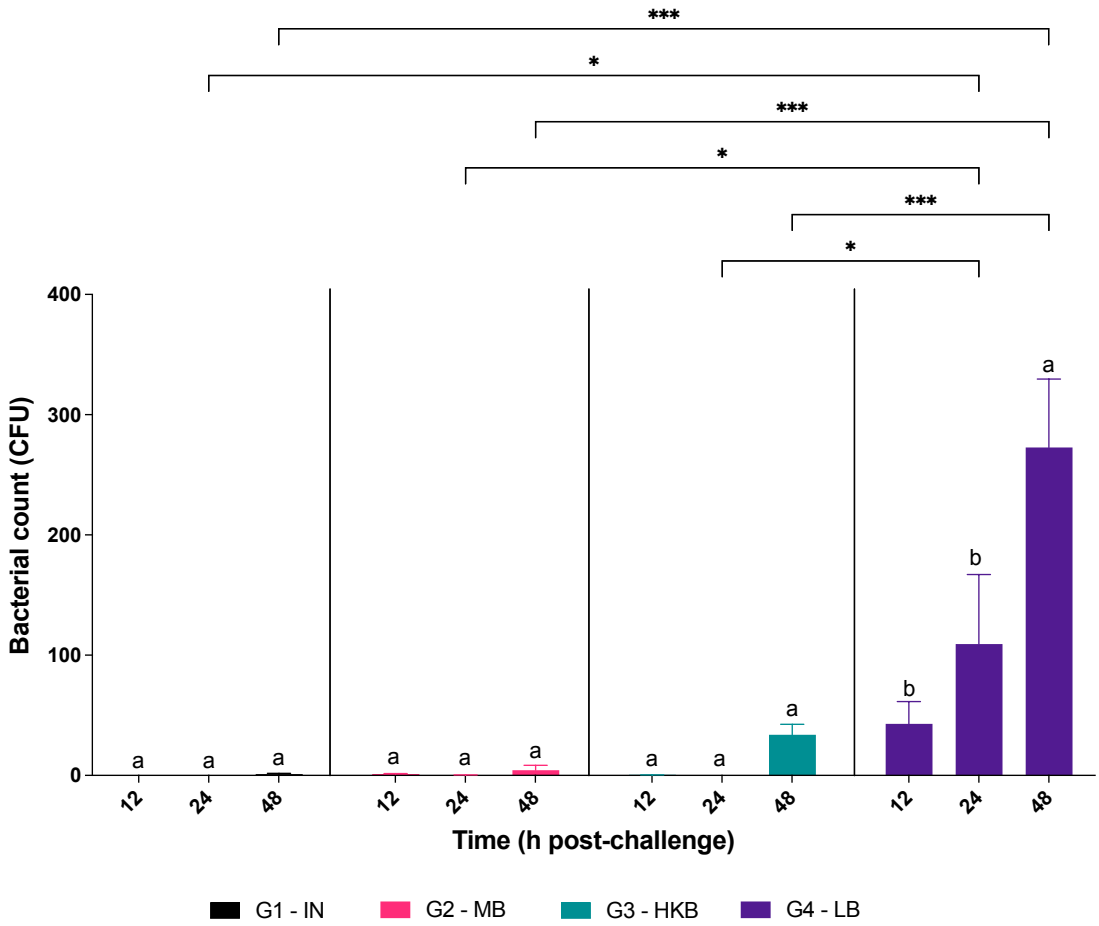


Figure 6.3. Quantification of Vibrionaceae colony forming units (CFU) obtained from haemolymph of the G1 – no injection (control), G2 – marine broth (injection control), G3 heat-killed and G4 live bacteria challenged groups at 12, 24, 48-hpc. All values represent average of 10 replicates per treatment. Error bars represent standard error of means (SEM). The hatching bracket between 2 bars represent significant differences between groups at the same timepoint. The single asterisk (*) represents a significant difference at $p < 0.05$, double asterisks (**) are for a difference at $p < 0.001$ and triple asterisks (***) are for a difference at $p < 0.0001$. The different letters (e.g., a and b) indicate comparisons between timepoints within the same groups.

6.3.3 Haemocyte count and haemocyte viability

The total number of haemocytes (THC) and haemocyte viability (% live haemocytes) detected in mussel haemolymph after 12, 24 and 48 hpc are shown in Figs. 6.4a and 6.4b, respectively. Within the first three control groups, samples at 12 hpc resulted in the largest number of haemocyte counts when mussels were not treated (G1 = mean ± SE, $3.9 \times 10^6 \pm 5.7 \times 10^5$ cells/mL), injected with marine broth (G2 = $3.6 \times 10^6 \pm 8.7 \times 10^5$ cells/mL), and injected with heat-killed bacteria ($3 \times 10^6 \pm 5.9 \times 10^5$ cells/mL). A decrease in haemocyte counts was determined as time progressed with 48 hpc showing the lowest number of haemocytes.

The opposite was true when assessing the group injected with live *P. swingsii* (G4), where the lowest number of haemocytes were detected at 12 hpc, and the highest number of

haemocytes detected at 48 hpc (Fig. 6.4a). Multiple comparison analysis (Bonferroni's test) revealed no significant differences within the group injected with *P. swingsii* (G4). Two-way ANOVA revealed no significance related to treatment ($p = 0.3126$), whereas sampling timepoint had a statistically significant effect ($p = 0.0310$). There was a significant interaction between treatment groups and time ($p = 0.0343$, 2-way ANOVA) as seen in the no injection control group at 48 hpc.

For haemocyte viability, similar levels of haemocytes were measured at 12 hpc within all treatments (G1: 72 ± 3.3%; G2: 75 ± 2.8%; G3: 66 ± 3.7%; G4: 66 ± 4.1%). All treatments (G1-G4) showed similar patterns as time progressed with an increase in haemocyte viability seen at 24 hpc followed by a decrease at 48 hpc (Fig. 6.4b). The 2-way ANOVA revealed a non-statistically significant effect of the treatment group ($p = 0.3529$) and the sampling timepoint ($p = 0.2144$) for the percentages of cell viability. The effect of treatment did not differ between timepoints (Timepoint*Treatment, $p = 0.942$).

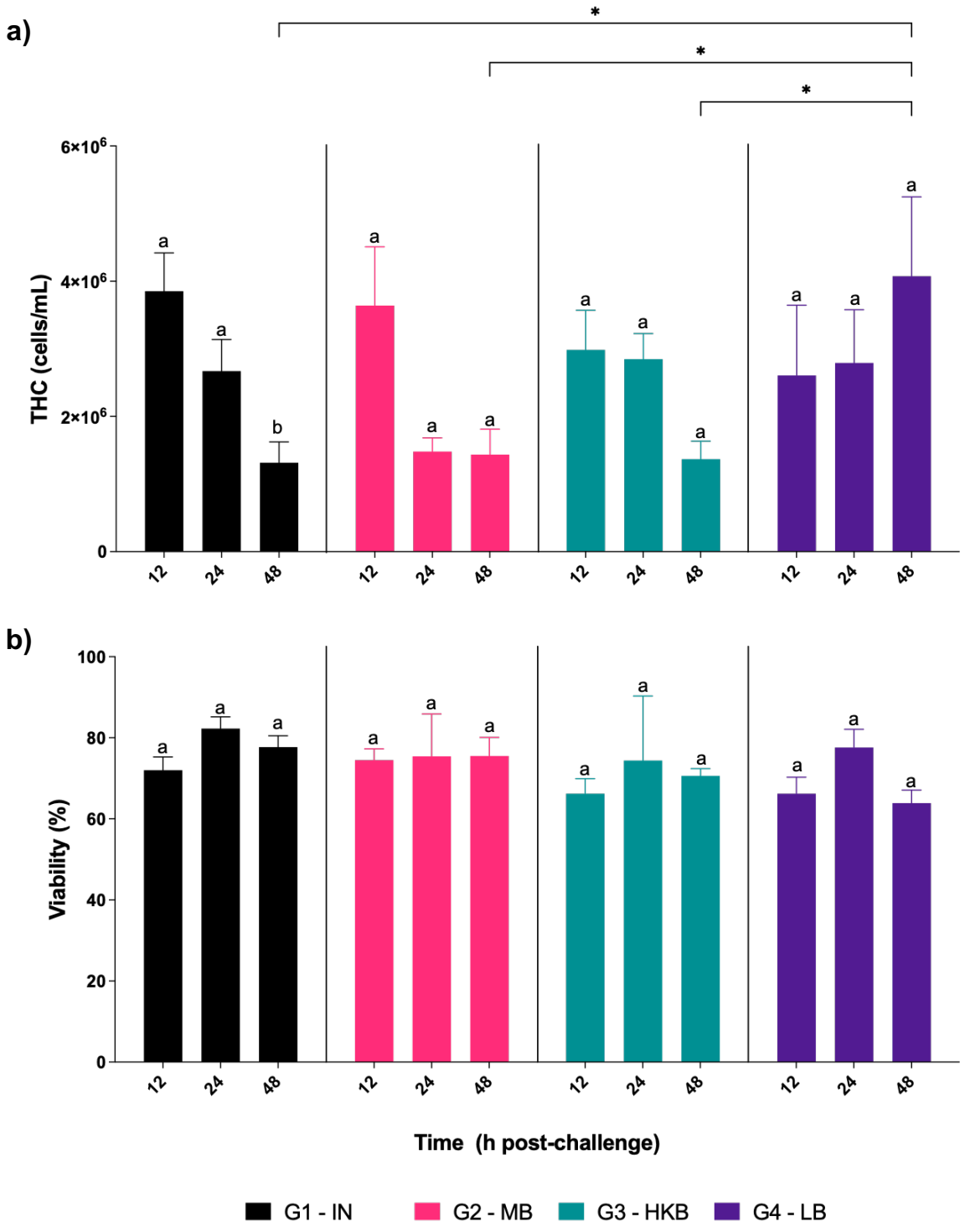
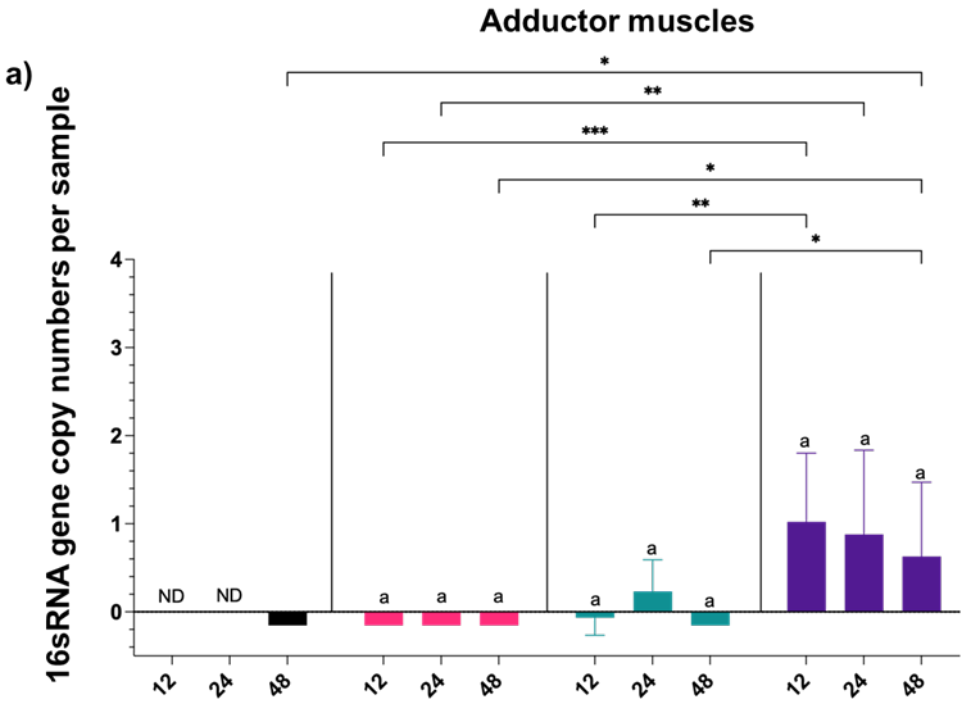


Figure 6.4. a) Total haemocyte counts, and **b)** haemocyte viability changes of Greenshell™ mussels in the G1 - no injection (control), G2 - marine broth (injection control), G3 heat-killed and G4 live bacteria challenged groups at 12, 24, 48-hpc. All values represent average of 10 replicates per treatment. The hatching bracket between 2 bars represent significant differences between groups at the same timepoint. The single asterisk (*) represents a significant difference at $p < 0.05$, double asterisks (**) are for a difference at $p < 0.001$ and triple asterisks (***) are for a difference at $p < 0.0001$. The different letters (e.g., a and b) indicate comparisons between timepoints within the same groups. All values represent average of 10 replicates per treatment. Error bars represent standard error of means (SEM).

6.3.4 qPCR

Following qPCR analyses, positive amplification signals were obtained from adductor muscle (Fig. 6.5a) tissue at 24 hpc in mussels injected with heat-killed *P. swingsii* (G3) and at 12, 24 and 48 hpc in mussels injected with live *P. swingsii* (G4). In the gill tissue (Fig. 6.5b), positive amplification signals were seen at 24 hpc in mussels injected with marine broth, at 24, and 48 hpc in G3, and at 12, 24 and 48 hpc in G4. The digestive gland tissue (Fig. 6.5c) showed positive amplification signals at 24 hpc in G3 and at 12, 24 and 48 hpc in G4. The bacterial DNA in mussel tissues injected with heat-killed bacteria (G3) was on average 5.81 gene copies per mussel (across tissues collected = 76.25 copies per mg of tissue) for all samples collected through the experiment. In mussels injected with live *P. swingsii* (G4), the average bacterial load was 29-fold greater - 163.2 copies per sample (2,141 copies per mg of tissue).

This difference in bacterial DNA between G3 and G4 was highly significant across all sampling times (12, 24 and 48 h) for all tissues analysed (2-way ANOVAs; $p < 0.001$, respectively). Across treatments, time did not have a significant effect on bacterial load and there was no significant interaction between time and treatment (2-way ANOVA; $p > 0.05$). Similarly, the bacterial load was not significantly different for the tissue types evaluated (adductor muscle, gill, and digestive gland) (2-way ANOVA; p-value > 0.05).



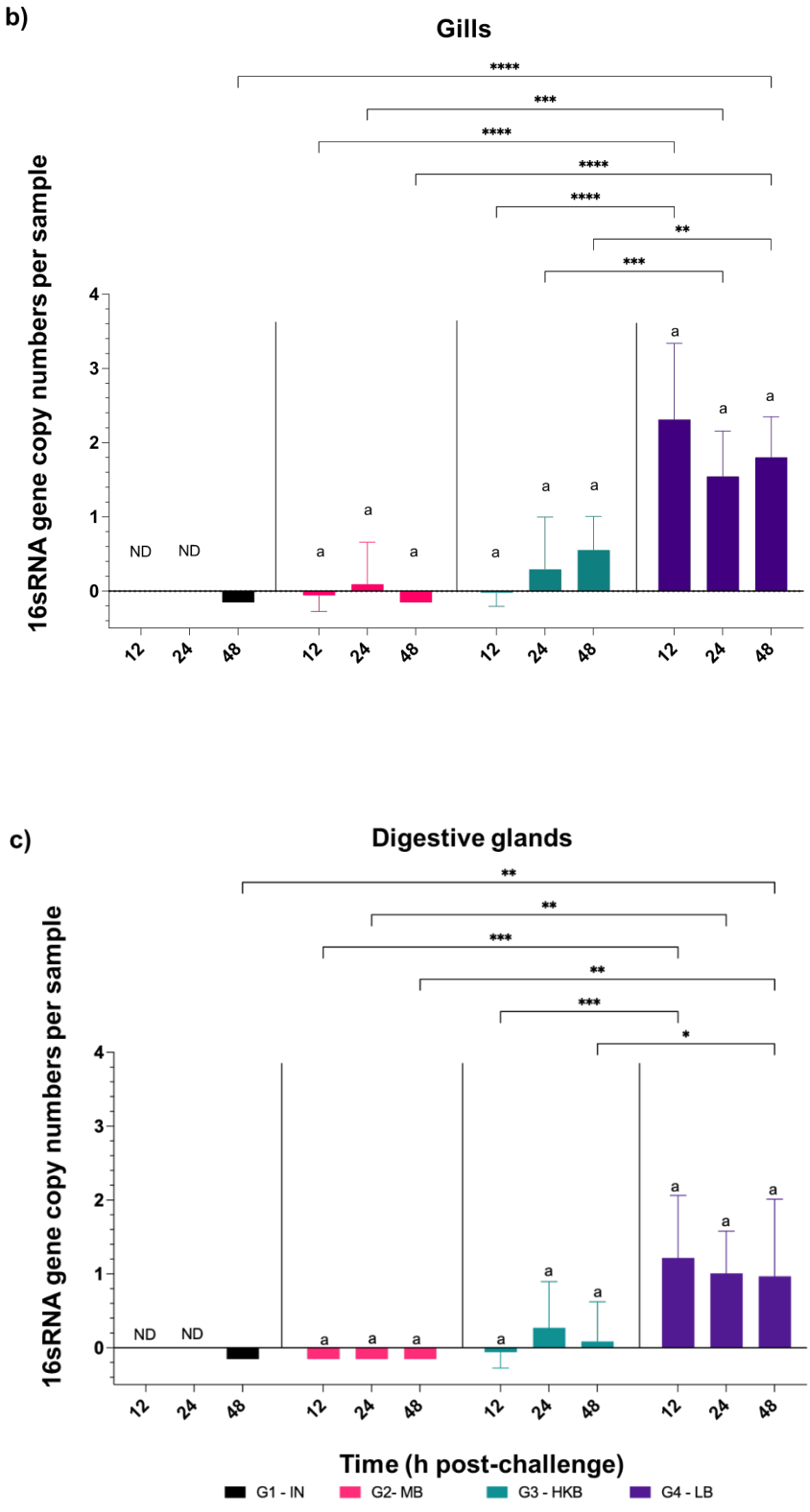


Figure 6.5. Bar graphs of log-transformed bacterial loads, which represents the concentration + 0.70 copies/sample (0 values replaced with minimum dataset value, followed by data transformation to log scale as measured by qPCR analyses from a) adductor muscle, b) gill and c) digestive gland tissue samples across treatments (G1 – no injection control, only sampled at 48h; G2 - marine broth injection; G3 - heat-killed bacterial injection, and G4 - live bacteria injection) at 12, 24, 48-hpc. All values represent average of 5 replicates per treatment.

6.3.5 Histopathology

Histopathological assessments were performed on all Greenshell™ mussels sampled over time (12, 24 and 48 hpc) and across treatment groups (G1-G4), as summarised in Table 6.1. In brief, the histopathological assessments focused on the body condition, distribution, and intensity of inflammation in the tissues, gonad development, digestive gland, connective tissues, gills, and mantle.

Variable infiltrates of inflammatory cells and haemocytes were present within the connective tissues of all mussels examined during the study, as seen within the control group (Fig. 6.6a), the marine broth injected control group (Fig. 6.6c), the heat-killed bacterial injection group (Fig. 6.6e) and the live bacterial injection group (Fig. 6.6j). The digestive glands in all mussels examined had non-specific changes consisting of variable digestive gland atrophy and early degenerative changes affecting the basophilic cells of the digestive glands with occasional inflammation affecting the glands directly (Fig. 6.6b, d, f). The gill tissues across treatments presented normal with occasional low levels of haemocyte infiltrates (Fig. 6.6k). The adductor muscle of mussels subjected to experimental injection (G2, 3 and 4) showed evidence of tissue degeneration and haemocyte infiltration presumed to be associated with the site of injection (Fig. 6.6h). The mantle showed variable inflammation with scattered nodular to diffuse haemocyte infiltrates (Figs. 6.6g, j) across all treatments. One mussel sampled (1/5) (G4T1) from the live *P. swingsii* injection group at 48 hpc had a focal area of marked inflammation within the connective tissue associated with short rod-shaped bacteria (Fig 6.6h), and one mussel (G4T2) had an incidental finding of birefringent organism resembling microsporidian like organism (Fig. 6.6l). Occasional bacteria were recognised within areas of muscle necrosis associated with the injection site of the live *P. swingsii* (Fig. 6.6h). Mussels within G4 also showed moderate to good body condition and female gonadal tissue (Fig. 6.6i) and normal gills (Fig. 6.6k).

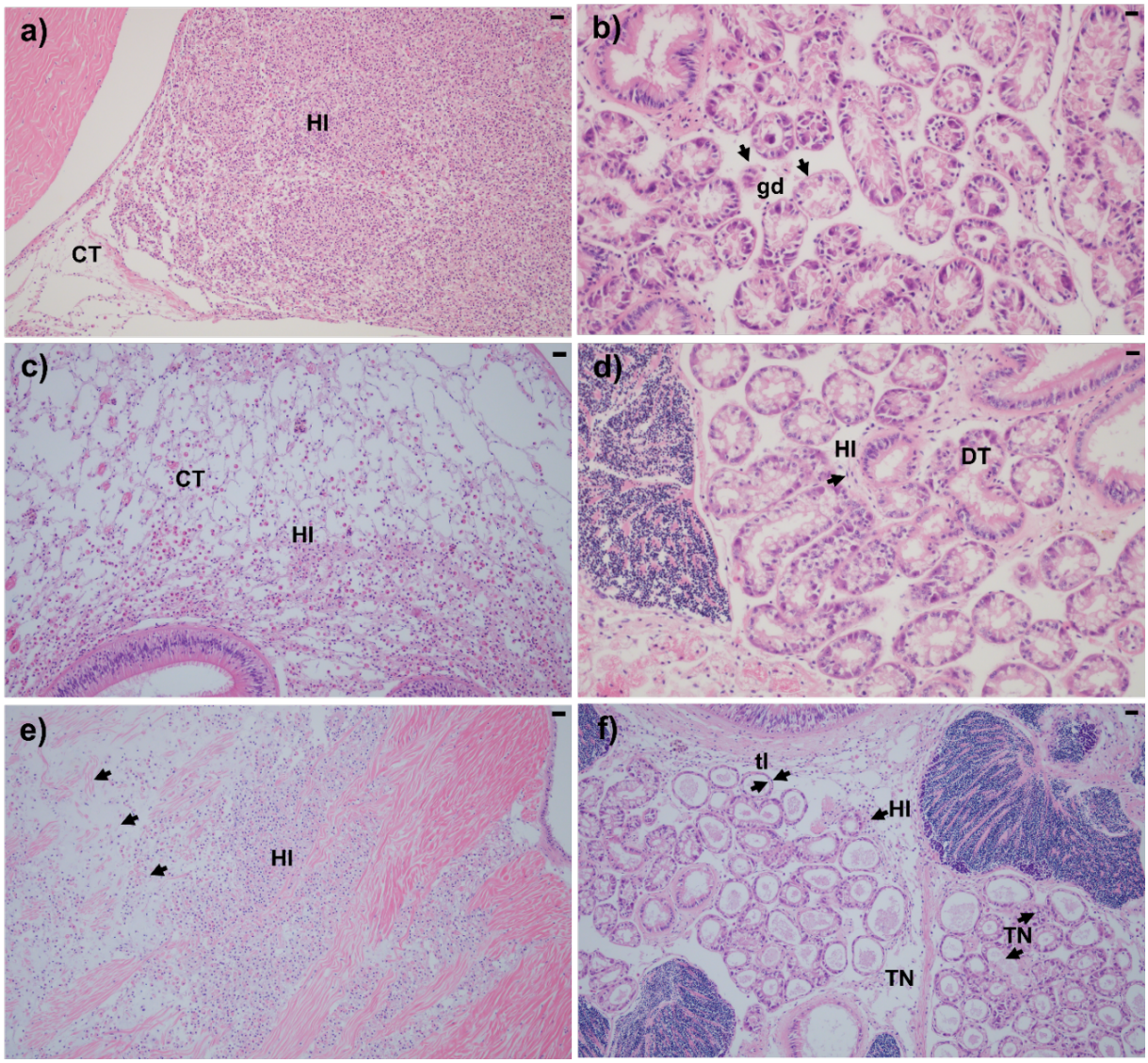


Figure 6.6. Histopathological alterations observed in *P. canaliculus* following a, b) no injection (G1); c, d) injection with marine broth (G2) and e, f) injection with heat killed bacteria (G3). **a)** Marked haemocyte infiltration (HI) with nodular distribution in the connective tissues (CT). **b)** Digestive gland disruption (gd, arrows) rounding of basophil cells and digestive epithelial cells detaching from basement membrane. **c)** Haemocyte infiltration (HI) with mild to moderate inflammation of the connective tissue (CT). **d)** Mild digestive gland atrophy with focal mild inflammation affecting the gland and lumen. **e)** Mantel with dense haemocyte infiltration (HI) and oedema of the connective tissue. **f)** Marked atrophy of digestive glands; with disruption of glandular epithelium and haemocyte infiltration (HI, arrow) among the digestive glands.

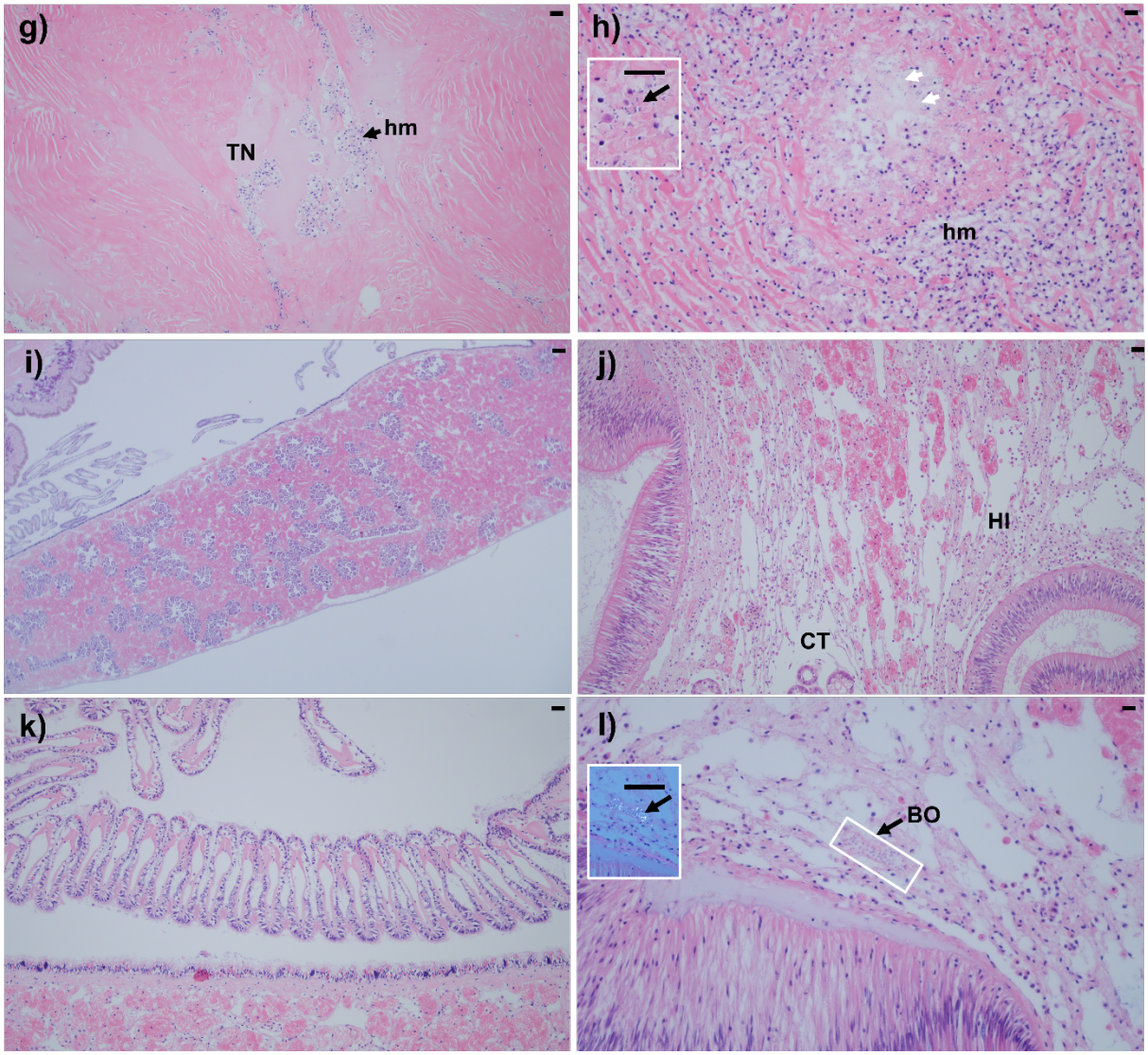


Figure 6.6. cont. Histopathological alterations observed in *P. canaliculus* injected with live bacteria (G4). **g)** Necrotic tissue (TN) with haemocyte (hm) aggregation, and fluid accumulation within the adductor muscle. **h)** Tissue degeneration (white arrows), haemocyte (hm) aggregation around the infection site. Inset (Scale bar = 50 µM); bacteria free in connective tissue. **i)** Example of mussel with moderate to good body condition and female gonadal tissue. **j)** Haemocyte infiltration (HI) and mild inflammation of the connective tissue (CT). **k)** Normal gills. **l)** Birefringent organism (BO). Inset (viewed with polarising filter (polarisers), Scale bar = 50 µM). Scale bar used throughout = 200 µM.

Table 6.1 Summary of the histopathological assessments of Greenshell™ mussel tissues from four treatment groups (no injection control, only sampled at 48h; marine broth injection; heat-killed *P. swingsii* injection and live *P. swingsii* injection) across sampling timepoints per 5 mussels observed (x/5).

Time post challenge	**12h**			**24h**			**48h**			
Treatment	**G2**	**G3**	**G4**	**G2**	**G3**	**G4**	**G1**	**G2**	**G3**	**G4**
Haemocytes infiltrations within tissue types										
Gills	2/5 NAD 3/5 (1+S)	5/5 (1+S)	5/5 (1+S)	5/5 (1+S)	5/5 (1+S)	5/5 (1+S)	4/5 NAD 1/5 (1+S)	5/5 (1+S)	3/5 (1+S) 2/5 (NAD)	5/5 NAD
Mantle	2/5 NAD 1/5 (1+S) 1/5 (1+F) 1/5 (1+N)	1/5 (1+S) 2/5 (1/2+D) 1/5(1+M) 1/5(1/2+)	2/5(1+S) 2/5 (1+M) 1/5(1+F)	3/5 (1+S) 2/5 (1/2+M)	2/5 (1/2+M) 1/5(1+M) 1/5 (2+) 1/5 (2/3+M)	2/5 (1+M) 1/5(1/2+NAD)2/5 (1+S)	2/5(1+S/N) 1/5(1+S/D) 1/5 (2+D) 1/5 (2/3+ NAD)	3/5 (NAD) 1/5 (1/2+S) 1/5 (1/2+M)	3/5 (1+S) 2/5 (NAD	2/5 (1+S) 3/5 (NAD
Digestive gland	5/5 (NR)	4/5 (HI) 1/5 (NR)	3/5 (HI) 2/5 (NR)	5/5 (NR)	2/5 (HI) 3/5 (NR)	1/5 (2+HA) 1/5 (2+HI) 3/5 (NR)	3/5 (HI) 1/5 (PR) 1/5(NR)	4/5 (HI) 1/5 (NR)	2/5 (HI) 3/5 (NR)	1/5 (HI) 4/5 (NR)
Dilation of digestive gland										
Mild (1+)	4/5	1/5	3/5	0	2/5	0	3/5	4/5	4/5	4/5
Mild to moderate (1/2+)	0	4/5	1/5	1	2/5	4/5	1/5	1/5	0	0
Moderate (2+)	0	0	0	0	1/5	1/5	1/5	0	0	0
Moderate to heavy (2/3+)	1/5	0	0	0	0	0	0	0	0	0
Heavy (3+)	0	0	1/5	0	0	0	0	0	0	0
Not represented	0	0	0	0	0	0	0	1/5	1/5	1/5
Body condition score										
Poor (1+)	0	0	0	0	1/5	1/5	0	0	0	0
Poor to moderate (1/2+)	1/5	4/5	0	1/5	1/5	1/5	0	0	1/5	0
Moderate (2+)	0	0	0	2/5	2/5	2/5	2/5	2/5	1/5	1/5
Moderate to excellent (2/3+)	0	0	3/5	2/5	0	1/5	3/5	1/5	1/5	4/5
Excellent (3+)	4/5	1/5	2/5	0	1/5	0	0	2/5	2/5	0
Inflammation based on connective tissues of mantle and gill (1st area) & digestive gland tubules (2nd area)										

Mild (1+)	0	0	4/5	3/5	3/5	0	2/5	4/5	1	4/5
Mild to moderate (1/2+)	2/5	2/5	0	2/5	0	4/5	0	1/5	0	0
Moderate (2+)	3/5	2/5	1/5	0	2/5	0	2/5	0	0	0
Moderate to heavy (2/3+)	0	1/5	0	0	0	1/5	1/5	0	0	0
Heavy (3+)	0	0	0	0	0	0	0	0	0	1/5
Adductor muscle degeneration and necrosis with haemocyte infiltrates										
Minimal	1/5 (MD +HI)	0	3/5 (MDN +HI)	4/5 (MDN +HI)	3/5 (MDN +HI)	1/5 (MDN +HI)	0	0	0	0
Mild (1+)	2/5 (MD +HI)	0	1/5 (MDN +HI)	0	1/5 (MDN +HI)	4/5 (MDN +HI)	1/5 (MD)	0	1/5 (MD+HI)	0
Mild to moderate (1/2+)	0	0	0	0	0	0	0	0	0	0
Moderate (2+)	0	0	1/5 (MDN +HI)	0	0	0	1/5 (MD)	0	1/5 (MD+HI)	2/5 (MD+HI)
Moderate to heavy (2/3+)	0	0		0	0	0	0	0	0	0
Heavy (3+)	1/5 (MD +HI)	0		0	0	0	0	0	0	0
Not represented	1/5	5/5		1/5	1/5	0	0	5/5	3/5	3/5
Presence or absence of bacteria										
Absence	5/5	5/5	3/5	1	4/5	1	0	1	4/5	4/5
Presence	0	0	2/5	0	1/5	0	0	0	1/5	1/5
Questionable	0	0	0	0	0	0	0	0	0	0
Apicomplexan-like/ Microsporadia like / Haplosporidian / microcell like organisms	2/5	1/5	2/5	0	0	3/5	1/5	0	0	0

Abbreviations: G1 = No injection, G2 = Marine broth injection, G3 = Heat-killed bacteria injection, G4 = Live bacteria injection; NAD = Nodular and diffuse; S = Scattered; F = Focal; N = Nodular; D = Diffuse; M = Multifocal; P = Poorly represented; NR = Not represented; HI = Haemocyte infiltration; HA = Haemocyte aggregation; MD = muscle degeneration; MDN = muscle degeneration and necrosis.

6.4 DISCUSSION AND CONCLUSIONS

Herein we discuss for the first-time results based on bacterial progression of a *Photobacterium swingsii* challenge in Greenshell™ mussels, *Perna canaliculus*. Our study revealed that injection with *P. swingsii* induced host response and mortalities while mussels injected with heat-killed *P. swingsii* and marine broth survived. This further supports the hypothesis that *P. swingsii* is pathogenic to mussels and despite the limitations posed by the challenge of mussels using injection of the adductor muscle, we found that live bacteria induced pathogenesis, while heat-killed bacteria did not. This indicates that the mortalities are due to bacterial proliferation rather than the injury caused by the injection itself.

Mussel response

No mortalities were observed in the control groups investigated in the current study, as previously seen in control treatments utilising *P. canaliculus* as an animal model (Azizan et al., 2022). Haemocyte viability was measured to quantify the amount of live or dead cells in a haemocyte population of mussels (Tresnakova et al., 2023), and our results found no major changes in haemocyte viability status recorded amongst control groups or timepoints. Similarly, insignificant changes were detected in haemocyte viability profiles in *P. canaliculus* subjected to temperature and pathogen stress, along with the corresponding controls (Azizan et al., 2023b).

Total haemocyte counts (THC) in molluscs are generally an indicator of organism health and immune status (Ericson et al., 2023c), with increasing circulating haemocytes associated with the presence of a stressor (Venter et al., 2021). At the first sampling point, (12 hpc), the number of haemocytes in the three control groups were the highest, potentially because of experimental holding (husbandry stress), while the lowest THC were detected at 48 hpc. Interestingly, the opposite THC response was seen in the group of mussels infected with live *P. swingsii*, where the lowest number of haemocytes were detected at 12 hpc, and the highest at 48 hpc. A previous study on Greenshell™ mussels reported a decrease in haemocyte concentration in the *Vibrio* spp. treated group (compared to controls) after 24h of infection (Ericson et al., 2022). Then again, when injecting clams *Ruditapes philippinarum* with live bacteria a loss of haemocytes was seen in the early phase of infection (Parisi et al., 2019). A loss of haemocytes at the onset of infection are believed to be due to the clearance of high initial bacterial loads, migration of haemocytes from the haemocoel to the injection site, lysis of haemocytes, or apoptosis of haemocytes after phagocytosis of bacteria (Mateo et al., 2009, Parisi et al., 2019). However, in the current study, as time progressed, the THC increased

after exposure to bacterial infection, suggesting that the haemocytes multiplied as a systemic response to stress (i.e., *P. swingsii* injection).

Colony forming units (CFU) were counted as a key predictor of bacterial presence (Quinn et al., 2022). Greenshell™ mussels injected with a live dose of *P. swingsii* displayed the highest CFU (compared to the other three groups), with CFU also increasing with time, resulting in the highest concentrations of bacterial colonies at 48 hpc. Thus, by two days post-challenge, bacteria within the haemolymph of *P. canaliculus* had not been cleared from the mussels' circulation system. It has been previously reported that *V. splendidus* bacteria were present in the haemolymph of *M. galloprovincialis* for 24 hpc, while *V. anguillarum* was still present in the haemolymph of the same species at 48 hpc (Parisi et al., 2008a). Ultimately, the kinetics of bacterial clearance remains variable based on the bacteria and bivalve species (Canesi et al., 2001). It should also be kept in mind that CFU were only measured in the mussels that survived the infection process, making the results a measure of mussels that are in the process of fighting the *P. swingsii* injection.

Mussel survival is a primary determinant of relative fitness (Shields et al., 2008). It is used as an end-point measure in the current study to determine the effect of *P. swingsii* on mussel physiology. The higher mortality observed in mussels injected with live *P. swingsii* potentially occurred due to the inability of mussels to clear bacteria from the haemolymph. In contrast, heat-killed bacteria did not cause any mussel mortalities in the current study. This outcome of zero mortality, has been previously reported in *Ruditapes philippinarum* injected with heat-killed *V. tapetis*, while the mortality of clams injected with live bacteria was attributed to the actions of the bacteria rather than influence of extracellular virulence factors (Allam et al., 2002).

Quantifying P. swingsii

For the first time, bacterial quantification from *P. swingsii* specific PCR is reported in this study. Herein, mussels injected with live and heat-killed *P. swingsii* showed detectable levels of bacteria, albeit higher in the group receiving the live dose of *P. swingsii*. Variations of *P. swingsii* DNA loads were detected within the adductor muscle, gill and digestive gland tissues investigated at all timepoints, indicating that *P. swingsii* spread into the internal organs potentially resulting in systemic infection. The quantification of *P. swingsii* DNA loads before 12 hpc remains an interesting aspect for future studies. In the current study we did not observe any clear trend of reduced or increased quantified bacteria overtime, as only three time points were studied, making it impossible to declare links to bacterial clearance as time continues. It is believed that a longer exposure duration, larger sample sizes, or shorter sampling

timepoints, would allow for a more precise quantification of bacterial loads which can be used to establish changes in bacterial load overtime. Amongst the tissues analysed in the present study, the bacterial DNA detected in the gill tissue was the greatest, which might hint at its important role in the immune response during pathogen challenge (Li et al., 2017). Indeed, the quantification of *P. swingsii* DNA loads in tissue samples will aid future research on this topic considering mussel tissues role in antimicrobial mechanisms (Bachère et al., 2015).

P. swingsii within mussel tissues

Histological findings from this study showed aggregation of haemocytes along with haemocyte infiltration, in the cross-section of the whole animal, suggestive of inflammatory cells migrating to a point in response to a stimulus. This observation of inflammation is not specific to a cause, but could indicate an unspecified adaptation response of mussels to laboratory conditions (Cajaraville et al., 1991), inadequate nutrition (Sokolova et al., 2012), natural physiological processes, such as spawning (Wendling and Wegner, 2013) or breakdown products (Van de Braak et al., 2002). Inflammation can also be a consequence of bacterial infection (Buckley et al., 2017, Jarc and Petan, 2019, Pudgerd et al., 2021), as seen in *P. viridis* exposed to *V. alginolyticus* (Laith et al., 2021), yet the effects of *P. swingsii* are difficult to discern from the current data, perhaps reflecting the short duration of the exposure or injection dose administrated. Incidental histological findings included detection of birefringent organism within the male gonad germinal cells and focally in the connective tissue of one mussel. These findings are likely associated with the fact that mussels were sourced from a commercial open seawater aquaculture facility and not a pathogen free colony/culture. They do not appear to have adversely affected the findings of this study.

In this study, inflammation in the gills was minimal within all experimental groups with no significant differences between them. The occurrence of gill inflammation, has been reported in clams with no link to damage to gill epithelia, but rather as a consequence of environmental variables (e.g., waterborne particles) (Costa et al., 2013). Resultantly, small changes in gill structure are to be expected, as seen in the current study, as gills are delegate structures, directly affected by external stimuli. Changes within the digestive glands, associated with dilation of the digestive lumen, atrophy of the gland epithelium and dissociation of the cells were detected across treatments and timepoints. Within bivalves the disruption of some digestive cells are considered a normal physiological process of digestion (Usheva et al., 2006). Also the housing of mussels in an aquarium setup has previously shown disintegration of digestive cells within the epithelium due to manipulation and aerial exposure (Dimitriadis and Koukouzika, 2003). Thus, the morphological changes expressed by the digestive gland

from all the mussels in the current study can be attributed to the disintegration phase of digestion and/or the effect of holding in the laboratory.

The adductor muscle tissues around the site of injection across treatments were characterised by muscle degeneration and necrosis with haemocytic infiltration. Such changes can be due to trauma from the injection where the possible hydrostatic pressure of injecting material/fluid, results in sterile tissue degradation (Wang, 2018). The same lesions were evident in all treatments without any specific link to *P. swingsii* injection. Bacterial injections into adductor muscle are known to cause local inflammation at the injection site (Allam et al., 2002). Previous studies on *Macrobrachium rosenbergii*, *Litopenaeus vannamei* and *Crassostrea gigas* reported necrosis of the muscle cells infiltrated with accumulating haemocytes following infections with *Vibrio* (*V. alginolyticus* and *V. splendidus*) (Gay et al., 2004, Liu and Chen, 2004, Ajadi et al., 2019). Injuries to tissue are seen as a breach of the first defence barrier (Gay et al., 2004), resulting in a general stress response as seen in the adductor muscle samples of the injected mussels under investigation. In *M. galloprovincialis* a recruitment of haemocytes along with changes in the volume of muscular fibres were reported at the bacterial site of injection. Yet, recovery of the muscle tissue organisation followed by 48h of injection (Parisi et al., 2019). In *P. canaliculus* the effect of injection was still evident following 48h, requiring more research to define the tissue recovery period.

In conclusion, this evaluation of *P. swingsii* pathogenesis in *Perna canaliculus* shows physiological changes due to bacterial injection overtime. At 48 hpc, mussel injected with *P. swingsii* showed the highest mortality rates, haemocytes counts and bacterial colony forming units. The bacterial quantification supported systemic infection of mussel tissues with *P. swingsii* overtime. The histopathological results showed injury obtained due to injection of treatments to the adductor muscle, along with the activation of an unspecific inflammatory response or the activation of defence mechanisms via cell proliferation. No clear link was established between the qPCR results (*P. swingsii* DNA load) and the presence of inflammatory cells.

To gain a better understanding on the susceptibility of mussels toward *P. swingsii* infection, further studies will require an assessment of the entire immune system including humoral factors, since cellular factors alone do not provide a complete picture of infection outcomes. The species-specific qPCR developed in this study can potentially be used for wider surveillance programmes to study the epidemiology of *P. swingsii* on mussel farms. Ultimately, this study supports efforts to strengthen biosecurity management and aquatic surveillance workstreams.

Chapter 7: Metabolite changes of *Perna canaliculus* following a laboratory marine heatwave exposure: Insight from metabolomics analysis

The sea, once it casts its spell, holds one in its net of wonder forever.“
- Jacques Yves Cousteau -

After examining pathogen infection, this chapter focuses on another stressor: thermal stress. Specifically, it investigates into the metabolomic response of Greenshell^{TM} mussels during a five-day laboratory-based marine heatwave exposure.

This chapter has been submitted for publication:

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Abstract

Temperature is considered to be a major abiotic factor influencing aquatic life. Marine heatwaves are emerging as threats to sustainable shellfish aquaculture, affecting the farming of New Zealand's green-lipped mussel [*Perna canaliculus* (Gmelin, 1791)]. In this study, *P. canaliculus* were gradually exposed to high-temperature stress, mimicking a five-day marine heatwave event, to better understand the effects of heat stress on the metabolome of mussels. Following liquid chromatography-tandem mass spectrometry analyses of haemolymph samples, key sugar-based metabolites supported energy production via the glycolysis pathway and TCA cycle by 24 h and 48 h of heat stress. Anaerobic metabolism also fulfilled the role of energy production. Antioxidant molecules acted within thermally stressed mussels to mitigate oxidative stress. Purine metabolism supported tissue protection and energy replenishment. Pyrimidine metabolism supported the protection of nucleic acids and protein synthesis. Amino acids ensured balanced intracellular osmolality at 24 h and ammonia detoxification at 48 h. Altogether, this work provides evidence that *P. canaliculus* has the potential to adapt to heat stress up to 24 °C by regulating its energy metabolism, balancing nucleotide production, and implementing oxidative stress mechanisms over time. The data reported herein can also be used to evaluate the risks of heatwaves and improve mitigation strategies for aquaculture.

Key words

Aquaculture; Marine heatwave; Metabolomics; Mussels; New Zealand; *Perna canaliculus*; Pathways; Temperature, Stress.

7.1 INTRODUCTION

An organism's optimal physiological functioning occurs within certain temperature limits, determined by thermal conditions experienced over evolutionary timescales. Beyond these limits, physiological function declines, and without mitigating action, mortality occurs (Smith et al., 2023). In New Zealand (NZ), seawater temperatures are currently reported as several degrees above normal conditions, with more frequent and severe events projected (Stevens et al., 2022). In line with these projections, extreme events are likely to have an impact on the rich and diverse marine ecosystems, aquaculture facilities and commercial and recreational fishing grounds found around NZ (Behrens et al., 2022). In particular, elevated summer seawater temperatures and marine heatwaves (MHW) are causing mortalities in farmed green-lipped mussels (*Perna canaliculus*) (Ericson et al., 2023c). Considering that mussel farming is the cornerstone of the NZ aquaculture industry (Stenton-Dozey et al., 2021) and

that the industry aims to grow significantly over the coming decade (FISHERIES`NEW`ZEALAND, 2022), data are needed to support efforts in safeguarding aquaculture production against marine heatwaves.

In broad terms, MHW are defined as periods where temperatures exceed the 90th percentile of the local climatology for five days or more (Smith et al., 2023). It is believed that MHW could have greater ecosystem and evolutionary impacts within marine systems than the more gradual effects of climate change. Here, heatwaves are said to compound the effects of underlying warming trends with limited opportunity for organisms to adapt or acclimate, while the slower effects of warming allows more time to process the change with adaption or acclimation to follow (Babcock et al., 2019).

Laboratory-based thermal studies on *P. canaliculus* support the use of 16 °C as a control condition, with mortalities starting to occur when seawater temperatures reach 24 °C (Ericson et al., 2023c). Changes in sea surface temperatures affect marine organisms at all levels of biological organisation, including, behavioural, molecular, biochemical and physiological responses (Georgoulis et al., 2021). Typically, the metabolic response to thermal stress includes an increase in energy production to maintain homeostasis, utilising various metabolic pathways and oxidation of multiple energy substances, such as proteins and lipids (Xu et al., 2020a). In *P. canaliculus*, metabolomics has been used to create a blueprint of metabolite pathways affected in response to thermal stress. Some of the key responses are (1) increases in tricarboxylic acid (TCA) cycle intermediates and metabolites feeding into the TCA cycle to sustain energy production via oxidative phosphorylation, (2) the activation of anaerobic metabolic pathways to support rapid energy production, (3) the use of substrate-level phosphorylation enabling the synthesis of adenosine triphosphate (ATP) directly from the phosphorylation of adenosine diphosphate (ADP), and the utilisation of the fatty acid oxidation to supply ATP molecules (Delorme et al., 2021b, Muznebin et al., 2022a, Ericson et al., 2023c). What has not been established is the short-term responses implemented by mussels at the metabolite level during a MHW event. This data which explains the physiological response of mussels to a stressor can be utilised to understand their resilience in a changing environment. This information can also be used to improve farming and processing efficiency, which adds value for the aquaculture industry (Venter et al., 2023). Such data can be obtained utilising metabolomics approaches, targeting the phenotype of an organism, and expanding on what is happening on a metabolic and physiological level (Alfaro and Young, 2018). Metabolomic analysis hold the promise to simultaneously monitor precursors, intermediates, and products of metabolic pathways, acting as a discovery tool to detect metabolites affected by stressors (Young and Alfaro, 2018).

Considering all the above, the objective of this study is to investigate the metabolic changes that occur in *P. canaliculus* (green-lipped mussel) when exposed to a laboratory-based MHW for a period of five days. By gaining a comprehensive overview of these metabolic changes, the study aims to enhance our understanding of the physiological responses of *P. canaliculus* to heat stress and its implications for overall mussel health and survival. We hypothesise that the metabolic changes observed will reflect adaptations to heat stress, including shifts in energy metabolism and alterations in key metabolic pathways. Moreover, the data reported herein can be used to support aquaculture industry initiatives and policies to evaluate the risks of MHW and improve mitigation strategies.

7.2 MATERIALS AND METHODS

7.2.1 Animal husbandry and haemolymph collection

Adult *Perna canaliculus* (n=150) were collected from a Marlborough Sounds marine farm and placed in a flow-through seawater system at ambient temperatures (± 16.04°C) located at Cawthron Institute's Te Wero facility in Nelson, New Zealand. Mussels were held for six days to acclimate to holding conditions and fed *Tisochrysis lutea*. Water flow rates were kept at 2–3 L/min and aerated with air-stones. Seawater temperature, ammonia, nitrite, and nitrate levels were monitored daily.

For experimental purposes, 96 animals were divided into eight tanks (i.e., 12 animals per tank). Mussels in tanks one to four were kept at ambient condition of 16°C, serving as the control temperature for this experiment (correlating to mean temperatures experienced in the wild in the summer in the Marlborough Sounds). Mussels within tanks five to eight were slowly exposed to an increase in seawater temperature of 1°C per day until 24°C was achieved, whereafter the set condition at 24°C was maintained for the duration of the experiment. Temperatures were achieved via a heat exchanger connected to a hot/cold loop, controlled by solenoid valves (Vaportec LTD, Napier, New Zealand), and monitored daily (Fig. 7.1). Once experimental temperatures were achieved, sampling started.

At days 1, 2, 3, 4, 5 (24, 48, 72, 96, 120h post exposure, respectively) of the experiment, haemolymph was collected from two animals per tank, per condition (resulting in 8 mussels per timepoint, per temperature). Prior to haemolymph sampling, the animals were patted dry with paper towels and weighed to the nearest 0.01 g and the shell lengths were measured to the nearest 0.10 mm along the longest axis, using callipers. The animals were gently opened

on the ventral posterior side to access the posterior adductor muscle. Using a pre-chilled 23-gauge needle attached to a 1 mL sterile syringe, haemolymph was collected and placed into a microcentrifuge tube. A volume of 400 µL (with 20 µL of 10 mM L-alanine-2,3,3,3-d_4 internal standard) were transferred to a microcentrifuge tube and snap-frozen using liquid nitrogen and stored at –80˚C until subsequent use (Ericson et al., 2023c).

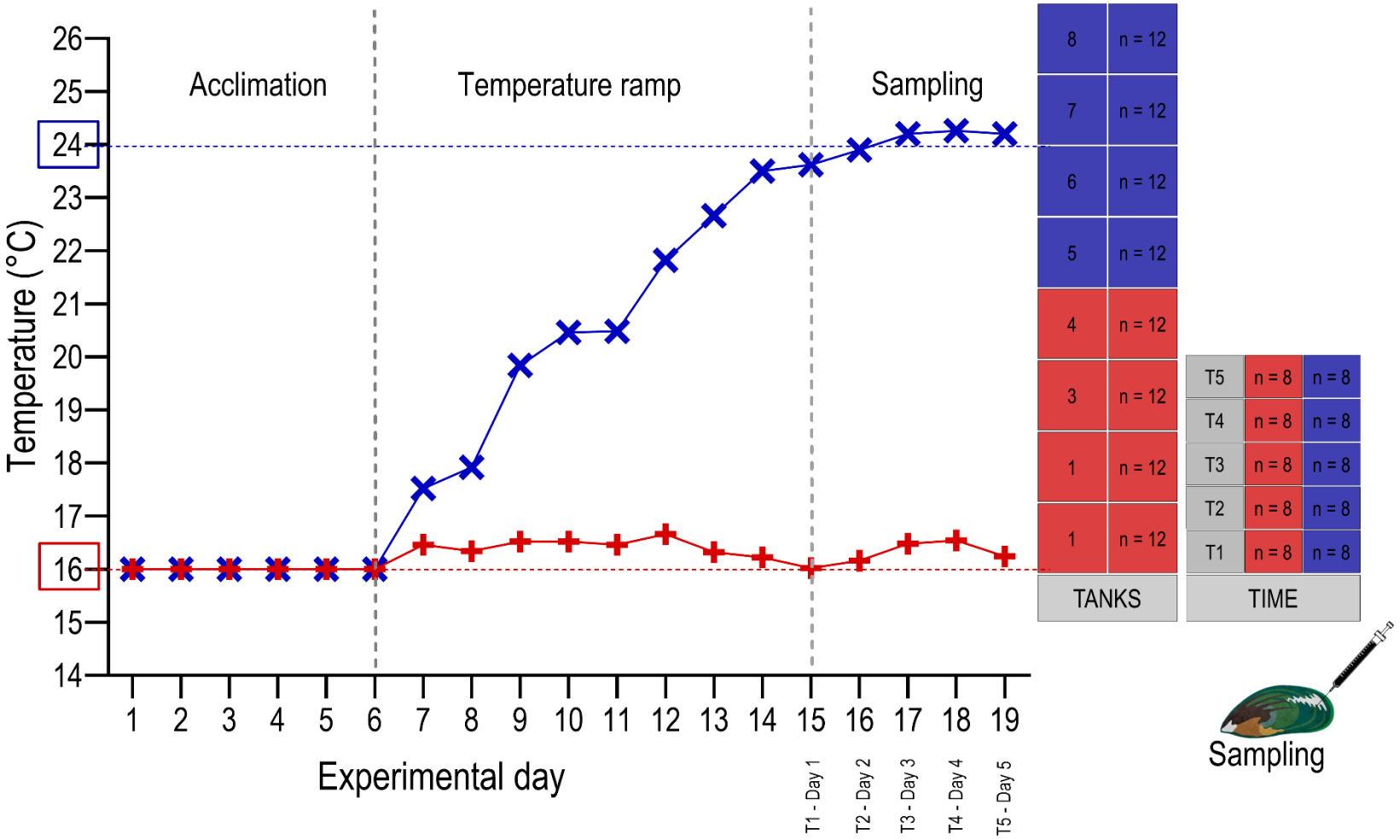


Figure 7.1. During the marine heatwave exposure, mussels were divided into eight tanks, with the first half of the tanks kept at ambient temperature of 16°C (red line) and the second half of the tanks experiencing a moderate temperature increase over time, resulting in a target temperature of 24 °C (blue line), whereafter haemolymph sampling was carried out for five days at the target temperatures.

7.2.2 Metabolomics sample preparation, analysis and data processing

Frozen haemolymph samples were dried under vacuum for 4 h at 0 °C. A two-step sequential extraction method was used for metabolite extraction. In brief, a volume of 500 µL cold methanol:water solution (50% MeOH:50% H_2O) was added to the dried sample and vortexed for 1 min, followed by centrifugation at 3500 rpm for 5 min at −9 °C. The supernatant was collected and transferred to a new tube, whereafter, the remaining sample was re-extracted by adding 500 µL cold methanol:water solution (80% MeOH:20% H_2O), vortexed and centrifuged (as above). Supernatants were collected from the re-extracted sample and pooled with the first collection, placed back in the −80 °C freezer overnight, and dried in a SpeedVact concentrator (Venter et al., 2021). A volume of 200 µL 50% MeOH:50% H_2O was added to the dried sample, vortexed, and centrifuged (10 min, 10,000 rpm), whereafter, 100 µL was transferred to a vial containing a pulled point glass insert for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses.

Quality control (QC) samples were included within the biological sample batches and prepared by taking a 50 µL of each biological sample, thoroughly mixed into a homogenous pooled sample (Broadhurst et al., 2018), and treating them as experimental samples. The QC samples were injected at regular intervals throughout the analytical run of the analysed batches to measure repeatability and identify any potential batch effects in the data.

Metabolomics analyses were conducted by running samples on an Agilent 1260 LC coupled to an Agilent 6470 triple quadrupole (QQQ) mass spectrometer. Agilent MassHunter Workstation Data Acquisition (Version 10.0) was used for compound calibration and data acquisition. For all analyses, the column was kept at 40 °C, and 2 µL of sample was injected. The flow rate was kept at 0.25 mL/min. For ion-pairing chromatographic separation, an Agilent ZORBAX Extend C18 column was used with the mobile phases prepared as follow: Mobile phase A: 97% water, 3% methanol, 10 mM tributylamine, 15 mM acetic acid, and 5 µM medronic acid (Agilent infinity lab deactivator additive). Mobile phase B: 10mM tributylamine, 15mM glacial acetic acid, and 5 µM medronic acid. Channel A and C delivered mobile phase A and B, respectively, while channel B delivered isopropanol, and channel D delivered acetonitrile to wash the column at the end of the run. The following gradient was used: 0–2.5 min, 100% A at 0.25 mL/min; at 7.5 min, 80% A; at 13.0 min, 55% A; at 20.0 min, 1% A and kept till 24.0 min. From 24.1–27.0 min, the 99% C was changed to 99% D. From 27.1–31.4 min, the flow rate with 1% A and 99% D was increased to 0.8 mL/min flow rate; from 32.3–40.0 min, the flow rate was steadily decreased and switched back to 100% A and reduced back to 0.25 mL/min. The mass spectrometer source parameters included the following: A gas

temperature of 150 °C, Gas flow of 10 L/min, Nebulizer gas pressure of 45 psi, Sheath gas temperature of 325 °C, Sheath gas flow of 12 L/min, Capillary voltage2000 V, Delta electron multiplier voltage (Delta EMV) 200 V. Dynamic MRM scans were used with 0.07 min peak width and acquisition time of 24 min. Delta retention time of plus and minus 1 min, fragmentor voltage of 40 eV, and cell accelerator voltage of 5 eV were incorporated into the method (as stipulated in the Agilent "MassHunter Metabolomics Dynamic MRM Database and Method", G6412-90006, Agilent 5991-6467EN application note). Tuning and calibration of the QQQ was achieved with Agilent ESI Low Concentration Tuning Mix.

The data were pre-processed with Agilent MassHunter Workstation Quantitative Analysis Software (Version 10.0). Two unique transitions were monitored per individual metabolite to provide spectral matching in addition to retention time, resulting in metabolite identities with the highest level of confidence (Sumner et al., 2007a, Schymanski et al., 2014). To remove non-biological variation, the data were normalised using the mass spectrometry total useful signal normalisation method (Venter et al., 2019). The data were generalised log transformed to alleviate the dependency of the variance on the compound concentrations (van den Berg et al., 2006). Two-way ANOVA was used to determine the influence of time (days 1–5) and temperature (16 and 24 °C) on the metabolite response of mussels (between subjects, $p < 0.001$). Herein a Venn diagram was generated to summarise the statistically significant changes in metabolite levels associated with each factor, along with their interactions (Figure 7.2). The metabolite response pertaining to time and temperature was future analysed and visualised in a blocked manner (Chong et al., 2019) with principal component analysis (PCA) (Figure 7.2). Statistically significant metabolites with an interaction effect, as indicated by the metabolite interaction patterns between temperatures (of the control and treatment groups) over time

7.3 RESULTS AND DISCUSSION

Exposure to environmental stressors often leads to elevated costs of basal metabolism, with increased energy requirements as the main outcome (Shang et al., 2023). This was demonstrated in the present study, where LC-MS/MS data of *P. canaliculus* haemolymph were used to investigate the metabolic processes of mussels exposed to 24 °C compared to the control group of mussels held at 16 °C over a five-day period. The metabolite response was a consequence of mussel exposure to a gradual increase in seawater temperature, which reached a target temperature (24 °C), followed by holding and monitoring at that temperature for 5 days. A total of 182 metabolites were detected, with 16 metabolites affected by temperature, 58 metabolites affected by time, and 61 metabolites showed significance due to

an interaction effect between time (T1-5) and temperature (16, 24 °C) (Fig 7.2a, Table S1). From the PCA score plot representing temperature (Figure 7.2b), a moderate overlap in scores were seen, showcasing the lower impact of this experimental factor on the metabolism of mussels. The relevant metabolic variation due to temperature is mainly captured by PC1, explaining 41.4% of the variance. The PCA score plot relating to time (Figure 7.2c) showed the smallest grouping within the first timepoint and a shift in metabolite response as time progresses. Herein timepoint five showed a clear grouping from the other timepoints, depicting an opposite metabolite response. Of importance for discussion in this manuscript is the metabolites relating to an interaction between temperature and time. These metabolites were mapped and interpreted in terms of pathways relating to (A) sugar metabolism; (B) glycolysis; (C) single-carbon metabolism and sulphur containing amino acids; (D) TCA cycle; (E) urea cycle; (F) aromatic amino acids; (G) branched chain amino acid metabolism; (H) pyrimidine metabolism and (I) purine metabolism (Figure 3). In the following sections, we discuss the overtime metabolite response shown by an increase or decrease in metabolite abundance as reflective of the heat-stressed mussels (in comparison to the controls).

Sugar and carbohydrate metabolism to support energy storage or utilisation

Typically, carbohydrates are the main source of energy metabolism and play a vital role in cell homeostasis under stressful conditions (Tagliaferro et al., 2022). The overall response in the sugar-based metabolites (Fig. 7.3, pathway A) of the heat stressed mussels under investigation was a decrease in metabolite concentration after 48 h, followed by an increase at 72 h. Metabolites, such as, cellobiose, glucose-1-phosphate, fructose-1.6-biphosphate, galactosamine, arabinose-5-phosphate, arabinose and xylitol increased after three days at a constant temperature of 24°C. It can be argued that mussels experienced an energy deficit by 72 h, due to the presence of heat stress, and increased these sugar metabolites to support increased glycolysis functioning (Fig.7.3, pathway B). The conversion of various types of sugar metabolites (glucose-1-P, fructose 1.6-biP, xylitol, xylulose-5-P) as an influx to the glycolysis pathway (Salway, 2004, Alam et al., 2022) in response to heat stress are herein reported for the first time in *P. canaliculus*. Ultimately glucose flux supported substrate-level phosphorylation in the current study, where ATP was directly phosphorylated from ADP, as previously described in thermally stressed *P. canaliculus* (Delorme et al., 2021b). An increase of glucose was also seen in the haemolymph of *Mytilus edulis*, to meet increased tissue energy demands in response to temperature stress (Matoo et al., 2021). The accumulation of sugars may also help to stabilise proteins against heat stress (Chen et al., 2019) and support the biosynthesis of defensive compounds induced by stress (Aguilera-Sáez et al., 2019). Additionally, sugar metabolites are capable of inducing antioxidant activity against oxidative stress (Chen et al., 2021b). Furthermore, the current study indicates that the sugar-based

metabolites entering the top intermediates of the glycolysis pathway had similar metabolite levels at both 16 and 24°C by day five, suggesting that an equilibrium had been reached between the depletion and utilisation of sugar metabolites in response to thermal stress. The xylitol-based metabolites showed opposite temperature trends by the end of the experiment, with both temperature groups ending with different metabolite concentrations. Both L-arabinose and xylose are used as carbon sources by a variety of microorganisms (Desai and Rao, 2010) and plants (Seiboth and Metz, 2011), while the functions of these metabolites have not been characterised in mussels to date.

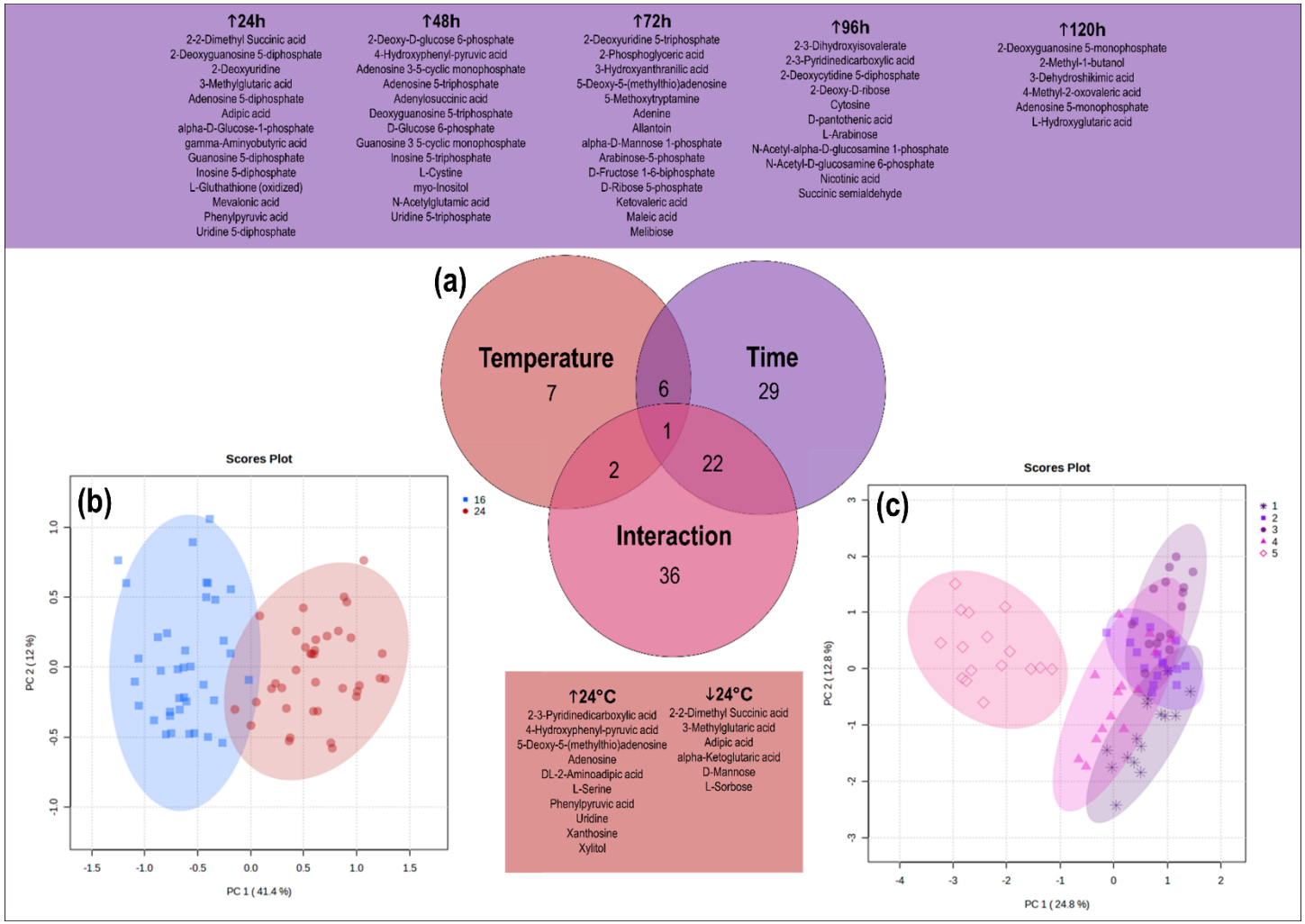
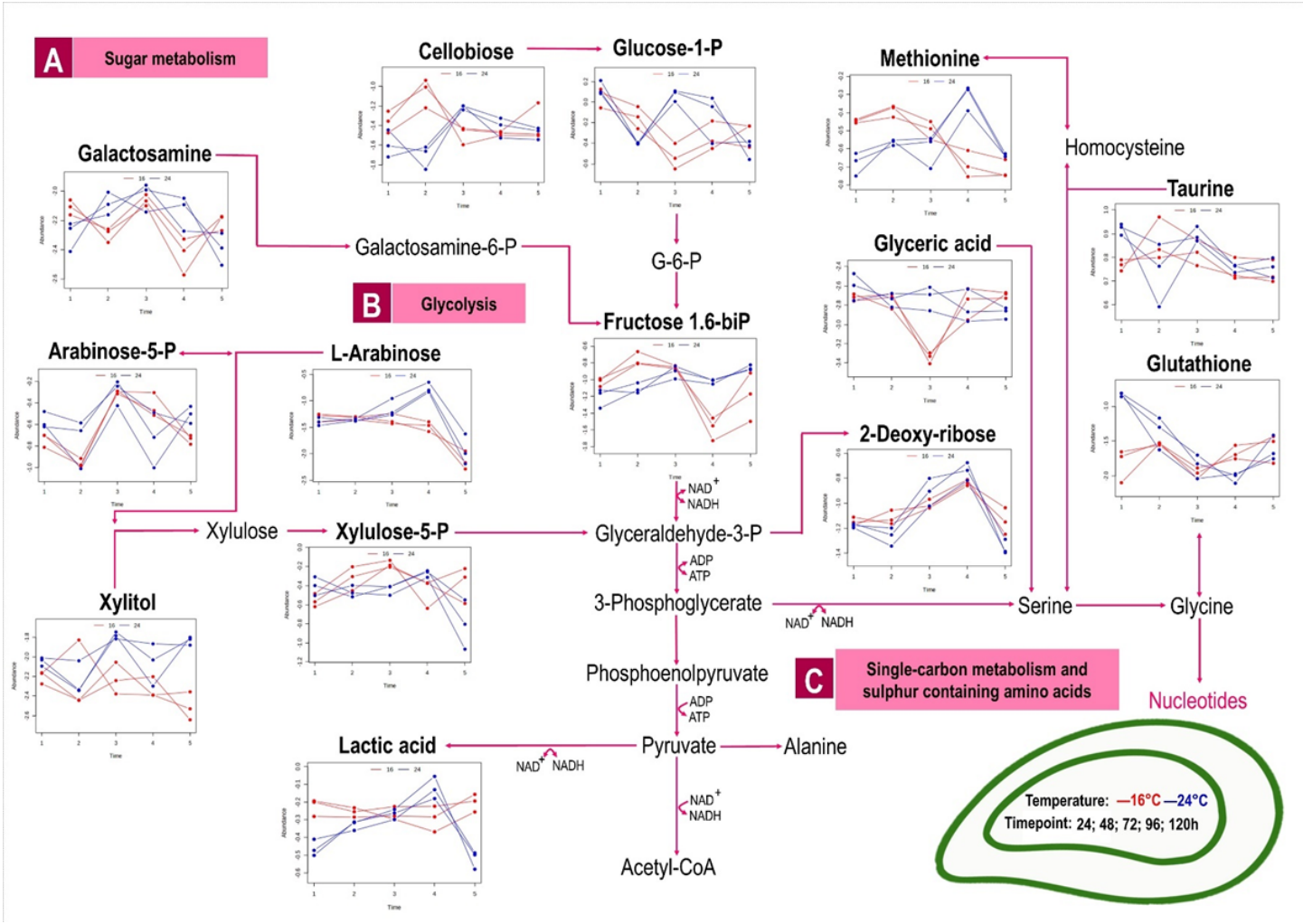
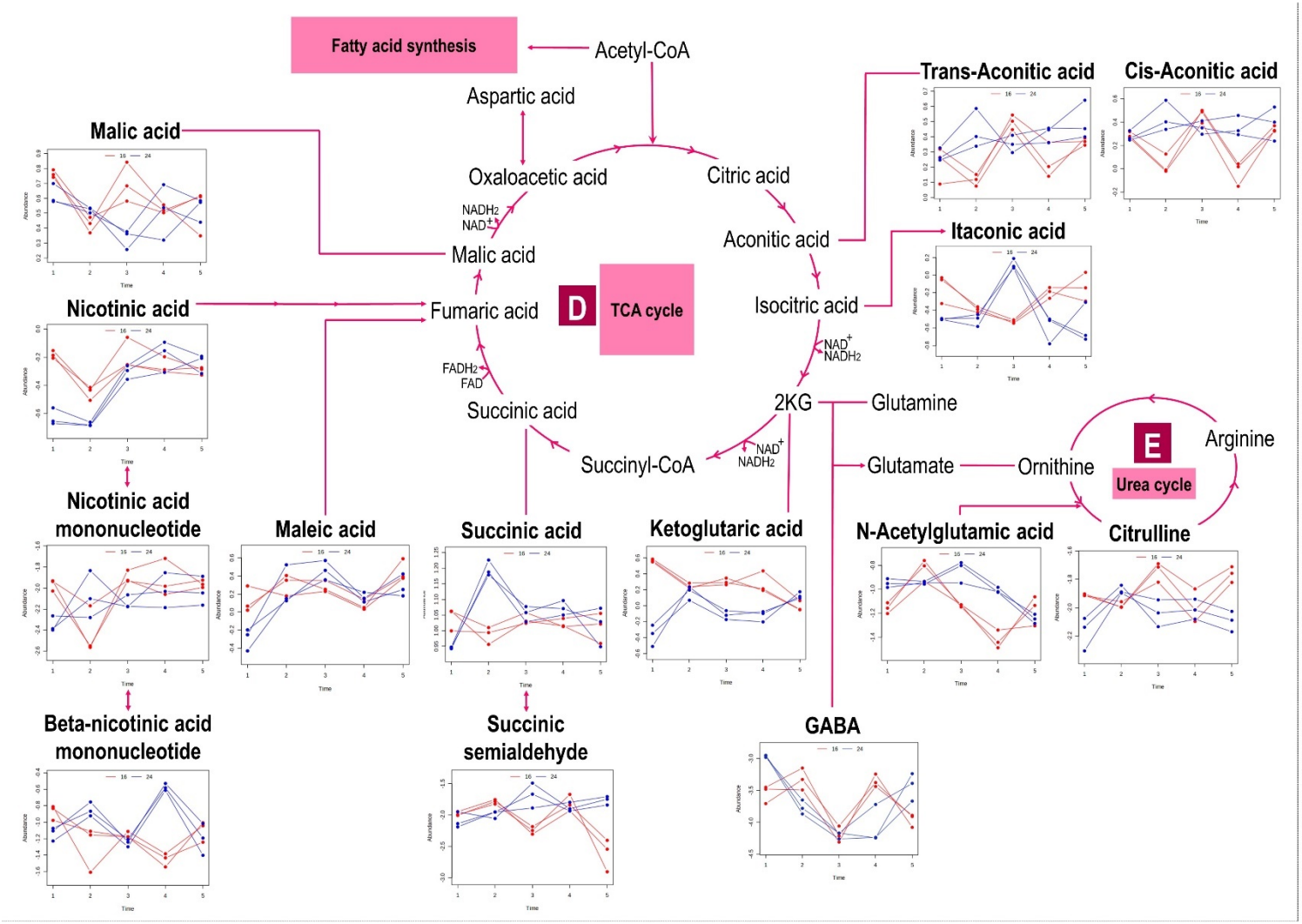
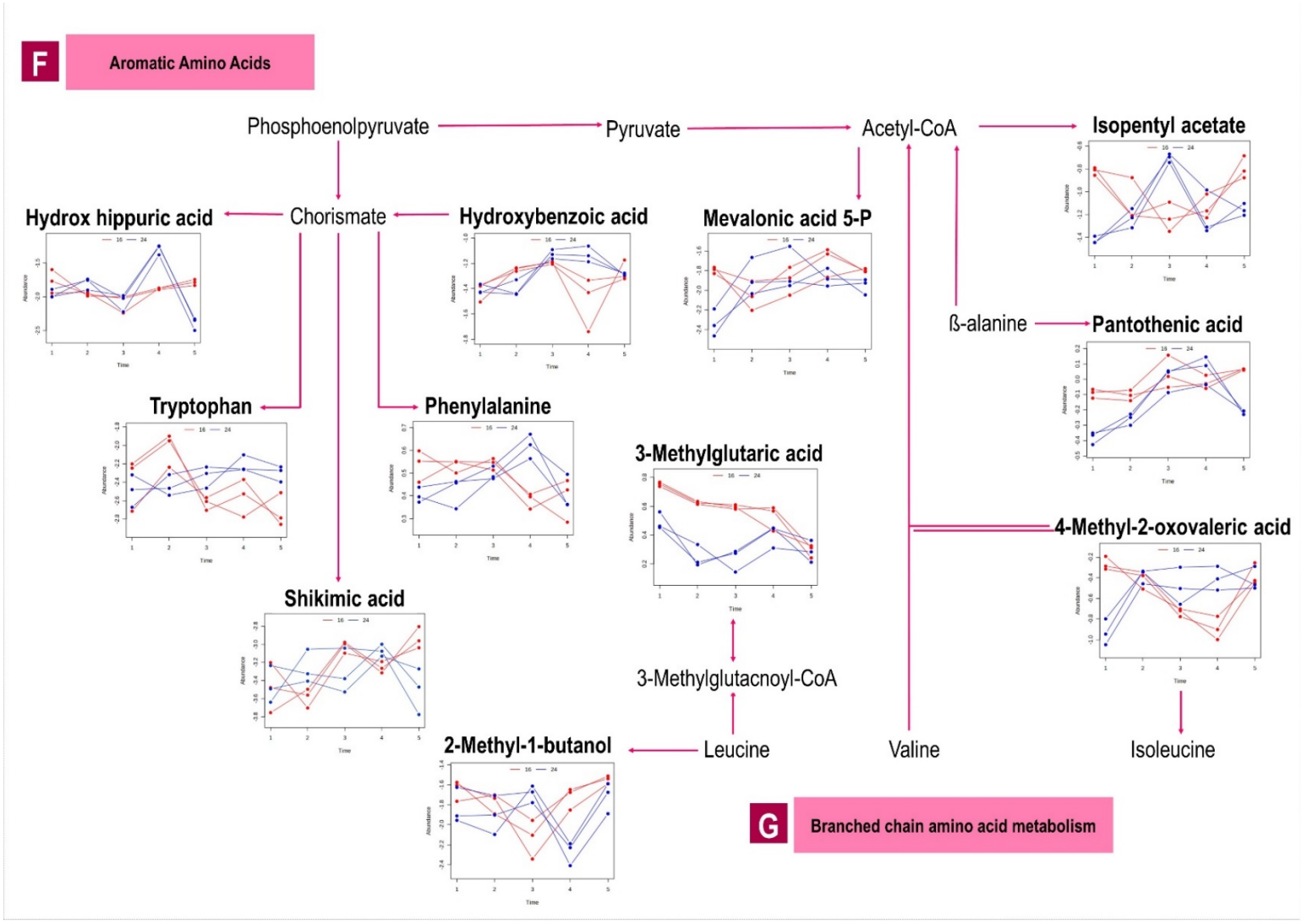
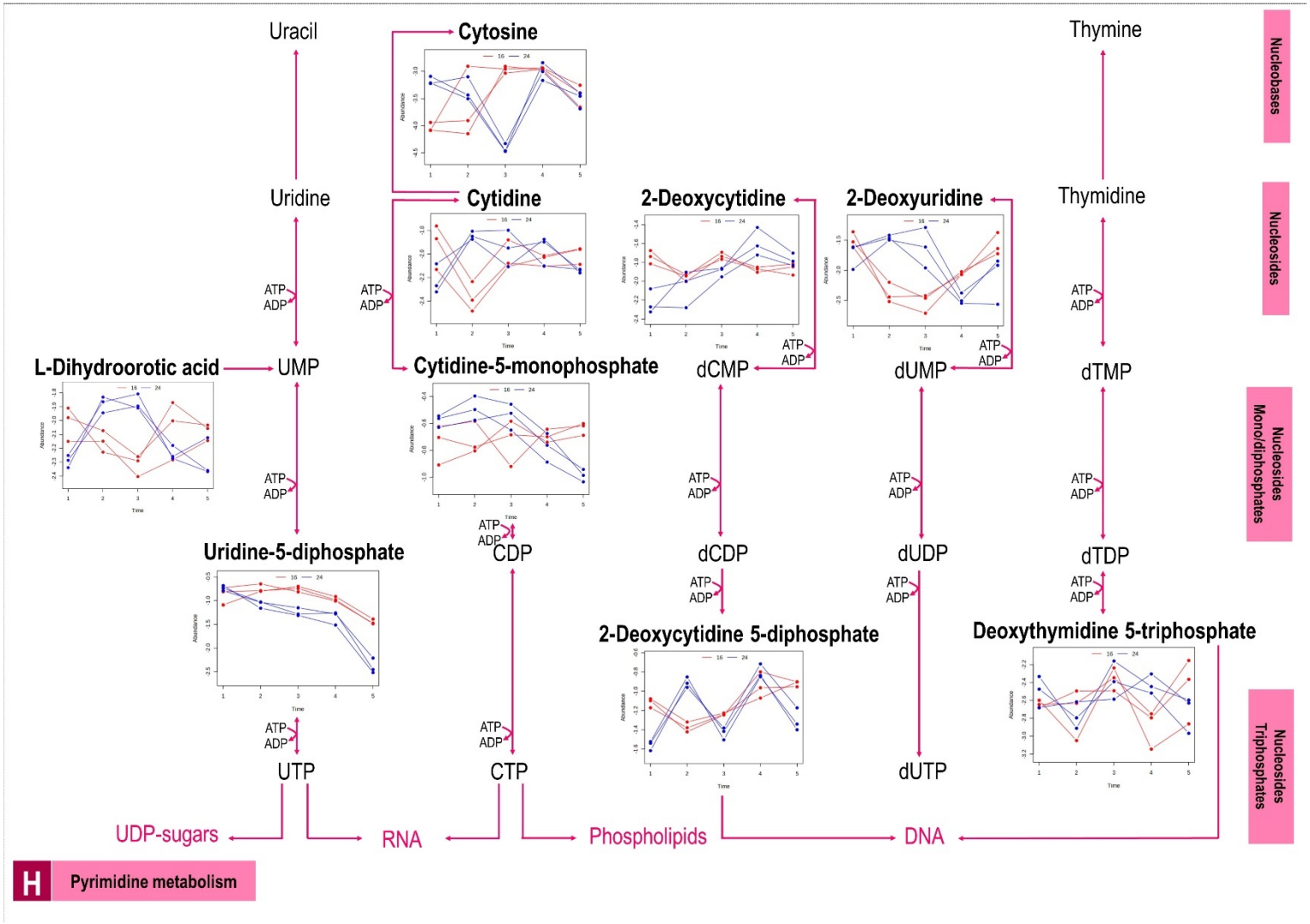


Figure 7.2. (a) Overview of metabolomics results as a Venn diagram focusing on significantly different metabolites due to the effect of temperature, time, and an interaction effect. Additionally, PCA plots are shown of mussels (b) exposed to two temperatures (16 and 24 °C) and (b) sampled at five timepoints (24, 48, 72, 96, 120 h).


A
Sugar metabolism
Cellobiose
Glucose-1-P
Galactosamine
Galactosamine-6-P
G-6-P
B
Glycolysis
Fructose 1.6-biP
Arabinose-5-P
L-Arabinose
Xylulose
Xylulose-5-P
Glyceraldehyde-3-P
Xylitol
3-Phosphoglycerate
Phosphoenolpyruvate
Lactic acid
Pyruvate
Alanine
Acetyl-CoA
NAD+
NADH
ADP
ATP
Methionine
Homocysteine
Taurine
Glyceric acid
Glutathione
2-Deoxy-ribose
Serine
Glycine
Nucleotides
C
Single-carbon metabolism and sulphur containing amino acids
Temperature: —16°C —24°C
Timepoint: 24; 48; 72; 96; 120h


Fatty acid synthesis
Acetyl-CoA
Aspartic acid
Malic acid
Oxaloacetic acid
Citric acid
Trans-Aconitic acid
Cis-Aconitic acid
NADH2
NAD+
Malic acid
Aconitic acid
Itaconic acid
Nicotinic acid
Fumaric acid
D
TCA cycle
Isocitric acid
NAD+
NADH2
FADH2
FAD
Succinic acid
2KG
Glutamine
Succinyl-CoA
NAD+
NADH2
Glutamate
Ornithine
E
Urea cycle
Arginine
Nicotinic acid mononucleotide
Maleic acid
Succinic acid
Ketoglutaric acid
N-Acetylglutamic acid
Citrulline
Beta-nicotinic acid mononucleotide
Succinic semialdehyde
GABA


F
Aromatic Amino Acids
Phosphoenolpyruvate
Pyruvate
Acetyl-CoA
Isopentyl acetate
Hydrox hippuric acid
Chorismate
Hydroxybenzoic acid
Mevalonic acid 5-P
ß-alanine
Pantothenic acid
Tryptophan
Phenylalanine
3-Methylglutaric acid
4-Methyl-2-oxovaleric acid
Shikimic acid
3-Methylglutacnoyl-CoA
2-Methyl-1-butanol
Leucine
Valine
Isoleucine
G
Branched chain amino acid metabolism


Uracil
Cytosine
Thymine
Nucleobases
Uridine
Cytidine
2-Deoxycytidine
2-Deoxyuridine
Thymidine
Nucleosides
ATP
ADP
L-Dihydroorotic acid
UMP
Cytidine-5-monophosphate
dCMP
dUMP
dTMP
Nucleosides Mono/diphosphates
Uridine-5-diphosphate
CDP
dCDP
dUDP
dTDP
2-Deoxycytidine 5-diphosphate
Deoxythymidine 5-triphosphate
UTP
CTP
dUTP
Nucleosides Triphosphates
UDP-sugars
RNA
Phospholipids
DNA
H
Pyrimidine metabolism

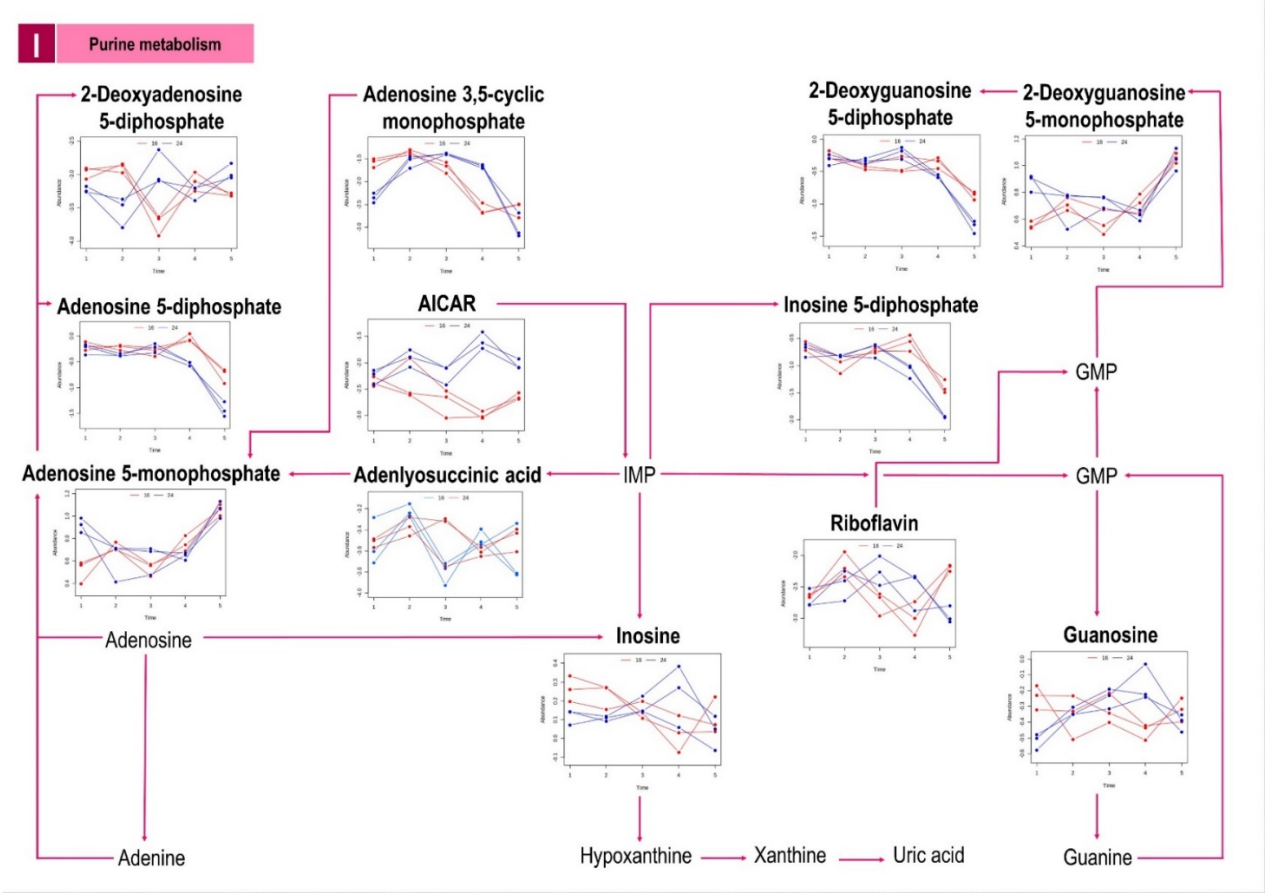


Figure 7.3. Perturbed metabolites from carbohydrate metabolism (**A**), the glycolysis pathway (**B**), and single carbon metabolism (**C**) detected in haemolymph of *P. canaliculus* following exposure to 16 °C (control) and 24 °C (heat stress) and five sampling timepoints (24, 48, 72, 96, 120 h). Perturbed metabolites connected to the TCA (**D**) and urea cycle (**E**) detected in haemolymph of *P. canaliculus* following exposure to 16 °C (control) and 24 °C (heat stress) and five sampling timepoints (24, 48, 72, 96, 120 h). Perturbed metabolites linked to amino acid (F, G) metabolism detected in haemolymph of *P. canaliculus* following exposure to 16 °C (control) and 24 °C (heat stress) and five sampling timepoints (24, 48, 72, 96, 120 h). Perturbed metabolites associated with pyrimidine metabolism (H) detected in haemolymph of *P. canaliculus* following exposure to 16 °C (control) and 24 °C (heat stress) and five sampling timepoints (24, 48, 72, 96, 120 h). Perturbed metabolites associated with purine metabolism (I) detected in haemolymph of *P. canaliculus* following exposure to 16 °C (control) and 24 °C (heat stress) and five sampling timepoints (24, 48, 72, 96, 120 h). Lines represent two different temperatures at 16 °C (control, blue lines) and 24 °C (heat stress, red lines) with 3 mussels/replicates per timepoint, per temperature.

TCA cycle as central hub for energy metabolism and redox balance

In the current study, a steady supply of ATP production during thermal stress was further secured via changes within the TCA cycle (Fig. 7.3, pathway D). Affected metabolites, such as itaconic acid, ketoglutaric acid, succinic acid and malic acid remained higher in the control group of mussels (16°C) after 24h of the experiment. It was only after 48h that the metabolite concentrations of the temperature stressed group (24°C) were similar to the 16°C group or higher. Increased TCA cycle intermediates were previously found in *P. canaliculus* following a 3 h acute thermal challenge (Dunphy et al., 2018) and after 60 min of severe heat shock (Delorme et al., 2021b). Considering that the metabolite pathways preceding the TCA cycle (i.e., glycolysis, pentose-p-pathway) did not show increased metabolites at timepoint one (24 h), an influx of intermediates is not experienced within the TCA cycle from the start. Yet, as time proceeded, the effect of temperature stress was seen as additional metabolite products were used to support rapid ATP production, funnelling more intermediates to the TCA cycle by 48, and 72 h of temperature stress. Increased succinic acid can occur via the reversal of the second half of the TCA cycle, to reduce equivalents for the synthesis of nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD) (Venter et al., 2023). At the remaining timepoints, a reduction of succinic acid was seen (compared to T2 which was the highest), in the temperature stressed group, which can be attributed to the upregulation of the aspartate-succinate pathway (more succinate was thus used) to support energy production in the presence of thermal stress (Venter et al., 2022). Notably, alpha (α)-ketoglutaric acid was the only TCA cycle intermediate in the heat stressed group which presented lower than the control group (albeit like the controls at timepoint five). Alpha-ketoglutaric acid is a rate determining intermediate of the TCA cycle, pivotal to energy metabolism (Wu et al., 2016), with functions in antioxidative defence, amino acid homeostasis, signalling and genetic regulation (Legendre et al., 2020). Additionally, the role of α-ketoglutaric acid in detoxification of reactive oxygen species (ROS) is potentially supported by other metabolites detected in the current study, such as itaconic acid, gamma aminobutyric acid (GABA), methionine and glutathione (Fig.7.3), suggesting that thermal stress initiated a rapid release of ROS in the mussels at 24°C.

Metabolites countering oxidative stress

Oxidative stress is caused by an imbalance between production and accumulation of ROS in cells and tissues, and the inability of a biological system to detoxify them (Li et al., 2020). Typically, antioxidant compounds scavenge ROS or indirectly act to upregulate antioxidant defences or inhibit ROS production (Gulcin, 2020). The production of ROS in response to thermal stress along with an increase in antioxidant metabolites and total antioxidant capacity

is well documented for *P. canaliculus* (Delorme et al., 2021b). In the current study, glutathione, an important antioxidant molecule with the ability to scavenge ROS, was detected in the highest concentration at the first sampling timepoint within the thermally stressed mussels. Possibly, temperature stress resulted in increased levels of glutathione, supporting glutathione ratios despite heat-induced oxidative stress (Verlecar et al., 2007). As time progressed, the levels of glutathione decreased in the current study, indicating the utilisation of this antioxidant to counter ROS production, as seen in *P. viridis* (Verlecar et al., 2007). Towards the last timepoint (T5), glutathione of the thermally stressed mussels under investigation, stabilised within ranges of the control group, possibly to support maintenance of the cellular redox status. Reduced methionine levels detected in the heat stressed mussels (T1-3) in the current study, reflected the use of methionine for transsulfuration and use within the glutathione pathway to support oxidative stress mechanisms as previously recorded in heat stressed *P. canaliculus* (Ericson et al., 2022). Additionally, itaconic acid showed the highest increase at 72h within the current study, potentially attributing to inhibition of mitochondrial ROS production in heat stressed mussels (Noe and Mitchell, 2019). Increased itaconic acid has previously been reported in *P. canaliculus* in response to thermal stress (Ericson et al., 2022), and remains a metabolite of interest within mussel metabolism (Nguyen et al., 2019b). As was the case for glutathione, GABA was detected at the highest concentration at the start of the current experiment in mussels kept at 24°C. GABA accumulation controls redox homeostasis in multiple organisms by supporting antioxidant status (Liang et al., 2022). Resultingly, the increased GABA can be ascribed to a ROS scavenging role during thermal stress. Many species have been shown to benefit from GABA's antioxidative properties, including juvenile *Litopenaeus vannamei* (Xie et al., 2017) and juvenile Chinese mitten crab (*Eriocheir sinensis*) (Zhang et al., 2022).

Anaerobic energy supply

The switch between aerobic and anaerobic metabolism is common in intertidal mussels (Hui et al., 2020), with the activation of anaerobic metabolism also previously supported in *P. canaliculus* in the presence of thermal stress (Dunphy et al., 2015). Within this study, the first evidence to support anaerobic metabolism is the affected metabolite, lactic acid (Fig. 7.3). Compared to the control group, lactic acid in the heat stressed group was reduced at timepoint one and two, suggesting the utilisation (depletion) thereof by tissues to support high energy production (Song et al., 2019). Next, lactic acid production increased when the demand for ATP and oxygen exceeded supply (Rabinowitz and Enerbäck, 2020), as seen at 96h of mussel exposed to 24°C. Likewise, increased levels of succinic acid can indicate oxidative stress in shellfish when oxygen availability is limited (Young et al., 2016), and supported a shift towards anaerobic metabolism via the succinate pathway, as previously detected in temperature

stressed *P. canaliculus* (Venter et al., 2023). The upregulation of carbohydrate metabolism with the production of alcohols and carbon dioxide as end products are often seen in oxygen deprived scenarios (Ratnawati et al., 2023), where succinic acid, ethanol and lactic acid are produced anaerobically (Dittrich et al., 2009). In the current study, both isopentyl acetate and 2-methyl-1-butanol can be considered as by-product of glycolysis, supporting energy production in anaerobic conditions, with highest concentrations found when intermediates of the glycolysis pathway were increased (T3). Amino acids can also serve as substrates or intermediates of anaerobic energy metabolism (Zurburg and De Zwaan, 1981), as seen by the peak of N-acetylglutamic acid (Fig. 7.3E) levels at 72 h, followed by a decrease towards the end of experiment. The transamination role of ketoglutaric acid is herein suggested, as seen in the mussel, *Mytilus edulise* (L.), when exposed to seasonal changes (Kluytmans et al., 1980). Upregulation of nicotinic acid (Fig. 7.3D) and its derivatives (nicotinic acid mononucleotide and ß-nicotinic acid mononucleotide) were seen as a peak at 96 h (T4) within the stressed group of mussels. Nicotinic acid (vitamin B3) contributes to important cofactors (like NAD^+) to regulate stress resistance and metabolism (Sauve, 2008). Nicotinic acid can also be converted to fumaric acid (TCA cycle) during catabolism (Das et al., 2023), and even though fumaric acid was not a statistically significant metabolite in the current study, the role of fumaric acid to improve ATP production during thermal stress cannot be ignored (Dong et al., 2022). Fumaric acid has been implicated as a biomarker of anaerobic metabolism in oysters (*Ostrea edulis*) (Eymann et al., 2020), and clams (*Mercenaria mercenaria*) (Hu et al., 2023) underpinning the involvement of this section of the metabolism with anaerobic energy production during heat stress in *P. canaliculus*. Nicotinic acid also plays an important role in the synthesis of precursors for pyrimidine nucleotides (Young et al., 2017), as shown in *M. coruscus* (Liang et al., 2023) and now in *P. canaliculus*.

Purine and pyrimidine metabolism

Purine and pyrimidine metabolism (Figs. 7.3, pathways H and I) were also altered in heat stressed *P. canaliculus* in the current study. Although no clear overall pattern can be described within the purine and pyrimidine affected metabolites, decreases hereof can indicate the restriction of substrates involved in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) turnover and repair mechanisms, as seen in *M. galloprovincialis* exposed to pollutants (Dumas et al., 2020). Towards the end (T5-120 h) of the heat stress challenge many purine metabolites (adenosine 5-diP, adenosine 3,5-CMP, adenylosuccinic acid, inosine, riboflavin, inosine 5-diP, 2-deoxyguanosine 5-diP) from the heat stressed mussels were decreased to the lowest point across all sampling times. Purine metabolites might have been utilised to protect organs and tissues against heat stress (Zhang et al., 2021), in *P. canaliculus*. Purine changes may also lead to impaired tissue maintenance, as reported in freshwater mussels subjected to

translocation stress (Roznere et al., 2017). Furthermore, the balance between energy demand and supply comes into question, as purine metabolism plays an essential role in energy supply (Liang et al., 2023), hinting at an energy deficit in the thermally stressed *P. canaliculus* following a five-day exposure to 24°C. The involvement of the purine salvage pathway has been highlighted as an ATP replenishment mechanism in *M. galloproviancilais* subjected to thermally induced hypoxia (Georgoulis et al., 2022); an area for further investigation in all mussel species. Increased levels of the purine metabolites, adenosine 5-monophosphate (AMP) and 2-deoxyguanosine 5-monophosphate in the heat stressed mussels at 72 h of the experiment. These purine metabolism changes co-insides with the increases found in glycolysis and TCA cycle metabolites, emphasising the protective roles of purine metabolites to support energy production. In addition, these two purine metabolites can provide energy and delay the accumulation of $NADH^+$ and H^+, which supports glycolysis and TCA cycle functioning (Haskó et al., 2004).

The pyrimidine metabolites, L-dihydroorotic acid and uridine-5-diP, detected in the current study (Fig. 7.3H), directly support the synthesis of uridine 5-monophosphate (UMP) and other pyrimidine nucleosides for the synthesis of DNA and RNA (Wang et al., 2021c). These pyrimidines were decreased by 120 h of heat stress, suggesting utilisation of these to produce nucleotides, and subsequent increased protein synthesis in relation to elevated temperature (Truebano et al., 2010). An increase in pyrimidine bases also occurred due to heat stress, mainly after 48h of thermal exposure. The pyrimidine metabolites, cytidine, 2-deoxyuridine, cytidine-5-monoP and 2-deoxyxytidine 5-diP showed an increased response (at T2), possibly indicating the activation of a tolerance mechanism to protect nucleic acids and protein synthesis (Das et al., 2017) against heat stress in *P. canaliculus*. Ultimately, exposure to a higher temperature may cause an increased requirement in thermally stressed mussels for pyrimidine metabolism due to increased RNA and DNA turnover, and potential cell damage encountered (Podrabsky and Somero, 2004).

Amino acid metabolism

The use of free amino acids to balance intracellular osmolality has been previously reported in *M. edulis* exposed to an array of stressors (Ellis et al., 2014), and comes to light in the current investigation. For example, changes in taurine (Fig. 7.3) levels can support osmoregulation functions (Meng et al., 2013). Taurine concentrations were the highest at 24 h, similar to other metabolites providing antioxidant functions, supporting the use of taurine to provide antioxidant and cytoprotective roles to mussels during thermal stress as seen in *M. galloprovincialis* (Georgoulis et al., 2022). Affected aromatic amino acids tryptophan, and phenylalanine (Fig. 7.3F), showed an increase at 96 h in mussels held at 24°C in the current

study, likely due to amino acid biosynthesis in response to stress (Shang et al., 2023). Then again, shikimic acid also showed an increase in metabolite response at 96 h of heat stress, serving as a precursor to aromatic amino acids, supporting the synthesis of proteins, vitamins, and structural blocks of electron carriers (Wu et al., 2022). The amino acid citrulline (Fig. 7.3, pathway E), was increased up to 48 h in the heat stressed mussels under investigation. Citrulline increased similarly in a recent study on heat stressed clams, as a means to balance the nitrogen and ammonia ions and prevent toxicity (Hu et al., 2023). Resultantly, the function of the urea cycle as a collection point for nitrogenous waste (Azizan et al., 2021) is demonstrated where citrulline concentrations decreased towards the last few sampling points (T4 and T5), to likely support ammonia detoxification and cellular osmoregulation (Wu, 2009). Increased pantothenic acid levels were seen in thermally stressed mussels in the current study 96 h into the experiment, possibly as a method to replenish coenzyme A (CoA) levels and maintain energy production in heat stressed mussels. Pantothenic acid (vitamin B5) is the precursor of CoA production, which is the essential cofactor of cellular metabolism involved in anabolic and catabolic reactions of lipids, carbohydrates, proteins, ethanol, bile acids and xenobiotics (Czumaj et al., 2020). Then again, amino acids also support de novo synthesis of purine nucleotides (Pareek et al., 2021)

Understanding mussel metabolite thermal response in an aquaculture context

When organisms are exposed to temperature ranges that exceed their optimum limits the outcome is survival or death, and even if survival is the result, the fitness of the organism will potentially be affected (Chen et al., 2019). The likelihood of surviving a marine heatwave event depends on a wide range of factors including the integrated thermal history of the mussel (Siegle et al., 2018), nutrition status, genetics, life-stage, pathogen-exposure, and reproductive stage (Ericson et al., 2023a). No studies to date (including the present study) have demonstrated significant mortality of *P. canaliculus* at 24°C in otherwise healthy mussels (Ericson et al., 2022), except when they are held at 24°C for many months (Ericson et al., 2023c) Seawater temperatures of 26°C, are detrimental to *P. canaliculus* survival and mortality occurs after several days at this temperature. This suggests that there is a tipping point at ~26°C where *P. canaliculus* cannot meet the physiological demands associated with this increase in temperature (Ericson et al., 2023a). From a metabolic perspective, *P. canaliculus* has the potential to adapt to heat stress up to 24°C for five-days by activating costly defence and repair mechanisms, which reallocate energy away from organismal growth towards maintenance. The investigation of temperature induced alterations at metabolite levels allows a background upon which management decisions can be founded and will support the development of tools that will further improve aquaculture techniques and mussel health. The use of metabolomics for the assessment of routine monitoring of mussel health in wild and farmed populations has applications beyond research. For example metabolite biomarkers can help to monitor the effects of environmental stressors on mussels (Waller and Cope, 2019). Considering that seasonal forecasting is nowadays being used to warn about marine heatwaves and inform management responses (Stevens et al., 2022), proactive steps can also be taken to promote mussel health. From the current study, it is clear that mussels exposed to a marine heatwave required additional energy for organismal functioning via aerobic and anaerobic processes, and upregulation of various metabolites to counter oxidative stress production. Hypothetically, these needs can be filled by ensuring adequate oxygen delivery to mussels and by supplementing their diets with amino acids. Dietary and nutritional management strategies have shown promising results to restore the effects of thermal stress in fish (Islam et al., 2022a). Strategies of increasing aeration on mussel farms have been investigated within the context of ocean acidification, and warrants future research to implement dropper aeration or integrated multi-trophic aquaculture to improve oxygen content in the face of climate stressors (Law et al., 2020). Open ocean aquaculture (Heasman et al., 2020), suspending mussel lines deeper in the water column and selective breeding for thermotolerance can also be used as mitigation strategies (Ericson et al., 2023a). Heat hardening (the transient response that improves thermal tolerance, and a plastic trait that can

be modified by acclimation to different thermal regimes) can be utilised to condition mussels to environmental conditions. This strategy has enhanced mitochondrial redox potential for oxidative defence capacity and respiration in *M. galloprovincialis* (Georgoulis et al., 2022) and remains a promising prospect for *P. canaliculus*.

7.4 CONCLUSIONS

Climate change has led to an increase in the frequency, duration, and intensity of marine heatwaves, which can cause physiological stress in musselsThese changes often involve synergistic processes and depend on the complex stoichiometric relationships of the host ecology, environmental context, and direct and indirect pathogens. While our investigation primarily aimed to assess a specific factor of heat stress associated with climate change, we acknowledge the hypothetical metabolic evidence provided regarding the complex dynamics of environmental interactions. Nevertheless, the current study presents new data concerning the potential adaptive response of *P. canaliculus* to a five-day marine heatwave exposure, along with metabolic insight on the dynamic nature of energy production in mussel haemolymph metabolites. Data from the current study may support management responses to mitigate the impacts of marine heatwaves on farmed *P. canaliculus*. In summary, mussels subjected to heat stress utilised sugar-based metabolites up to 48 h post-exposure, whereafter, a deficit of the carbohydrate sources likely promoted an increase of glucose production by 72 h to ensure ATP via substrate-level phosphorylation and the TCA cycle. Upregulation of the TCA cycle and the aspartate-succinate pathway followed by 48 h and 72 h of exposure to possibly allow rapid ATP production. The presence of oxidative stress within the thermally stressed mussels is likely at 24 h, with increased antioxidant molecules detected to scavenge ROS. However, the cellular redox status stabilised as time continued. Purine metabolism was depleted by 120 h, and pyrimidine bases were upregulated by 48 h of thermal exposure. Affected amino acids could potentially be involved in osmolality, biosynthesis, and ammonia detoxification functions. Ultimately, when marine heatwaves occur, *P. canaliculus* shows capabilities to respond and acclimate to thermal stress by regulating their energy metabolism, balancing nucleotide production, and implementing oxidative stress mechanisms over time. This study hypothesises that *P. canaliculus* has the capacity to respond to and survive short-term marine heatwave events, but their ability to rapidly acclimate to repeated events with longer timeframes remains unclear and warrants further investigation

Chapter 8: Interactive effects of elevated temperature and *Photobacterium swingsii* infection on the survival and immune response of marine mussels (*Perna canaliculus*): a summer mortality scenario

"Science knows no country because knowledge belongs to humanity and is the torch which illuminates the world."
- Louis Pasteur

From previous chapters, temperature stress and bacteria both induce stress in shellfish, therefore this chapter investigates the interactive effects of elevated temperature and *P. swingsii* infection on mussel survival and immune responses.

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The New Zealand Greenshell™ mussel (*Perna canaliculus*) is an economically important aquaculture species. Prolonged increases in seawater temperature above the long-term average pose a significant threat to mussel survival and health, potentially increasing susceptibility to bacterial infections. Using challenge experiments, this study examined the combined effects of increased seawater temperature and bacterial *Photobacterium swingsii* infection on animal survival, haemocyte and biochemical responses of adult mussels. Mussels maintained at three temperatures (16, 20 and 24°C) for seven days were either not injected (control), injected with sterile marine broth (injection control) or *P. swingsii* (challenged with medium and high doses) and monitored daily for five days. Haemolymph and tissue samples were collected from the low, medium and high temperature groups, at 24, 48, 72, 96, 120 hours post-challenge and analysed to quantify bacterial colonies, haemocyte responses and biochemical responses. Mussels infected with *P. swingsii* exhibited mortalities at 20 and 24°C, likely due to a compromised immune system, but no mortalities were observed when temperature was the only stressor. Bacterial colony counts in haemolymph decreased over time, suggesting bacterial clearance following by the activation of immune signalling pathways. Total haemocyte counts and viability data supports haemocyte defence functions being stimulated in the presence of high pathogen loads at 24°C. Oxidative stress responses, measured as total antioxidant capacity and malondialdehyde (MDA) levels, were higher in infected mussels (compared to the controls) after 24h and 120h post-challenge at the lowest (16°C) and highest temperatures (24°C), inferring the presence of oxidative stress due to temperature and pathogen stressors. Overall, this work indicates that mussels may be more vulnerable to bacterial pathogens under conditions of elevated temperature, such as those predicted under future climate change scenarios.

Keywords

Biomarker; Greenshell™ mussel; immune response; *Perna canaliculus*; *Photobacterium*; temperature; summer mortality.

8.1. INTRODUCTION

Endemic Greenshell™ mussels (*Perna canaliculus*) are the most valuable aquaculture export in New Zealand (NZ) (Castinel et al., 2019), with annual revenues of over NZD 303 M in 2021 (Miller et al., 2023). Environmental changes in NZ coastal areas, such as marine heatwaves, are increasingly common and result in mortalities and increased disease outbreaks in aquaculture farms (Heasman et al., 2020). Shellfish mortalities are generally associated with increasing summer temperatures (termed ‘summer mortalities’) and have been reported in different bivalve species such as *P. canaliculus* (Newton and Webb, 2019), *Mytillus*

galloproviancialis (Anestis et al., 2010), *Crassostrea giga*s (Malham et al., 2009, Samain, 2011) and *C. virginica* (Encomio and Chu, 2005). The exact causes of summer mortalities often remain unclear, but thermal stress caused by increasing seawater temperatures along with pathogen proliferation at these temperatures often contributes significantly to mussel mortality events (Li et al., 2020). Mussel-growing areas along the Coromandel coast (north-eastern NZ) and in other northern regions of NZ are experiencing higher sea surface temperatures (SSTs) in recent years (Stevens et al., 2021). Depending on the thermal intensity and duration, this warming can lead to marine heatwaves (MHWs), which are periods where SSTs exceed the seasonally varying 90th percentile for at least five consecutive days (Oliver et al., 2017). In the Marlborough Sounds, a key NZ mussel farming location, SSTs above 20°C are currently recorded during summer months, which is beyond the normal range for optimum mussel growth and survival (Broekhuizen et al., 2021). Notably, the projected increase in warming events in the next century will impact mussel aquaculture across most regions in New Zealand (Srinivasan et al., 2021).

The physiological effects of thermal stress on mussels are complex and have been widely studied. For example, *M. galloprovincialis* acclimated at 18 and 25°C showed significant differences in certain metabolites specific to environmental temperature variations (Frizzo et al., 2021). In a short-term (24-hours) laboratory experiment, *P. canaliculus* showed signs of physiological stress at 24°C (Ericson et al., 2022). In a chronic (15-months) heat stress exposure experiment, the overall biological performance of *P. canaliculus* was impacted by elevated temperature, with a survival tipping point noted between 21 and 24°C after many months; net mortality was 6, 10, and 100% at 17,21, and 24°C, respectively (Ericson et al., 2023c). Additionally, Delorme et al. (2021b) reported the accumulation of non-viable haemocytes in *P. canaliculus* following exposure to severe heat stress (30°C for 60 min). These studies reveal that the temperature range of 24–30°C exceed the optimum limits of fitness and survival of Greenshell^{TM} mussels. Few studies have investigated the combined impact of heat-stress and other drivers on Greenshell^{TM} mussel physiology. For example, food limitation in juvenile *P. canaliculus* had been shown to reduce the ability of mussels to cope with a subsequent heat stress (Delorme et al., 2020a). However, further investigation is needed to elucidate the precise nature of interactions among stressors, particularly the interacting effects of temperature and pathogens.

Field and laboratory studies have demonstrated that increased seawater temperature hastens the susceptibility of bivalves to pathogens, such as Ostreid Herpes virus (OsHV-1) (Segarra et al., 2010) and a range of *Vibrio* species (Newton and Webb, 2019). Microbe pathogenicity such as bacterial virulence factors and innate host immune response are thought to play a

major role in the survival and growth of wild mussel populations (Alfaro et al., 2019b). In contrast, at high seawater temperatures, certain bivalve species may present adaptations that allow them to improve their survival in the face of certain pathogens (Delisle et al., 2018). Previous laboratory studies have identified *Vibrio* spp. (*Vibrio splendidus* and a *Vibrio coralliilyticus neptunis*- like isolate [DO1]) as pathogenic to *P. canaliculus* larvae (Kesarcodi-Watson, *et al.*, 2009). In addition, *Photobacterium swingsii* (family of *Vibrionaceae*) isolated from moribund mussels were used in a laboratory challenge experiment which confirmed that this bacterium causes high mortalities in adult *P. canaliculus* (Azizan et al., 2022). Furthermore, thermal stress and *Vibrio* sp. DO1 stressors resulted in oxidative stress, inflammation, and changes in amino acid metabolism in adult *P. canaliculus* (Ericson et al., 2022). These studies provide new insights into the effects of pathogenic bacteria on *P. canaliculus*. However, the rate of bacterial proliferation and the host's response to bacterial stress over time is currently unknown.

Key physiological responses to pathogens and thermal stress occur within the fluidic (haemolymph) and cellular (haemocytes) components of the invertebrate circulatory system (Muznebin et al., 2022a). Haemolymph and haemocytes are responsible for supplying organs and tissues with essential nutrients and are crucially involved in immunological responses and homeostatic processes (Rolton and Ragg, 2020). Biomarkers such as total haemocyte count (THC) and haemocyte viability have been shown to be useful to monitor changes due to temperature variations in *Mytilus edulis* (Mackenzie et al., 2014), *M. coruscus* (Wu et al., 2016), *P. virdis* (Wang et al., 2011) and *P. canaliculus* (Delorme et al., 2021b). In addition, THC and haemocyte viability have been widely applied to evaluate the health state of animals in response to pathogen exposure, in both natural environments and laboratory experiments (Ford et al., 1993, Oubella et al., 1996, Allam et al., 2006, Nguyen et al., 2019c, Ericson et al., 2022). Haemolymph also contains antibacterial factors and lysosomal components, which function together with the haemocyte cytotoxic and phagocytic process to ensure the clearance of pathogenic bacteria (Bettencourt et al., 2009). In mussels (*M. galloprovincialis)* infected with *V. splendidus*, no bacteria were observed in the haemolymph after 24-hours, which highlights the bacterial clearance efficiency of mussels (Parisi et al., 2019). In addition, Allam et al. (2002) reported bacterial clearance in the clam, *Ruditapes decussatus,* after three days of being infected with *V. tapetis*. In contrast, bacterial clearance in *Crassostrea virginica* and *R. philippinarum* has been reported to take around two-weeks (Froelich and Oliver, 2013). Data on bacterial clearance efficiency of mussels when exposed to multiple stressors remains scarce but adds value when evaluating a change in pathogenic resistance within mussel haemolymph.

Combined exposure to suboptimal temperatures and pathogen infections exacerbates cellular oxidative stress in marine invertebrates and fish (Abele and Puntarulo, 2004). Mussels can control increasing levels of reactive oxygen species (ROS) by activating cellular antioxidant defence systems (Boukadida et al., 2017), while antioxidants also protect cells from the negative effects of oxidative stress (Lesser, 2006). Oxidative stress biomarkers are used frequently in mussel studies to determine the imbalance in the production of ROS (Azizan et al., 2023d). Antioxidants (e.g., superoxide dismutase, catalase, glutathione peroxidase) help protect mussels against oxidative stress (Wenning and Di Giulio, 1988). Measurements of total antioxidant capacity (TAC) are useful for evaluating the overall ability of a biological system to counteract ROS (Namiesnik et al., 2008) and effectiveness of different antioxidants in mussel to reduce oxidative stress (Fraga et al., 2014). In previous reports, *P. canaliculus* has been shown to have decreased TAC in response to heat stress (Delorme et al., 2021b) and *Vibrio* sp. infection (Ericson et al., 2022).

Typically, generated ROS in infected cells is associated with the formation of lipid peroxides in cell membrane (Farmer and Mueller, 2013). When the free radical formations surpasses their rate of elimination by antioxidant system, oxidative damage occurs such as lipid peroxidation (LPO) (Gutteridge and Halliwell 2006). LPO breakdown products (e.g. malondialdehyde [MDA], lipofuscin particles) can thus be used as effective oxidative damage biomarkers (Taylor et al., 2017). Changes in lipid composition in severely stressed organisms may indicate changes in lipid fluidity and permeability, altering ion transport capacity, and eventually inhibiting metabolic processes in shellfish (Jimenez et al. 2016). Reports in freshwater mussels namely, *Coelatura aegyptiaca*, *Mutela rostrata* and *Chambardia rubens* (Said and Nassar, 2022) and green mussels, *Perna viridis* (Wang et al., 2018a) indicated that elevated levels of LPO products following thermal stress were indicative of high oxidative assault on cellular components. While temperature stress and pathogenic bacteria are both factors that induce oxidative stress in shellfish, it is unclear how the combination affects mussel survival and physiology.

The aim of the present study was to investigate the combined effect of temperature and bacterial infection (*P. swingsii*) on *P. canaliculus* survival, bacterial clearance, total haemocyte count, and haemocyte viability over a five-day challenge experiment. Additionally, oxidative stress markers (total antioxidant capacity and lipid peroxidation) were assessed to infer the redox biology of mussels when exposed to multiple stressors. These experiments provide an insight into the potential resilience or vulnerability of mussels to bacterial pathogens under elevated temperature. It is envisaged that the findings of this work will advance our knowledge of bivalve health, particularly from risk forecasting standpoint which will assist the development

of animal health strategies and policies for mitigating the risks of future summer mortality events.

8.2. METHODS AND MATERIALS

8.2.1 Experimental setup

The experimental setup consisted of a group of mussels that were used to monitor mortalities over a seven-day period (collectively referred to as the 'monitoring cohort'), and a separate group of mussels used to destructively sample animals over a five-day period (referred to as the 'sampling cohort'). The two groups of animals were treated in the same manner, except when stated otherwise.

Animal husbandry

Adult *P. canaliculus* (n=1 200; mean shell length [± SD] = 74 ± 6 mm) were collected from a Marlborough Sounds mussel farm in May 2022 (seawater temperature at the time of collection was around 16ºC). The animals were transported to the Cawthron Institute Te Wero facility in Nelson, New Zealand. In the laboratory, the mussels were randomly assigned to three racks of 36 X 8 L tanks (108 tanks in total housing 11 mussels per tank) equipped with UV filter, individual airlines, and with continuous recirculating seawater. Water temperature was kept at an ambient condition of 16°C for six days to allow mussel recovery after collection and transport. Mussels were fed with a mixture of 50:50 *Tisochrysis lutea* and *Chaetoecerous mulleri* microalgae. Water ammonia, nitrite, and nitrate levels were monitored throughout the recovery and the experimental period. Mortalities of the monitoring and sampling cohorts were checked and recorded daily. Mussels displaying uncontrolled gaping were assessed using the British Standard Squeeze method, i.e., if the mussels' shells stayed apart after 10 general squeezes the animals was classified as dead and removed from the system (Dunphy et al 2015).

Temperature exposure

Experimental temperature conditions were adjusted to achieve three temperature regimes (16, 20 and 24°C) with 36 tanks containing 11 animals per tank. The 16°C temperature represented the control temperature (mean summer temperatures in the Marlborough Sounds, and the temperature of the seawater at the time of mussel collection). The 20°C and 24°C temperature conditions were achieved by slowly increasing the seawater temperature by 1°C per day over 7 days. Temperatures were regulated via a heat exchanger connected to a hot/cold loop,

controlled by solenoid valves, and monitored daily (Fig. 8.1). Once the experimental temperatures were achieved, the bacterial administration section of the experiment was started.

Bacterial exposure

Prior to starting the injection challenge, a *P. swingsii* culture was prepared using CRYOBANK® bead cultures (Mast Group Ltd., UK) stored in glycerol and previously frozen at −80°C. A bead containing the *P. swingsii* culture was transferred into 50 mL marine broth (made up using 37.4 g of 2216 medium (Difco, USA), 1 L water; autoclaved for 15 min at 121°C). The culture was incubated at 22°C for 24h, then diluted in sterile fresh marine broth to obtain a suspension with an optical density (OD) of 1 at 600 nm. The culture was also plated on thiosulfate citrate bile sucrose agar (TCBS) plates up to dilutions of 1×10^{10} and plates were incubated for an additional 24h at 22°C to verify the CFU/mL of the bacterial stock culture (Azizan, et al*.,* 2022). This was done on day 13 to prepare a stock for injection into the monitoring cohort as well as on day 14 to prepare a stock for injection into the sampling cohort.

For the challenge experiment four experimental groups were implemented: 1) no injection control, 2) marine broth injection control, 3) injection of a medium dose of bacteria (10^7 CFU/mL), and 4) injection of a high dose of bacteria (10^9 CFU/mL). After 2-weeks of acclimation at the different temperature regimes, *P. swingsii* bacteria were injected into the posterior adductor muscle (bacterial administration starting on day 14 for injection of the monitoring cohort and on day 15 for injection of the sampling cohort). The no injection group was handled in the same manner as the injection groups, i.e., shells were opened and water was drained out, but without the action of injection (n=33 for monitoring cohort [11 mussels X 3 tanks] and n=66 for the sampling cohort [11 mussels X 6 tanks]). The marine broth (MB) injection control group was injected with 100 µL sterile marine broth into the posterior adductor muscle following opening of the shell with a blunt knife (n=33 for monitoring cohort and n=66 for the sampling cohort). The bacterial injection groups were injected with 100 µL of medium (1.3×10^7 CFU) and high (1.5×10^9 CFU) *P. swingsii* suspensions (in both instances n=33 and n=66 total for the sampling cohort) (Fig. 8.1).

After injection, each animal was returned to its tank. Treatments were handled sequentially until all groups were infected. Mussels within a specific temperature regime were handled at separate time (i.e., handling and infection started with mussels at 16°C then 20°C and lastly 24°C). Within a specific temperature regime, mussels were treated in a block-wise manner

starting with the no injection control group, followed by the marine broth injection group, and then the injection of a medium dose of bacteria and lastly injection of a high dose of bacteria. As soon as bacteria were present in the infected mussel tanks, the recirculating water system for the bacteria infected mussels was diverted to waste and clean seawater was added.

8.2.2. Mussel sampling

Within the sampling cohort a total of eight animals were sampled from the no injection, marine broth injection, and high dose bacterial injection groups, housed at low and high temperatures (16 and 24°C) at 5 timepoints; (24h, 48h, 72h, 96h and 120h hours post challenge [hpc]). At the time of sampling, mussels (following the same sampling sequence) were patted dry with paper towels, weighed to the nearest 1 g and the shell lengths were measured to the nearest 1 mm along the longest axis, using callipers. The animals were gently opened on the ventral posterior side to access the posterior adductor muscle. Using a pre-chilled 23-gauge needle attached to a 1 mL sterile syringe, approximately 500 µL haemolymph were collected and transferred to a pre-chilled microcentrifuge tube and split into a sub-sample for flow cytometry analyses and a sub-sample for bacterial plating (Fig. 1). The gill tissues (3x3 mm) of 8 mussels were dissected, placed in separated aluminium tin foils, and stored at –80°C for later total antioxidant capacity and lipid peroxidation analyses. Additionally, the sex of individual mussels sacrificed for haemolymph and gill sampling was identified by observing the colour of the gonad.

For flow cytometry analyses, 50 µL haemolymph were diluted equally with the same volume of autoclaved filtered seawater to create a working haemolymph stock. 50 µL of working haemolymph stock was added to 150 µL autoclaved filtered seawater, whereafter 1 µL of concentrated Muse® Count & Viability Kit (200X, MCH100104; Luminex) was added to the sample. The sample was vortexed, incubated at 18°C for 5 min, then analysed using a Muse Cell Analyzer (Ericson et al., 2021).

For bacterial plating, a 10 µL aliquot of pure haemolymph was spread onto TCBS agar using the spread plate technique (Demann, et al., 2019). All plates were incubated at 22°C for 24–48h before being counted manually to obtain CFU/mL of *P. swingsii* in the haemolymph.

For biochemical analyses (TAC and MDA, Section 8.2.3), gill samples from two biological replicates were pooled per treatment to account for potential sources of inter individual variability in the different experimental conditions, resulting in four pooled samples per treatment.

8.2.3 Biochemical assays

Total antioxidant capacity assay

Total antioxidant capacity (TAC) in gill tissue was analysed using a microplate antioxidant assay kit (CS0790, Sigma-Aldrich), following the manufacturer instructions and methods describe previously by Delorme et al. (2021b). Frozen gill tissue samples from two biological replicates were pulverised at liquid nitrogen temperatures using a liquid nitrogen-cooled mini mortar (Z756377, Merck). Approximately 100mg of frozen tissue powder from each sample were then transferred to pre-chilled 2 mL polypropylene bead-beating tubes containing ten 2.3 mm zirconia/silica beads. A volume of 500 µL of cold assay buffer was added to each sample and homogenised at low temperature using a MiniG bead beater and a cryo-block, which was chilled at –80°C before the homogenisation process. Samples were homogenised via two cycles of 45 sec at 1,500 rpm, then centrifuged at 4°C for 15 min at 17,000 × g. The supernatant was collected, placed in a new prechilled 1.7 mL microcentrifuge tube, and used for TAC analysis following the assay kit instructions. Absorbance was measured at 405 nm using a microplate reader. Calculations of antioxidant capacity in gill tissue were performed using a five-point calibration standard curve using Trolox (15–420 mM, R^2 = 0.995), and results are expressed as Trolox-equivalent antioxidant capacity (TEAC).

Lipid peroxidase assay

Lipid peroxidation (LPO) in gill tissue was measured according to procedure of Barrick et al. (2018). Frozen gill tissue samples (approximately 100 mg) were transferred to prechilled 2 mL polypropylene bead-beating tubes containing ten 2.3-mm zirconia/silica beads. A volume of 500 µL of cold assay Tris-HCl buffer was added to each sample to maximise the volume and was homogenised at low temperature using a cryo-block, which was chilled at –80°C before the homogenisation process to avoid oxidation process. Homogenisation was performed through two cycles of 45 sec at 1,500 rpm, then samples were centrifuged at 4°C for 15 min at 17,000 g. For each homogenate 90 µL were split in a 0.5 mL microcentrifuge tube for bicinchoninic acid (BCA) protein assay, and the remaining was divided into different microcentrifuge tubes for thiobarbituric acid reactive substance (TBARS) assay.

The protein concentration (mg/mL) was measured using the Pierce™ BCA Protein Assay Kit from ThermoFisher Scientific (23225). In brief, the BCA reaction mix was made according to the kit instructions (50:1, BCA Reagent A: B). A serial dilution of bovine serum albumin (BSA, 10 mg/mL) was used as protein standard. One µL of each BSA standard dilution or sample

was pipetted in a transparent 96-well plate in triplicate. Then, 50 µL of BCA reaction mix were added to each well with a multichannel pipette and briefly mixed using a plate shaker. Then, the plate was incubated at 37°C for 30 min. Finally, the protein concentration was measured spectrophotometrically at an absorbance of 560 nm. All measurements (standards and samples) were done in triplicate in each plate to evaluate the intra-assay variability.

The lipid peroxide content in gill tissues was determined using TBARS as described by Barrick et al (2018). The tissue homogenate (210 µL) was added to 1.2 mL of 1.2% phosphoric acid, 300 µL 154 mM potassium chloride (KCl) and 0.5 ml of 0.003M TBA. The mixture was made up to 2.2 mL then incubated for 45 min at 90°C. After cooling to room temperature, 300 µL of mixture were pipetted into a 96-well microplate and the absorbance was measured at 540 nm. As the standard, 1,1′, 3,3′-tetramethoxy propane (TMP) was used and treated in a similar way to the test mixture. The lipid peroxide concentration was expressed as nmoles TBARS released/mg. All measurements (standards and samples) were conducted in triplicate in each plate to evaluate the intra-assay variability.

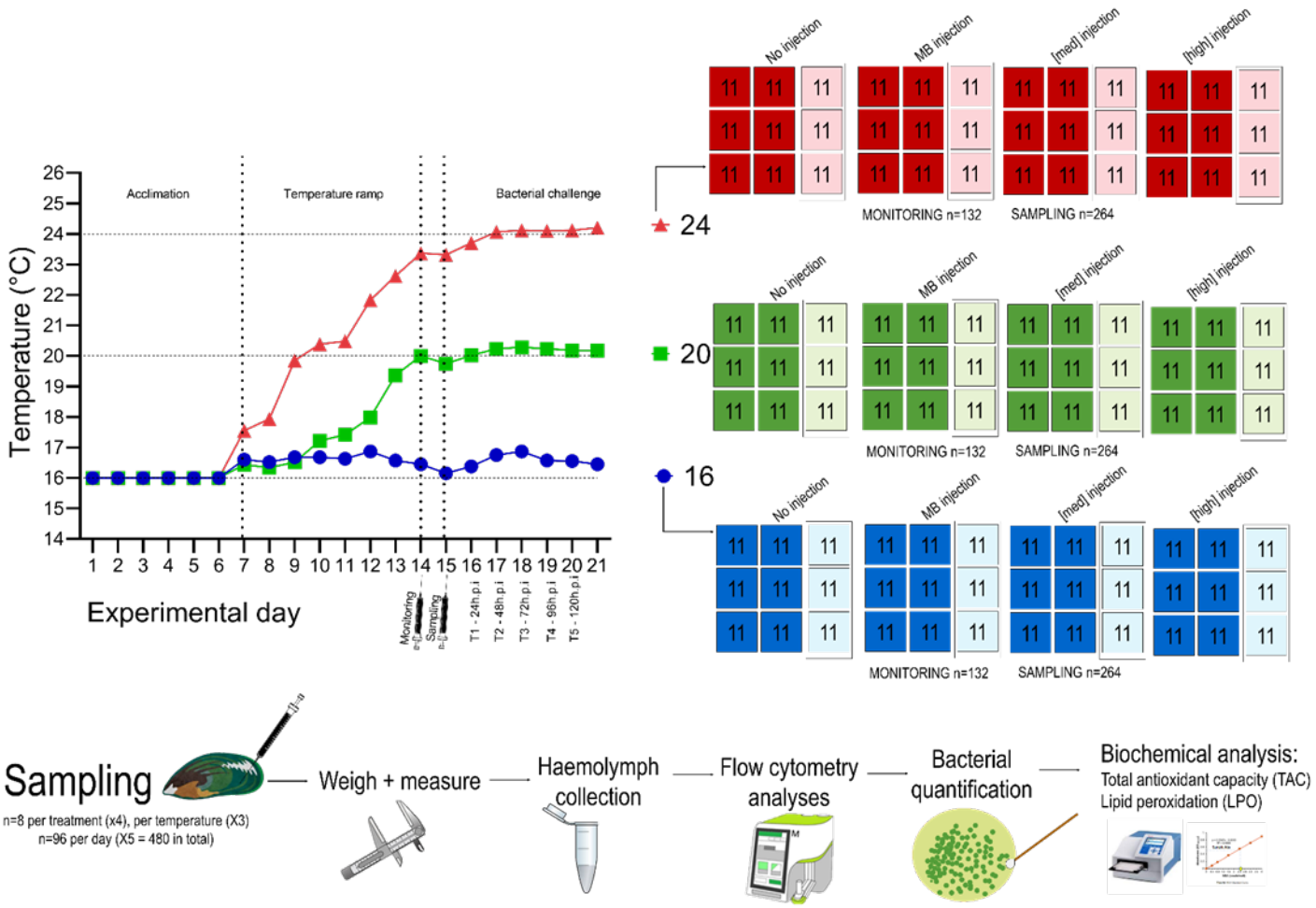

Figure 8.1. Experimental design for the thermal stress and *Photobacterium swingsii* challenge trial. Mussels were exposed to three different temperature regimes and exposed to a medium and high dose of bacteria, along with comparative controls. Haemolymph and gill samples from three treatment groups (no injection, marine broth injection, high dose of bacterial injection) at two different temperatures (16°C and 24°C) were collected from mussels at 24, 48, 72, 96, 120 post challenge (hpc), with total sampling numbers n = 238. Haemolymph samples were utilised for bacterial quantification and flow cytometry analyses. Gill samples were used for biochemical analyses. The lighter coloured tanks referred to the monitoring mussels and the darker ones are the sampling mussels.

8.2.4 Statistical analyses

Statistical analyses for survival, bacterial load, total haemocyte count, haemocyte viability and biochemical analyses data were carried out using R Studio software package (version 4.2.3) and a significance level of 0.05.

Kaplan-Meier survival analysis

Survival was analysed using a Kaplan-Meier analysis from the binary data (died or censored) of all mussels and treatments relating to the monitoring cohorts. To assess the significant differences ($\alpha = 0.05$) in survival probability curves among treatments, tests based on Chi-squared distribution (Log rank test) and pairwise comparisons were performed. A coxme model was also run with tank as a random factor, to test whether experimental tank influenced the results.

General linear model analyses

Mixed linear model tests were used to investigate the effect of temperature, treatment, timepoint and sex on the average bacterial load, total haemocyte count, haemocyte viability, and the oxidative stress response variables (TAC and LPO) investigated. In a preliminary analysis 'tank' was added as a random effect in generalised mixed effect models but did not show any significant effect so it was removed from subsequent models. We then constructed general linear models (GLM) with the same structure, and the model selection via AIC preferred the simpler GLMs. Therefore, all remaining analyses of the relationship between biological responses of the mussel [bacterial load, total haemocyte count, haemocyte viability, and the oxidative stress response variables (TAC and LPO)] and treatments, temperature and timepoints, were completed using general linear modelling (GLM). With the GLM models, if significant differences were found among treatments, a Tukey's posthoc test was performed. All data met assumptions for normality (Shapiro-Wilk Test, $p > 0.05$) and for homogeneity of variances (Levene's test, $p > 0.05$). All remaining analyses were therefore completed using general linear modelling (GLM). The following packages were used for visualising the data and modelling: 'tidyverse' (Wickham and Wickham, 2017), 'ggplot2' (Wickham et al., 2016), 'nlme' (Pinheiro et al., 2017), 'rstatix' (Kassambara, 2021), 'MASS' (Ripley et al., 2013), 'dplyr' (Jockers et al., 2020), and 'wesanderson' (Ram and Wickham, 2018). The power of the experiments (the likelihood that our tests would have detected a biologically meaningful effect had it existed) was determined by power analysis using the using pwr.f2.test in 'pwr' (Champely et al., 2018) package.

8.3 RESULTS

8.3.1 Survivability of mussels

For mussels within the monitoring cohorts, no mortalities occurred in the no injection or marine broth injected groups at any of the target temperatures (data not shown). The survival rate of different injection groups differed between seawater temperatures (Temperature*Group Log-rank: $X^2 = 127$, $p < 0.001$). At the medium bacterial dose, mussel survival at the end of the experiment was lower for the 24°C seawater treatment (52% survival rate; Fig. 8.2a) compared to 16°C seawater (88% survival rate; Fig. 8.2a) ($p = 0.004$). Survival rates were not different between mussels injected with the medium bacterial dose at 16°C (88% survival rate; Fig. 2a) and 20°C (73% survival rate; Fig. 8.2a) ($p = 0.209$). Within the high bacterial dose group, similar patterns were seen as in the medium injection group. Pairwise comparisons determined that mussel survival rates at 24°C (49% survival rate; Fig. 8.2b) were significantly different compared to the 20°C (79% survival rate; Fig. 8.2b) and 16°C groups (88% survival rate; Fig. 8.2b) ($p = 0.016$ and $p < 0.001$, respectively). Survival rates were also not different between mussels injected with the high bacterial dose at 16°C (88% survival rate) and 20°C (82% survival rate) (Fig. 8.2b; $p = 0.067$). The output from the coxme model showed that the variance attributed to tank as a random effect was <0.

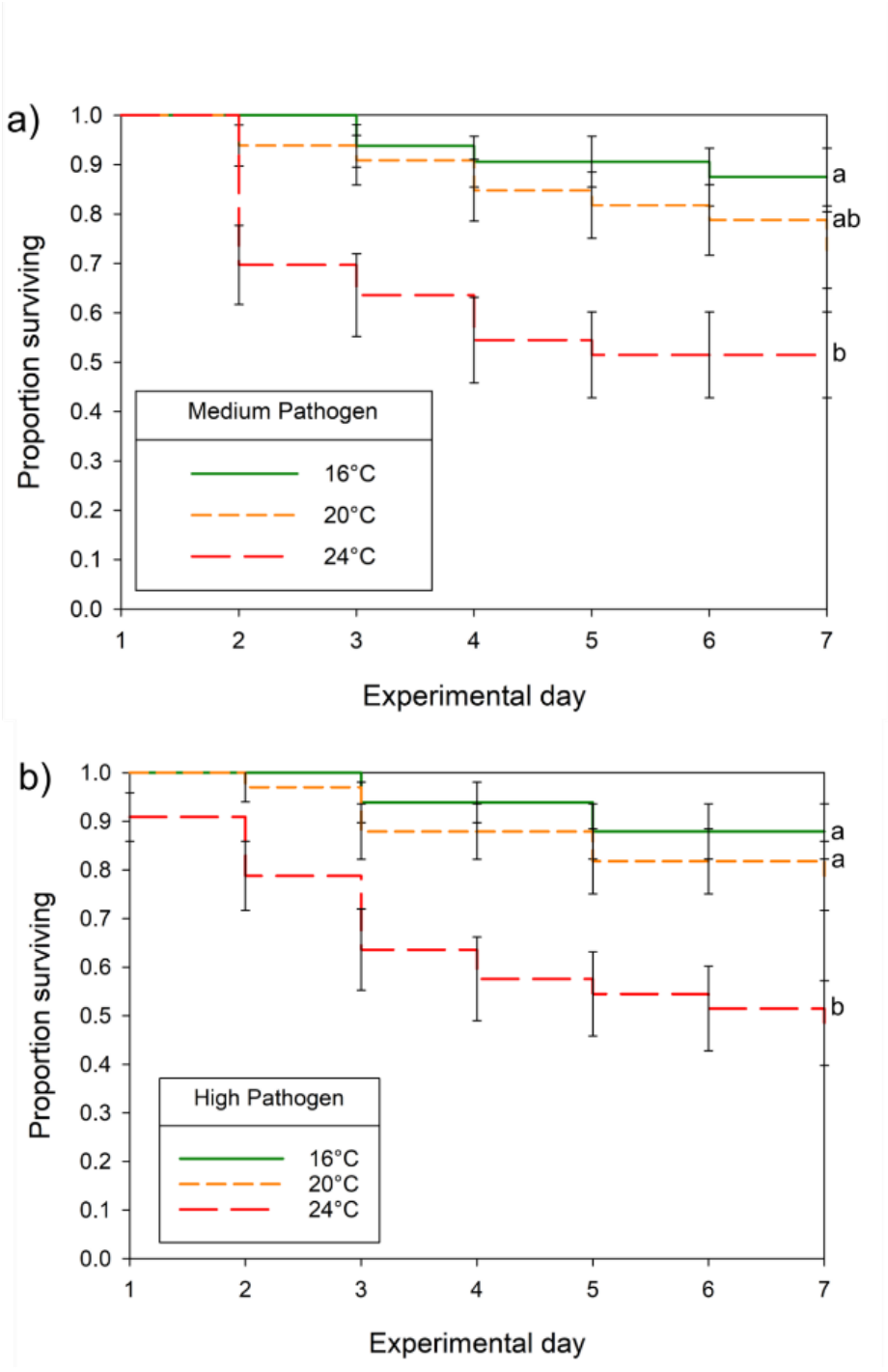

Figure 8.2. Kaplan-Meier survival trajectories (±SE) for *P. canaliculus* after injection with medium (a) and high (b) doses of *Photobacterium swingsii* at three different seawater temperatures 16°C (—— green line), 20°C (- - - orange line) and 24°C (— — red line).

8.3.2 Effects of *P. swingsii* and temperature on *P. canaliculus*

8.3.2.1 Bacterial counts

Haemolymph from mussels in the no injection and marine-broth injected groups showed no bacterial colonies at any of the sampling points (Fig. 8.3). Colony forming units (CFU) within the medium and high injection group were significantly higher ($p < 0.05$) when compared to the no injection control treatment. Significant interactions were observed in the CFU data between mussel haemolymph collected within the medium injection group at 48, 96, and 120 hpc, in comparison to the mussels in the no injection group sampled at 24 hpc (Table 8.1, Fig. 8.3). Also, significant interactions were observed for CFUs in mussels within the high injection group collected at 48 and 120 hpc.

Table 8.1 General linear model results for bacterial counts considering all experimental conditions, using the model: glm (formula = sqrt(Colonies) ~ Group * Timepoint , data = df_combined). Abbreviations: Injection of control-marine broth (MB), degrees of freedom (DF) and probability value (p). Significant values ($p < 0.05$) are illustrated as bold values.

	Estimate	Std. Error	Pr(>|t|)
(Intercept)	0.000	0.847	1.000
GroupMB injection	0.000	1.185	1.000
Group[med] injection	6.337	1.185	**<0.05**
Group[high] injection	6.780	1.185	**<0.05**
Timepoint48	0.000	1.185	1.000
Timepoint72	0.000	1.185	1.000
Timepoint96	0.000	1.185	1.000
Timepoint120	0.000	1.185	1.000
GroupMB injection:Timepoint48	0.000	1.667	1.000
Group[med] injection:Timepoint48	-4.353	1.667	**<0.05**
Group[high] injection:Timepoint48	-3.745	1.667	**<0.05**
GroupMB injection:Timepoint72	0.000	1.667	1.000
Group[med] injection:Timepoint72	0.524	1.667	0.753
Group[high] injection:Timepoint72	-2.655	1.667	0.112
GroupMB injection:Timepoint96	0.000	1.667	1.000
Group[med] injection:Timepoint96	-4.925	1.667	**<0.05**
Group[high] injection:Timepoint96	-1.452	1.667	0.384
GroupMB injection:Timepoint120	0.000	1.686	1.000
Group[med] injection:Timepoint120	-5.057	1.686	**<0.05**
Group[high] injection:Timepoint120	-5.940	1.667	**<0.05**

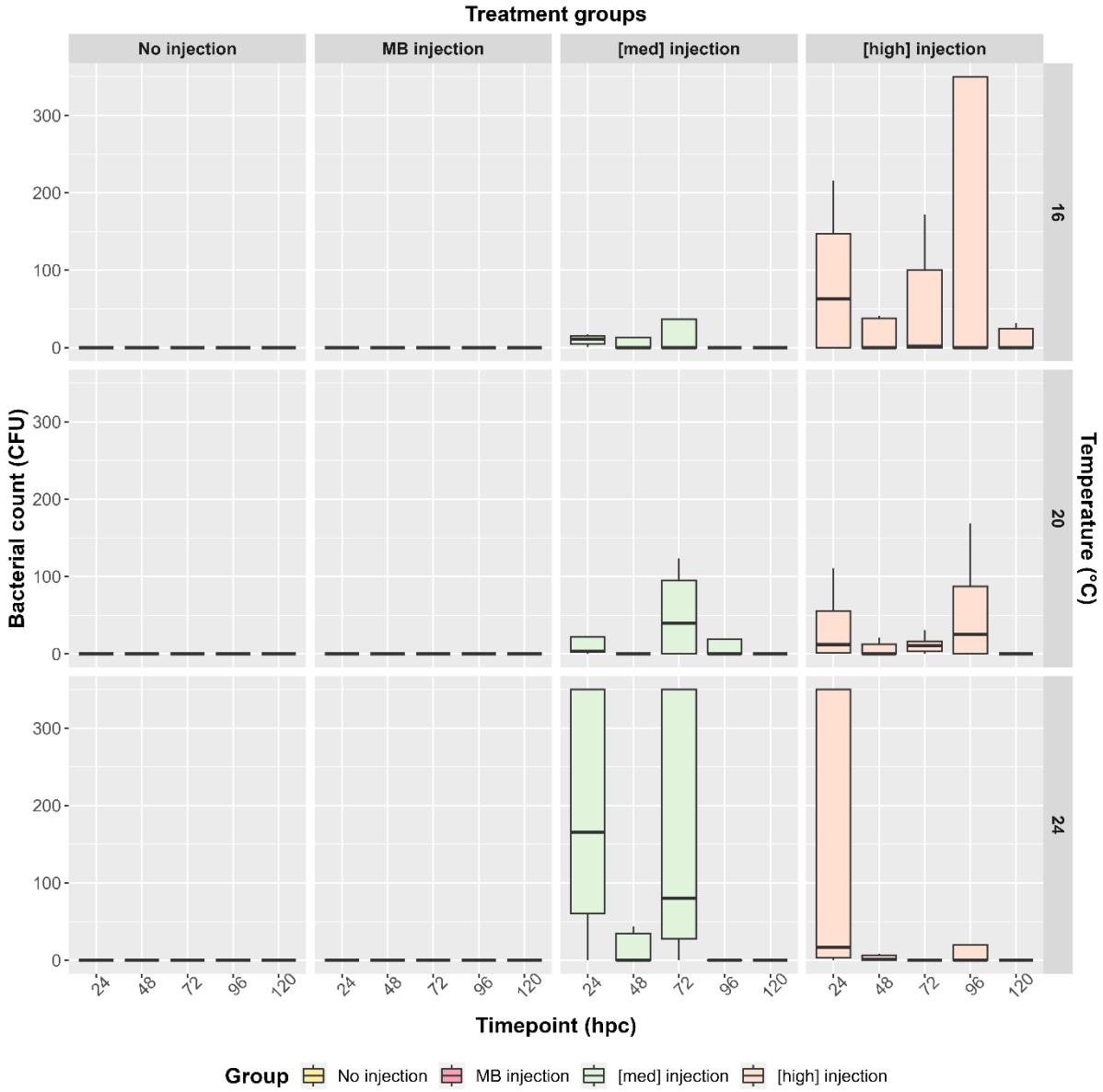

Figure 8.3. The combined effects of temperature, treatment and timepoints on bacteria count. Each panel compares the mean bacterial counts from *P. canaliculus* haemolymph following injection with a high and medium dose of *P swingsii,* marine broth (MB) injection control and no injection control, at low and high seawater temperatures (16, 20 and 24°C), across five timepoints (24, 48, 72, 96, 120 hpc).

8.3.2.2 Total haemocyte count (THC)

THCs from the medium and high injection group were significantly different ($p < 0.05$) when compared to the no injection control group. Mussels exposed to 24°C showed significant differences when compared to the baseline temperature at 16°C. The THC of mussels collected at 120 hpc were significantly lower ($p < 0.05$) compared to 24 hpc (baseline). Significantly higher interaction of THC was demonstrated between mussels held at temperature 20°C and collected at 120 hpc (Table 8.2, Fig. 8.4).

Table 8.2 General linear model results for total haemocyte counts (THC) considering all experimental conditions, using the model: glm (formula = log (THC) ~ Timepoint + Group + Temperature, data = df_combined). Abbreviations: Injection of control-marine broth (MB), degrees of freedom (DF)and probability value (p). Significant values ($p < 0.05$) are illustrated as bold values.

	Estimate	Std. Error	Pr(>|t|)
(Intercept)	15.097	0.150	**<0.05**
GroupMB injection	-0.047	0.100	0.640
Group[med] injection	-0.278	0.100	**<0.05**
Group[high] injection	-0.473	0.100	**<0.05**
Temperature20	-0.106	0.194	0.587
Temperature24	0.591	0.193	**<0.05**
Timepoint48	0.091	0.193	0.638
Timepoint72	-0.356	0.193	0.066
Timepoint96	-0.040	0.193	0.835
Timepoint120	-0.852	0.194	**<0.05**
Temperature20:Timepoint48	-0.139	0.274	0.612
Temperature24:Timepoint48	-0.431	0.273	0.115
Temperature20:Timepoint72	0.179	0.274	0.513
Temperature24:Timepoint72	-0.391	0.273	0.153
Temperature20:Timepoint96	0.229	0.274	0.404
Temperature24:Timepoint96	-0.189	0.273	0.489
Temperature20:Timepoint120	0.852	0.275	**<0.05**
Temperature24:Timepoint120	0.077	0.277	0.781

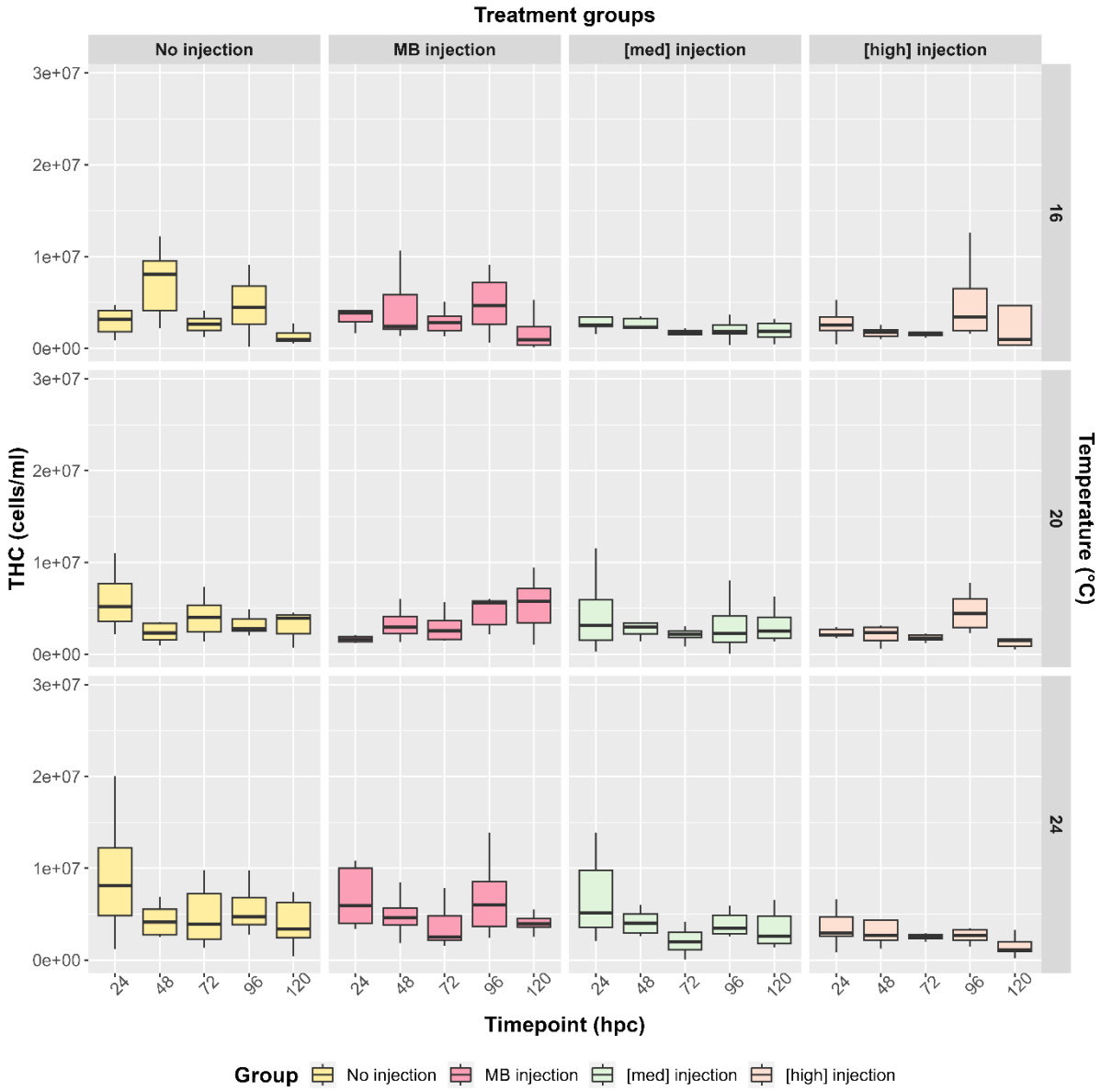

Figure 8.4. The combined effects of temperature, treatment and timepoints on total haemocyte count. Each panel compares the mean bacterial counts from *P. canaliculus* haemolymph following injection with a high dose of *P swingsii,* marine broth (MB) injection control and no injection control, at low and high seawater temperatures (16 and 24°C), across five timepoints (24, 48, 72, 96, 120 hpc).

8.3.2.3 Haemocyte viability

Haemocyte viability showed a significant difference ($p < 0.05$) between 24 and 48 hpc. Also, the high injection group significantly differed ($p < 0.05$) from the no injection group. No significant interaction was observed (Table. 8.3, Fig. 8.5).

Table 8.3. General linear model results for haemocyte viability considering all experimental conditions. considering all experimental conditions, using the model: log(Viability) ~ Temperature + Timepoint + Group, data = df_combined). Abbreviations: Injection of control-marine broth (MB), degrees of freedom (DF) and probability value (p). Significant values ($p < 0.05$) are illustrated as bold values.

	Estimate	**Std. Error**	**Pr(>|t|)**
(Intercept)	-0.043	0.015	**<0.05**
Temperature20	0.008	0.012	0.515
Temperature24	0.016	0.012	0.174
Timepoint48	0.028	0.015	0.063
Timepoint72	0.006	0.015	0.706
Timepoint96	0.007	0.015	0.637
Timepoint120	0.019	0.015	0.213
GroupMB injection	-0.021	0.014	0.133
Group[med] injection	-0.049	0.014	**<0.05**
Group[high] injection	-0.072	0.014	**<0.05**

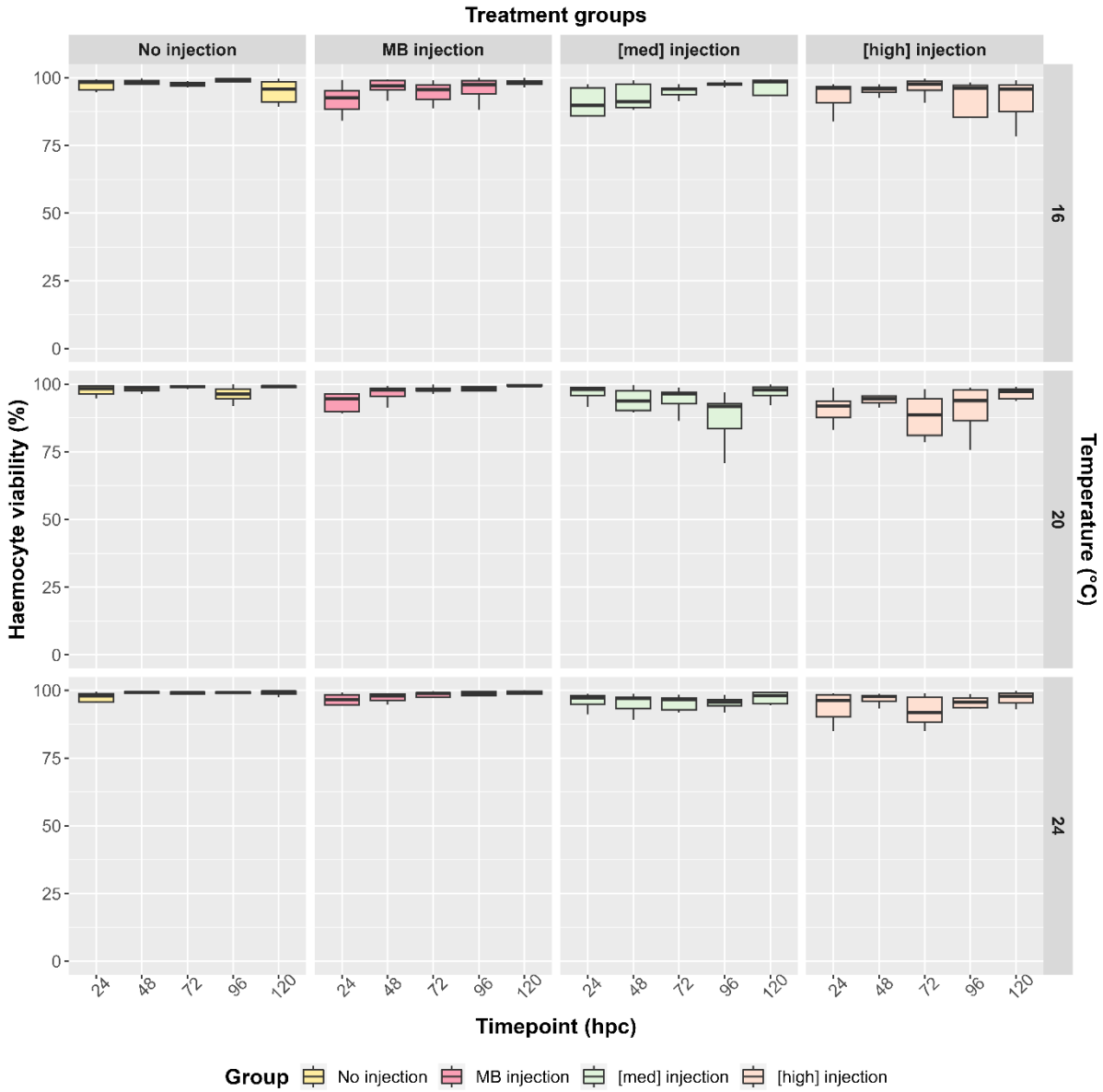

Figure 8.5. The combined effects of temperature, treatment and timepoints on haemocyte viability. Each panel compares the mean bacterial counts from *P. canaliculus* haemolymph following injection with a high and medium dose of *P swingsii,* marine broth (MB) injection control and no injection control, at low and high seawater temperatures (16, 20 and 24°C), across five timepoints (24, 48, 72, 96, 120 hpc).

8.3.3 Effects of sex on selected measurements

The sex of individual mussels were assessed, documenting 206 females, 228 males and 46 not identified mussels (Fig. 8.6a). Unidentified mussels had more CFU (178 ± 183 CFU) than males (24.7 ± 77.1 CFU) and females (20.4 ± 69.7 CFU) although this difference was not statistically significant (Fig. 8.6b). No significant difference in the THC (Fig.8.6c) and haemocyte viability (Fig. 8.6d) were found between sexes ($p > 0.05$).

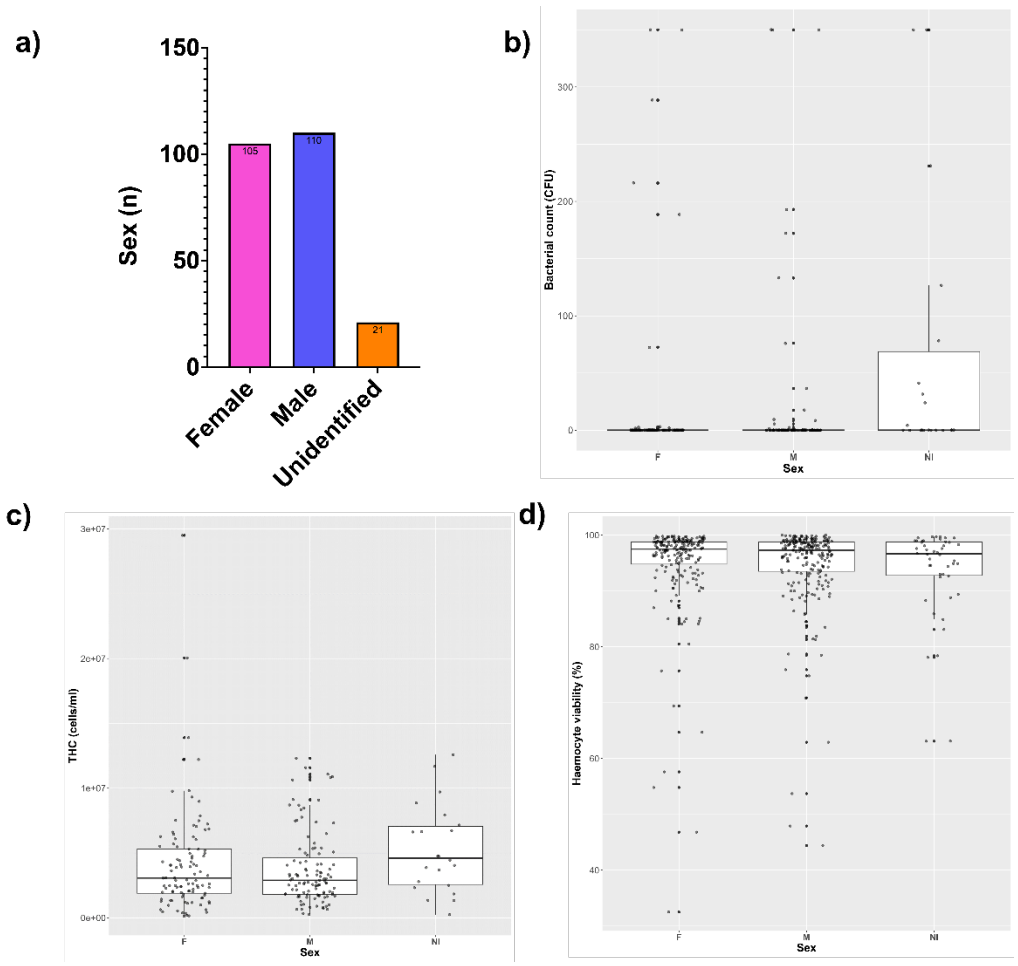

Figure 8.6. a) Proportion of female (F), male (M) and unidentified (NI) *P. canaliculus* in different treatment groups across timepoints. Individual mussel sex data (±SE,) collected from b) bacterial counts, c) total haemocyte counts and d) haemocyte viability.

8.3.4 Biochemical analyses

8.3.4.1 Total antioxidant capacity (TAC)

The mean total antioxidant capacity (calculated as trolox-equivalent antioxidant capacity [TAC]) of gill tissues were the highest in mussels injected with a high dose of *P. swingsii* (compared to the no injection and marine broth injection controls), at both 24 and 120 hpc and both 16 and 24°C (Fig. 8.7). TAC showed a significant higher ($p < 0.05$) between no injection group and high injection group. Also, TACs within the marine broth injection group were significantly lower ($p < 0.05$) when compared to the no injection control treatment. Mussels exposed to 24°C showed significant higher ($p < 0.05$) when compared to baseline temperature at 16°C. A significantly higher interaction ($p < 0.05$) was demonstrated for TACs in mussels within the high injection group collected at 120-hour post challenge (Table 8.4, Fig. 8.7).

Table 8.4. General linear model results for total antioxidant capacity (TAC) considering all experimental conditions, using the model: glm(TAC ~ Temperature + Group * Timepoint, data = df_combined). Abbreviations: Injection of control-marine broth (MB), degrees of freedom (DF) and probability value (p). Significant values ($p < 0.05$) are illustrated as bold values.

	Estimate	**Std. Error**	**Pr(>|t|)**
(Intercept)	124.083	7.120	**<0.05**
Temperature24	65.819	5.382	**<0.05**
GroupMB injection	-24.432	9.322	**<0.05**
Group[high] injection	63.125	9.322	**<0.05**
Timepoint120H	-0.055	9.322	0.995
GroupMB injection:Timepoint120H	7.387	13.183	0.578
Group[high] injection:Timepoint120H	33.415	13.183	**<0.05**

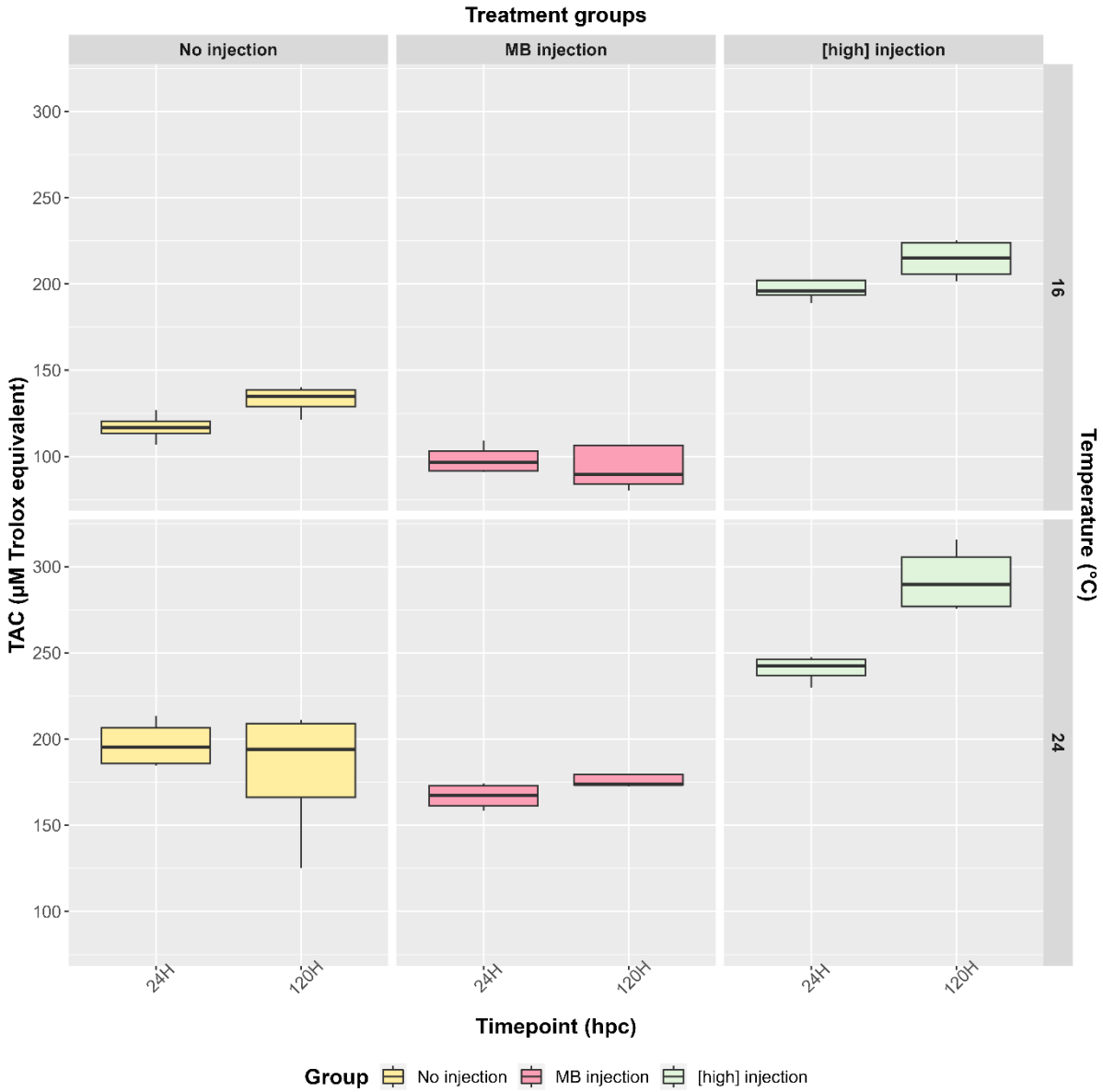

Figure 8.7. The combined effects of temperature, treatment and timepoints on Trolox-equivalent antioxidant capacity (TAC). Each panel compares the mean TAC from *P. canaliculus* haemolymph following injection with a high dose of *P swingsii*, marine broth (MB) injection control and no injection control, at low and high seawater temperatures (16 and 24°C), across two timepoints (24 and 120hpc).

8.3.4.2 Lipid peroxidation (LPO)

Malondialdehyde concentrations (representative of TBARS concentration) were the highest in gill tissues obtained from mussels injected with a high dose of *P. swingsi* at both temperatures (16 and 24°C) and timepoints (24 and 120 hpc) (Fig. 8.8). MDA levels within the high injection group were significantly increased ($p < 0.05$) when compared to the no injection control treatment. A significantly higher interaction ($p < 0.05$) was observed for MDA levels within mussels held at 24°C and receiving a high dose of infection in the gill tissues sampled at 120 hpc compared with mussels held at 16°C (Table 8.5, Fig. 8.8).

Table 8.5. General linear model results for lipid peroxidation (LPO) considering all experimental conditions, using the model: glm(formula = LPO ~ Timepoint * Group * Temperature, data = df_combined). Abbreviations: Injection of control-marine broth (MB), degrees of freedom (DF) and probability value (p). Significant values ($p < 0.05$) are illustrated as bold values.

	Estimate	Std. Error	Pr(>|t|)
(Intercept)	0.036	0.010	**<0.05**
Timepoint120H	0.002	0.014	0.884
GroupMB injection	-0.002	0.014	0.916
Group[high] injection	0.042	0.014	**<0.05**
Temperature24	0.012	0.014	0.418
Timepoint120H:GroupMB injection	-0.004	0.020	0.832
Timepoint120H:Group[high] injection	-0.017	0.020	0.414
Timepoint120H:Temperature24	-0.010	0.020	0.629
GroupMB injection:Temperature24	-0.006	0.020	0.774
Group[high] injection:Temperature24	-0.020	0.020	0.318
Timepoint120H:GroupMB injection:Temperature24	0.006	0.029	0.845
Timepoint120H:Group[high] injection:Temperature24	0.256	0.029	**<0.05**

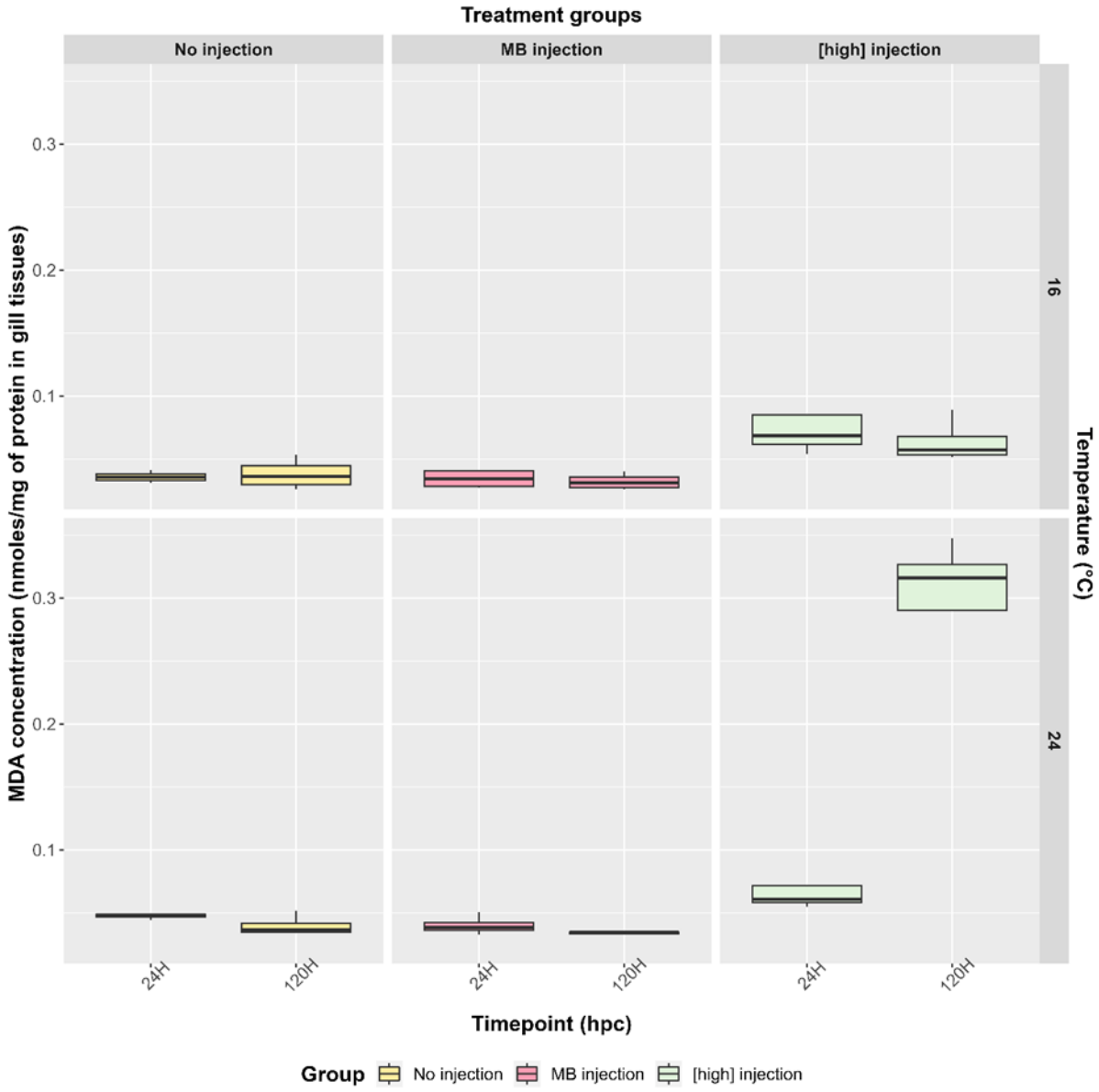

Figure 8.8. The combined effects of temperature, treatment and timepoints on lipid peroxidation (LPO) as a measure of MDA equivalents normalised by total protein (TBARS) determined from mussel gill samples. Each panel compares the mean TBARS from *P. canaliculus* haemolymph following injection with a high dose of *P swingsii*, marine broth (MB) injection control and no injection control, at low and high seawater temperatures (16 and 24°C), across two timepoints (24 and 120hpc).

8.4 DISCUSSION AND CONCLUSIONS

This study considers for the first time the combined effects of seawater temperature and bacterial challenge time on the host–pathogen relationship between *P. canaliculus* and *P. swingsii*. Following acclimation and a temperature ramping period, mussels were kept at the target temperatures (16, 20 and 24 °C) for five days, followed by different bacterial injection regimes. Using haemolymph samples, bacterial quantification and flow cytometry analyses

were performed, while gill tissues were used to determine the total antioxidant capacity and lipid peroxidation status. Building on previous *Vibrio* sp. pathogen and temperature experiments (Nguyen et al., 2018c, Ericson et al., 2022) conducted on *P. canaliculus*, this study gives further insight into infection physiology, and may inform risk management of future summer mussel mortalities.

Survival response

Temperature interactions with pathogenic bacteria and bivalve hosts have previously been associated with summer mortalities (Le Roux et al., 2015, Baker-Austin et al., 2017, Lupo et al., 2021). Also, bacterial (*Photobacterium* sp.*)* growth, adherence, and virulence factors are directly influenced by temperature (Reverchon and Nasser, 2013, Labella et al., 2017). In the current study, significant mussel mortalities occurred at 20 and 24°C only when *P. swingsii* was present, suggesting that the bacterial challenge drives mortality once thermal stress is already in place. We know that temperature stress causes mussel mortality over long time periods (> four-weeks) (Ericson et al., 2023c, Venter et al., 2023), opposed to short term exposures, which results in result in few mortalities (Delorme et al., 2021b, Ericson et al., 2022). This is confirmed in the current experiment where mussels subjected to temperature stress only survived following the five-day exposure period. The use of *P swingsii* as infectious agent (at doses of 10^7 and 10^9 CFU/mL) in *P. canaliculus* has previously resulted in mortalities when mussels were kept at 16°C (Azizan et al., 2022), supporting the notion that the bacterial (*P. swingsii*) challenge is primarily responsible cause for the mortalities observed. Yet, from the current investigation it is apparent that higher mortality occurs at higher temperatures, suggesting a synergistic effect when temperature stress and *P. swingsii* are present together. Our study parallels findings on Pacific oysters (*Crassostrea gigas*) where oysters infected with *Vibrio alginolyticus* at high temperatures (i.e., 33°C), had a higher mortality rate than oysters at a normal temperature (i.e., 23°C) (Li et al., 2023).

Bacterial quantification from host haemolymph

Mussels which survive infection have likely cleared the infecting pathogen from their system (Duneau et al., 2017). From the current experiment mussels injected with a high dose of *P. swingsii* kept at 16°C showed quantifiable numbers of bacterial colonies up to 120 hpc, suggesting that *P. canaliculus* has the capacity to retain viable pathogens for up to five days after infection when kept at 16°C. Little is known about the long-term survival of pathogens inside bivalves or the impact of their presence on the health status of the host. Previous studies have reported that *P. swingsii* can be lethal for adult and juvenile *P. canaliculus* (Azizan et al., 2022, Azizan et al., 2023c) and larval *M. edulis* (Eggermont et al., 2017). Bacterial quantification from mussel haemolymph kept at 24°C in this study showed quantifiable

bacterial colonies up to 96 hpc. A decline in bacterial counts within the first three sampling timepoints at 24°C was seen, suggesting the clearance of *P. swingsii* at this temperature when an abundance of bacteria was present (high dose). The faster clearance of *P. swingsii* at higher temperatures may be due to the bacterial susceptibility to haemolymph defence mechanisms or the stimulation of the haemocyte phagocytic reaction implanted by the mussels immune response in the early hours of the experiment (Canesi et al., 2002b, Parisi et al., 2008b). The infection of *M. galloprovincialis* with *V. splendidus*, (bacterial suspension 10^7), while at 20°C showed an increase in bacterial CFU at one- and six-hours post infection, with very few colonies detected at 24, 48 and 72 hpc (Parisi et al., 2019). Mussels in the current study were not sampled before 24h, raising some interest for future studies targeting shorter sampling times. Yet, at the timepoints when sampling took place in the current study, different bacterial quantities were detected, confirming that the kinetics of bacterial clearance may occur in a variable manner according to the particular *Vibrio*/bivalve interactions (Canesi et al., 2002b).

Mussel immune response by total haemocyte counts and haemocyte viability

The number of circulating haemocytes often reflects the magnitude of a systemic response to a stressors at a given time (Renwrantz et al., 2013), as seen in the current study in the presence of a high dose of *P. swingsii* . A decrease in total haemocyte counts (THC) overtime, where at both 16 and 24°C, THC declined up to 72 hpc within the high *P. swingsii* exposure group. In a previous study where *P. canaliculus* was infected with *Vibrio sp.* a decrease in haemocyte counts was seen (at 16 and 24°C), and attributed to the movement of haemocytes to different tissues to counter the bacterial infection, resulting in a lower number of haemocytes in the haemolymph (Ericson et al., 2022). In a different study (*P. canaliculus* infected with *P. swingsii*), an increase in THC from 12 to 48 hpc (housed at 16°C), possibly as a sign of stress (Azizan et al., 2023). Result suggested circulating haemocytes play a critical role in the encapsulation and phagocytosis of pathogens. The control treatments fluctuate within narrow limits, while the high dose infection group produced the lowest THC. This could be attributed to haemocyte mobilisation to different tissues and resulting in haemocyte count changes after exposure (Allam and Raftos, 2015). Moreover, Caza et al. (2020) demonstrated that haemocytes present in the circulation system of *Mytilus edulis desolationis* can exit the haemolymph after a few hours post infection, and move to the intervalvar fluid before being discharged into seawater. These external haemocytes can survive in seawater for a few hours and then enter healthy mussels and spread the bacteria within the host (Caza et al., 2020). Whether *P. swingsii* infected haemocytes can enter healthy *P. canaliculus* remains unknown, but this information would be useful to describe systemic infection in mussels.

In the present study, the haemocyte viability profiles showed small changes between treatments. Generally, the number of circulating haemocytes is not necessarily a reflection of the total size of the haemocyte population and can change rapidly due to associations between haemocytes and host tissues (Sokolova et al., 2004). Within the control treatments (no-injection and marine broth injection), the first sampling timepoint (24 hpc) mostly had the lowest haemocyte viability (at all temperatures), potentially due to handling stress. In a transport stress study on *P. canaliculus,* 24 hours of acclimation to laboratory conditions was deemed insufficient to achieve pre-transport haemocyte viability levels (Venter et al., 2021), highlighting that handling of mussels in the current study may have affected haemocyte viability. Within the high dose *P. swingsii* challenged group, haemocyte viability had more variation at a single sampling point, attributing to a diverse response pattern. Yet at both 16 and 24°C, the second timepoint (48 hpc) showed the highest haemocyte viability, with lower levels to follow at other sampling times. Lower haemocyte viability has been reported in bivalves due to *V. splendidus* (Cheikh et al., 2017), *Vibrio sp.* DO1 (*V. coralliilyticus*/*neptunius*-like isolate) (Nguyen et al., 2019c) and *V. tapetis* (Allam et al., 2006) infections. The decreasing numbers of circulating haemocytes found in the current study can probably be attributed to cell death induced by stressors, or signs of damage to the cell membrane (Tresnakova et al., 2023). The ability of bivalves to respond to environmental and pathogen stressors largely depends on the viability and functional capabilities of haemocytes but also on species of bivalve (Hégaret et al., 2003, Pruzzo et al., 2005). It is also suggested that haemocyte responses can be explained by the presence and composition of pathogen recognition receptors (PRRs) and antimicrobial peptides (AMPs) in mussel species (Le Guernic et al., 2020). PRRs in mussels may be recognised differently by haemocytes, and additional stressors (such as thermal stress), may lead to impairment of the recognition process (Canesi and Pruzzo, 2016). Consequently, haemocytes undergo a variety of immune responses, affecting the overall viability of the haemocytes, as seen in the current study. Knowledge of AMPs and PRRs in Greenshell™ mussels is still considerably lower than in blue mussels, and more research is necessary to establish a correlation to haemocyte viability.

Selected mussel oxidative stress markers

Total antioxidant capacity (TAC) and malondialdehyde (MDA) as a measure of lipid peroxidation (LPO) levels following *P. swingsii* infection at high (24°C) and control (16°C) temperatures provides insight into oxidative stress when dual stressors are present. Both TAC and LPO were successfully used to monitor oxidative stress in freshwater mussels exposed to temperature stress (Said and Nassar 2022), and green-lipped mussel spat from different seeding densities (Reyden et al., 2023). The present study showed increased TAC and MDA concentrations in the high dose *P. swingsii* injection group (compared to the controls) at 24

and 120 hpc at both 16 and 24°C. Moreover, injected mussels at 120 hpc at 24 °C had higher TAC and MDA concentrations than the injected group at 16°C. An increase in MDA was seen when infecting *Pinna nobilis,* with *Haplosporidium pinnae* (Box et al., 2020) and *Crassostrea virginica* with *Vibrio* sp. (Genard et al., 2011). The high levels of MDA along with a reduction in energy production prevented an adequate antioxidant response to resist the pathogen challenges in the above-mentioned studies. A similar outcome can be suggested for *P. canaliculus* where increased MDA levels were an outcome of increased LPO, which results from oxidative stress damage caused by temperature and pathogen stress. Temperature stress is believed to influence the immune status of molluscs, leading to increased overproduction of superoxide anion radicals ($O2^{\bullet-}$), as seen in *Perna viridis* where increased MDA levels were reported due to the generation of ROS in response to thermal stress (Wang et al. 2018).

Likewise, increased TAC concentrations were seen in the current study in *P. swingsii* infected mussels at both temperatures, reflecting a compensatory response due to increased oxidative stress levels (Matoo et al., 2013). Other studies have reported decreased TAC values due to the presence of stressors, suggesting the depletion of antioxidant defences following oxidative stress production (Kaloyianni et al., 2009, Rahman et al., 2019, Said and Nassar, 2022). Yet, an increase in TAC in response to stressors are not widely reported. For example, in *P. canaliculus* juveniles, an increase in antioxidant activities have been observed when mussels were exposed to air at different humidity levels (Truebano et al., 2010). Also, scallops showed higher baseline TAC concentrations than other bivalve species and are believed to have higher steady-state ROS production. Although, in oysters and clams, increased TAC concentrations were reported during oxygen deficient conditions, which appears to be an adaptation to tolerate rapid changes in oxygen availability (Ivanina and Sokolova, 2016). From the current study, an increase in TAC at the highest temperature and pathogen stress can be considered a response against oxidative stress, indicating that antioxidant defences were upregulated to counter the production of oxidative stress (Franco et al., 2016).

Dual stressors as trigger for summer mortality

For summer mortality events various environmental and physiological factors (temperature, nutrient levels, turbidity, salinity, bivalve growth rate and reproductive effort) have been identified as triggers (King et al., 2019). More often than not, mass mortalities are triggered by the synergistic effects of two or more factors (Soon and Zheng, 2020). Outcomes from the current study indicate that multiple stressors have a more severe impact on mussel survival and oxidative stress responses. Survival data from the present study showed higher

mortalities in mussels held at the highest temperature (24°C) infected with *P. swingsii* bacteria than the control groups. The increase of water temperature may lead to bacterial proliferation in water and bacterial accumulation in mussel tissues, leading to stress and mortality (Rahman et al., 2019). High levels of bacterial colonies were detected within the first 24 hpc in the haemolymph of mussels kept at 24°C when infected with *P. swingsii*. In addition, mussel oxidative stress markers were different in the treatment groups at the highest temperature. High water temperature is known to reduce immune responses of oysters and promote growth and virulence of pathogenic bacteria (Cowan et al., 2023). The same can be suggested for mussels where an interaction between *P. swingsii* and temperature stress is seen. This study also demonstrates that *P. swingsii* is an aquatic pathogen affects *P. canaliculus*. The current findings are essential to the development of effective animal health strategies and policies, required to progress risk assessment and management of aquatic diseases (Castinel et al., 2019). In this study, the importance of environmental conditions (i.e., temperature) on mussel survival and health is highlighted. Real-time environmental monitoring that predicts extreme temperature events remains key to mussel aquaculture and can allow stakeholders the opportunity to mitigate losses (Zhang et al., 2023b). Additionally, the collection of such data in the field via artificial intelligence could be a rapid and cost-effective solution to assist with disease management, water quality monitoring, and more (Bi et al., 2023).

In conclusion, this is the first report on the combined effects of temperature stress and *P. swingsii* infection on mussel survival, haemocyte parameters and antioxidant parameters, and provides new insights about physiological and biochemical responses of mussels exposed to two major environmental stressors. This five-day *P. swingsii* infection under elevated temperature exposure resulted in mussel mortality, haemocyte alterations and increases of both TAC and LPO levels. High temperature stress along with bacterial exposure can impose greater costs on mussel's physiological response and cellular redox status than a single stressor. Ultimately, the results will have a positive impact as pivotal information assisting with the development of animal health strategies and policies for mitigating risks.

Chapter 9: Investigating the effect of bacterial co-infections on juvenile and adult, green-lipped mussels (*Perna canaliculus*)

"The study of biology is the study of life itself, unlocking the secrets of how organisms function and interact with their environment."
- Jane Goodall -

This chapter now aims to assess metabolic response to effects of bacterial pathogen coinfection in mussels, utilising juvenile and adult Greenshell™ mussels exposed to a single pathogen and combination of multiple pathogens.

This chapter has been submitted for publication:

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The New Zealand Greenshell™ mussel (*Perna canaliculus*) aquaculture industry is being affected by summer mortality events associated with increasing seawater temperatures and pathogens. In this study, challenge experiments were conducted to investigate, for the first time, the effects of pathogen co-infection on survivability and haemolymph immune responses of juvenile and adult mussels. Animals were injected with marine broth (control), *Vibrio mediterranei*, *Photobacterium swingsii*, or a mixture of *V. mediterranei* and *P. swingsii*. Then, mussel survival was monitored for 72 hours, and haemolymph was sampled for bacterial quantification and metabolomics analyses at 24- and 48-hours post-challenge. Co-infected adults and juveniles showed 100% mortality. Bacterial colony counts in haemolymph decreased as infection time continued, especially in juveniles. The haemolymph metabolome of mussels exposed to single bacterial species and co-infection showed response changes largely within energy metabolism. Mussels infected with *V. mediterranei* exhibited increased metabolites linked to the glutathione pathway, branched chain amino acids, and others over time, supporting structural functions. Conversely, mussels infected with *P. swingsii* showed no metabolic differences over time. The co-infection group exhibited large decreases in important metabolites, such as fatty acids as an alternative energy source and amino acids to support immune functions and protein synthesis.

Keywords:

Bacterial co-infection; Green-lipped mussels; Metabolomics; *Photobacterium sp.*; *Vibrio* sp.

9.1 INTRODUCTION

Aquaculture is currently the fastest-growing food production sector in the world (Garlock et al., 2020). In New Zealand, the Greenshell™ mussel (*Perna canaliculus*) is the cornerstone of the country's aquaculture industry, with revenues estimated to be NZ$381 million in 2019 (Stenton-Dozey et al., 2021). However, over the past few years, mass mortality events have occurred at mussel farms throughout New Zealand, threatening the industry. During the summer months, increased seawater temperatures act as a stressor on mussels, impacting essential biological processes, such as metabolism, reproduction, growth, behaviour, immune response, and survival (Dunphy et al., 2018). Higher temperatures also augment the prevalence of bacteria in marine environments, including *Vibrio* species (Vezzulli et al., 2013). Although the exact reason for these mortalities is unknown, the combination of a weakened host immune system and increased bacterial loading suggest that pathogens may be the cause of these mortality events (de Burgh-Day et al., 2022).

Pathogens associated with mussel summer mortality are species, such as *Vibrio mediterranei* and *Photobacterium swingsii*. *V. mediterranei* is a well-researched pathogen, which has been isolated and identified as a dominant bacterium in ailing and dying fan mussels (*Pinna Nobilis*) during mass mortality events (Prado et al., 2020a). *P. swingsii* is known to be pathogenic in oysters (Fichi et al., 2015), abalone (Shi et al., 2017) and octopus (Gomez-Gil et al., 2011). *P. swingsii* and *V. meditteranei* have both been isolated and deemed as highly pathogenic strains in a previous study on Greenshell™ mussels (Azizan et al., 2022). As opportunistic pathogen, *V. mediterranei* appears to lay dormant in its host, but can cause disease when protective barriers are breached or when immunosuppression occurs (Prado et al., 2020a). Currently, there is limited information available specifically on the dormancy and coexistence patterns of *P. swingsii*. However, within the *Photobacterium* genus, some species for instance, *Photobacterium damselae* subsp. *piscicida* have been found to exhibit dormancy or enter into resting states under unfavourable conditions (Magarinos et al., 1994). During favourable conditions *P. swingsii* has exhibited the expression of virulence genes (*hsp60*, *zm*, *vcpA*, *toxR*, *ompU*, *mshA*, *chi*, *lip*, and *plp*), attributing to mussel mortalities in a challenge experiment, demonstrating pathogenic qualities of this bacterium (Azizan et al., 2022). Likewise, *V. mediterranei* has been reported to possess virulence mechanisms including adhesion, superoxide dismutase production, and toxin production (e.g., zona occludins toxin (*zot*)) (Reshef et al., 2008). Ultimately mussels host large quantities of opportunistic bacteria that can become problematic (related to quorum-sensing mechanisms / enhanced virulence factor production) when their host's defences are weakened (Eggermont, 2017), as seen during marine heatwave events (Ericson et al., 2023b). The mussel response in light of the combined stressors and multiple bacteria remains unknown and warrants further research.

Many previous studies have investigated the interactions between individual pathogens and their hosts. However, in the natural environment, it is more likely that mussels would be harboured by multiple pathogens simultaneously (Karvonen et al., 2019). Most cases of bacterial infections are caused by mixed infections, rather than one single species. Co-infections can alter disease dynamics by affecting pathogen transmission and virulence, host immune responses, and the effectiveness of disease control measures (Carella et al., 2020). Co-infections can also result in various outcomes, such as one or both pathogens being amplified, one or both pathogens being suppressed, or one pathogen being amplified and the other suppressed (Kotob et al., 2017). Currently, there is limited information on how bivalves respond to co-infections. Co-infections have been found to be the driver of mass mortality events in various species (Gay et al., 2004). Previous studies have found that one pathogen may promote proliferation of another pathogen, and can alter the progression and severity of disease (Arzul et al., 2012), pathogenesis and clinical outcomes, and influence the spread of

infections at a population level (Lassalle et al., 2007), creating disease outbreaks in corals (Ushijima et al., 2020), fish (Kotob et al., 2017), and other bivalves (Tracy et al., 2018). While most research investigating bacterial infections in mussels has been conducted on adult animals (Eggermont et al., 2014, Benabdelmouna et al., 2018, Rey-Campos et al., 2019b), it is believed that juvenile mussels are likely more susceptible to pathogenic infections . This can be attributed to smaller mussels having a lower tolerance to pathogens, since juveniles may not have fully developed immune systems (Coen and Bishop, 2015). Therefore, it is important that pathogen-mussel studies incorporate the hosts' different sizes and life stages.

Molecular, biochemical, and physiological biomarkers which respond to a stressful condition provide detailed insights into organism health (Delorme et al., 2021b). Metabolomics is an approach to detect changes in relevant metabolites within an organism, and can be used to describe complex biological systems at the metabolic level (Young and Alfaro, 2018), while also capturing a snapshot of the organism's physiological state at a given time (Alfaro and Young, 2018). Changes in metabolic pathways can inform how pathogen infections induce stress-related mechanisms and affect molecular regulatory processes (Li et al., 2020). To date, experiments studying *Vibrio* spp.-related infections in *P. canaliculus* using metabolomics have only focused on singular pathogenic infections (Nguyen et al., 2018c, Nguyen and Alfaro, 2020b, Ericson et al., 2022), highlighting that more research is needed to understand the responses of mussels during co-infections.

The aim of this study was to use a gas chromatography-mass spectrometry (GC–MS) based metabolomics approach to investigate physiological changes in the haemolymph of juvenile and adult *P. canaliculus* infected with *V. mediterranei*, *P. swingsii*, and a combination of both strains over a 48-hour timeframe. This study is the first to explore the mechanisms of pathogen co-infection in Greenshell™ mussels and support efforts to strengthen biosecurity management in New Zealand.

9.2 MATERIALS AND METHODS

9.2.1 Animal husbandry and bacterial culture

Mussels (n=200) were obtained from Kaiaua Marine Farms Ltd. (Firth of Thames, New Zealand) and transported to the Auckland University of Technology aquaculture facility. Upon arrival at the laboratory, mussels were divided into juveniles and adults based on size (juveniles 50-60 mm; adults 70-80 mm). The two groups were placed into separate seawater

re-circulating systems (temperature of 14°C; salinity of 35 ppt; and pH of 8.2) and acclimated for two-weeks prior to the start of the infection challenge.

Prior to the infection challenge experiment, isolates of *V. mediterranei* and *P. swingsii* (obtained from AUT's Aquaculture Biotechnology Research Group frozen culture library) were revived and suspended in sterile fresh marine broth to obtain a suspension with an OD_{600} of 1.0. The cultures were then quantified, and two single-species (*V. mediterranei* or *P. swingsii*) and a mixture (*V. mediterranei* and *P. swingsii*) solution were prepared with a concentration target of 10^7 CFU/mL to be used in the mussel challenge experiment. This bacterial concentration was selected as per previous mussel bacterial infection studies conducted in the same laboratory (Azizan et al., 2022).

9.2.2 Mussel bacterial challenge and sampling

Juvenile and adult mussels were divided into four experimental groups based on treatment (Fig. 9.1). Mussels were injected with 1) marine broth (MB), 2) *Vibrio mediterranei* (VM), 3) *Photobacterium swingsii* (PS), and 4) *Vibrio mediterranei* and *Photobacterium swingsii* (VM+PS). Additionally, the mussels from each of the four groups were divided into two cohorts: 1) monitoring cohort – used to track survival overtime (10 juveniles and 10 adults per treatment = 40 juveniles and 40 adults in total), and 2) sampling cohort – used to destructively sample mussels' overtime (20 juveniles and 20 adults per treatment = 80 juveniles and 80 adults in total).

To inject mussels, they were slightly opened, and a pipette tip placed between the valves. Then, a needle attached to a syringe was placed into the posterior adductor muscle. Injections were performed in the following treatment sequence: MB with 100 µL of marine broth, VM with 100 µL marine broth containing 10^7 CFU/mL *V. mediterranei*, PS with 100 µL marine broth containing 10^7 CFU/mL *P. swingsii*, and VM+PS with 100 µL marine broth containing a mixture of both *V. mediterranei* and *P. swingsii* (10^7 CFU/mL). Juveniles and adults in the monitoring and sampling cohorts were injected at about the same time within their treatments. After injection, the mussels were placed into individual 2-L tanks, aerated using air stones, and 50% of the seawater was replaced daily. The water temperature was monitored and maintained between 16 and 18°C. At 24 and 48 hours post-infection challenge, animals displaying uncontrolled gaping were assessed using the British Standard Squeeze method (Dunphy et al., 2015) – if the mussels did not adduct after ten squeezes they were classified as dead. Mussels were sexed during removal of the dead animals via visual observation of the colour of the gonads (white in males and orange in females). Observations on spawning occurrences

and water quality parameters (nitrite, nitrate, ammonia, and pH) were recorded before daily water exchanges.

At 24 and 48 hpc, haemolymph samples were aseptically obtained from each mussel (n=5 per treatment). A pipette tip was used to maintain the shell opening, and a pre-chilled needle (25-gauge) attached to a 3-mL syringe was inserted through the opening and into the posterior adductor muscle. Approximately 0.5-1.0 mL of haemolymph was sampled, of which 50 µL was transferred into a microcentrifuge tube for thiosulfate-citrate-bile salts-sugar (TCBS) agar plating, and 400 µL transferred into a cryo-vial and then flash frozen in liquid nitrogen for later metabolomics analyses (Ericson et al., 2022).

9.2.3 Bacterial clearance measurements

Aliquots of 10 µL of undiluted haemolymph were spread in duplicate onto TCBS agar media. Agar was used for heterotrophic plate counting because this selective medium provides better support for stressed cells and produces discrete (larger) colonies overnight. Dark milky green colonies were counted as total *V. mediterranei* colonies, light green colonies were counted as total *P. swingsii* colonies, and bacterial loads were estimated as a colony-forming unit (CFU) formed after 24-hour incubation at 22°C. Data obtained from two plates were expressed as mean ± standard error (SE) for each time point.

9.2.4 Metabolite profiling

Frozen haemolymph (400 µL), together with 10 µL of internal standard (10 mM L-alanine-2,3,3,3-d_4 concentration on column, prepared in water) were dried under vacuum for 4 h at 0°C. A two-step sequential extraction method was used for metabolite extraction by adding 500 µL of cold methanol: water solution (50% MeOH:50% H_2O) to the dried samples, vortexed for 1 min, and centrifuged for 10 min at 20,8003*g* at -9°C. The supernatants were collected and transferred to a new tube. The sample pellets were re-extracted by adding 500 µL cold methanol: water solution (80%MeOH:20%H_2O), followed by vortex and centrifugation (as earlier). After combining and freezing the supernatants, the samples were dried in a SpeedVac concentrator before derivatisation (Venter et al., 2021).

Extracted metabolites were derivatised by methyl chloroformate (MCF) alkylation using an established protocol (Smart et al., 2010). Dried extracts were resuspended in 400 µL 1M sodium hydroxide (40 g NaOH/1,000mL H_2O) and transferred to salinized borosilicated glass tubes containing 334 µL methanol and 68 µL pyridine. While keeping the samples on a vortex,

a volume of 40 µL MCF reagent (Sigma-Aldrich, M35304) was added to the samples and vortexed for 30 s, followed by a second volume of 40 µL MCF reagent for 30 s. Then, 400 µL of chloroform were added and vortexed for 10 s, followed by the addition of 800 µL 50 mM sodium bicarbonate (4 g NaHCO3/1,000 mL H_2O) and vortexed for a further 10 s. The mixture was centrifuged for 5 min at 1,1743 *g* at 6°C. The upper aqueous layer was discarded, and approximately 30 mg of anhydrous sodium sulphate was added to remove residual H_2O. The chloroform phase containing the MCF derivatives was transferred to 2-mL amber CG glass vials fitted with inserts. Quality control (QC) samples were included in every batch by preparing a pooled mixture of the haemolymph or tissue samples. These QC samples were included within the biological sample batches and treated no differently than the samples of interest. The QC samples were injected at regular intervals throughout the analytical run of the analysed batch to measure repeatability and identify any potential batch effects in the data. A separate standard amino acid mix (100 µL, 20 mM Sigma-Aldrich, 79248) and a sample blank containing 10 µL of the internal standard were similarly derivatised for QC purposes (Young et al., 2019).

Derivatised samples were analysed on an Agilent 7890A gas chromatograph (GC) coupled to an Agilent MSD5975C mass spectrometer detector (Agilent Technologies, CA, USA), with an electron ionisation (EI) source operated at 70 eV. The system was equipped with a DB-1701 GC capillary column (30 m × 250 µm internal diameter × 0.25 µm film thickness) (Agilent, Santa Clara, CA, USA). Helium was used as the carrier gas and was held at a constant flow of 1 mL/minute. Samples (1 µL) were injected under splitless mode with the injector temperature set at 290°C. The GC-oven temperature was initially held at 45°C for 2 min and then raised with a gradient of 9°C/min to 180°C; after 5 min, the temperature was increased from 40°C/min to 220°C. After a further 5 min, the temperature was increased from 40°C/min to 240°C and held for 11.5 min. Finally, the temperature was increased at 40°C/min until it reached 280°C, where it was held for a further 16 min. The interface temperature was set to 250°C, and the quadrupole temperature was set to 250°C. The mass spectrometer was operated in scan mode, starting after 5.6 min, with a mass range of 40–600 atomic mass unit (amu) and a scan time of 0.1 sec. Identification of compounds was carried out using mass spectra acquired in scan mode from 40 to 600 amu, with a detection threshold of 80 ion counts (Smart et al., 2010). A derivatised sample blank containing the internal standard, a standard amino acid mix, and a sample of pure chloroform solvent were also injected and analysed for QC purposes. MCF samples were derivatised and injected in four sub-batches over four consecutive days. Samples were completely randomised, and QCs were injected five times at regular intervals to account for potential within-batch signal drift (Young et al., 2019).

Deconvolution of chromatographic data and metabolite identification was performed using the Automated Mass Spectral Deconvolution and Identification System (AMDIS v. 2.66) software based on an in-house mass spectral library of MCF-derivatized commercial standards. Compound identifications were based on matches (> 70%) to both the MS spectrum of the derivatised metabolite and its respective chromatographic retention times. As such, the identified compounds can be assigned a Level 1 and 2 identification and the unknown features receiving a Level 3 confidence interval (Schymanski et al., 2014). MassOmics, version 2.5 (Guo et al., 2021), a Windows-based data extraction application, was used to generate a composite list of all metabolites detected in the dataset, containing metabolite identifications, mass spectral identification scores, the most abundant ion for each library match, the number of times each metabolite was detected in the whole dataset, and the retention time drift for each metabolite. A Microsoft Excel file containing peak height data for each metabolite was generated and manually checked for the presence of contaminants (e.g., MCF derivative artifacts). Data were blank-corrected, and aberrant records were removed. The data matrices of peak intensities were pre-processed for QC purposes and to meet the distributional requirements before statistical analyses using the web-based tool MetaboAnalyst 4.0 (Chong et al., 2018). Data were normalised against the internal standard (Venter et al., 2021).

9.2.5 Data analysis

Mussel weight, length, and number of bacterial colonies were analysed with two-way analysis of variance (ANOVA). The different treatment groups (marine broth control, *Vibrio mediterranei, Photobacterium swingsii,* and co-infection), time points (24, 48 hpc), and mussel life stage (juvenile and adult) were selected as within-subject factors, with dependent variables of weight (g), length (mm), and number of bacterial colonies. Tukey pairwise comparisons were used for all analyses to examine the significant differences among the factor levels. When interactions were non-significant these terms were removed from the model to test for single-order effects alone. Survival data were analysed by a Kaplan–Meier survival plot. All statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) with an alpha level of 0.05. For metabolomic data, univariate and multivariate analyses were conducted using MetaboAnalyst 5.0 (Chong et al., 2018). The data were generalised log (glog) transformed to alleviate the dependency of the variance on the compound concentrations and subjected to two-way analysis of variance (ANOVA) to determine the influence of animal life stage and sampling time (between subject, $p < 0.05$), as summarised in the Venn diagrams.

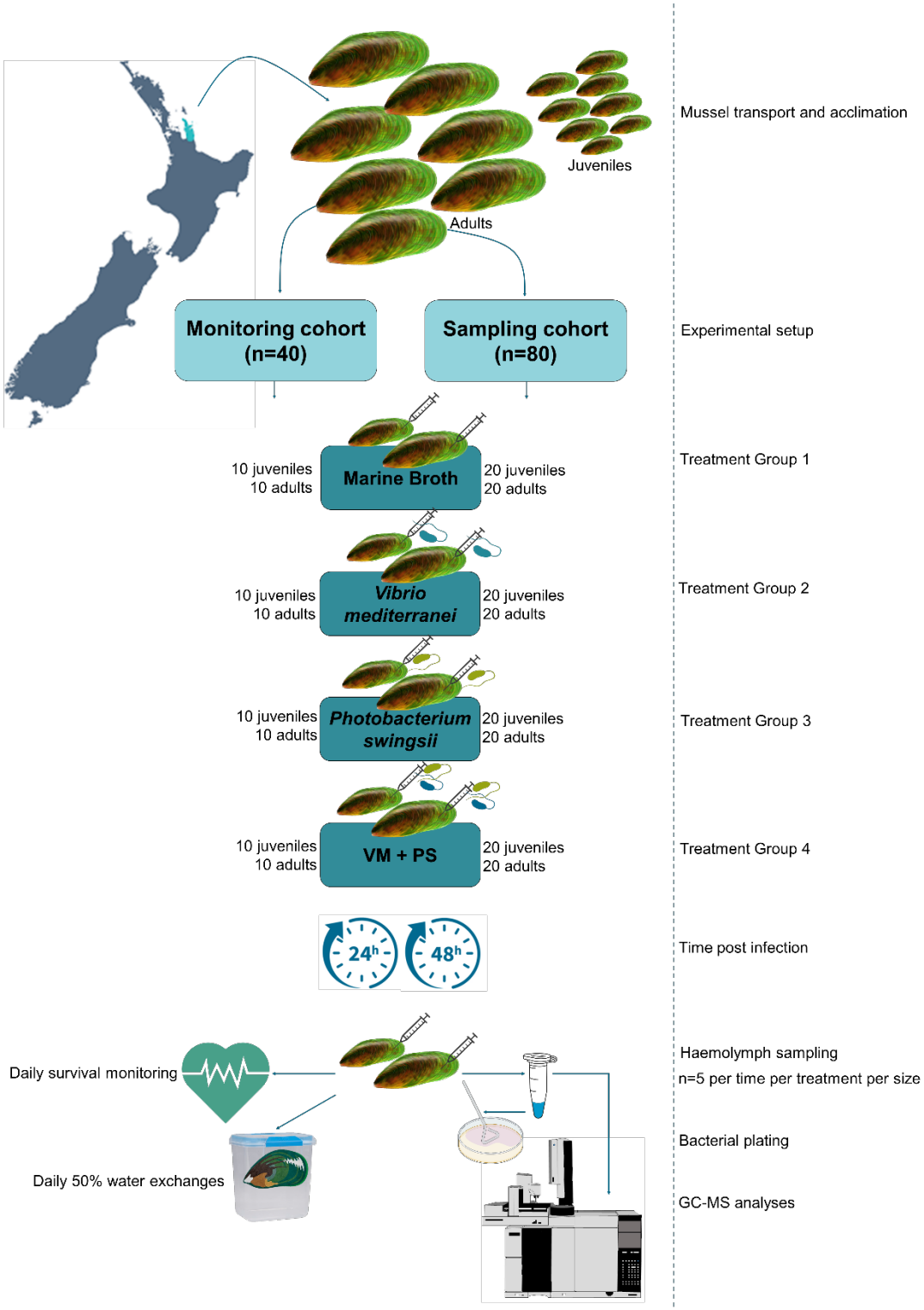

Figure 9.1. Schematic overview of the experimental and sampling design. Following mussel transportation and acclimation, juvenile and adult mussels were divided into a sampling and monitoring cohort, which were injected with either marine broth, *V., mediterranei*, *P. swingsii* or a combination of both bacteria. Mussels were sampled at 24 and 48 hpc. Then, haemolymph was snap-frozen for metabolomics analyses and used to quantify bacterial growth. Additionally, mussel survival was recorded from the monitoring cohort until 96 hpc.

9.3 Results

The mussel average (± SE) lengths were of 57 ± 7 mm for juveniles (n=80) and 72 ± 5 mm for adults (n=80). There were no significant differences ($p = 0.1374$) in shell length between the different treatment groups (MB, VM, PS, VM+PS) within juvenile or adult mussels. However, as expected, juvenile mussels were statistically shorter than adult mussels (two-way ANOVA, $p < 0.001$). There was no interactive effect between treatment groups and mussel stages on length ($p = 0.1416$). Average (± SE) weights were 15 ± 6 g for juveniles and 32 ± 5 g for adults. There were no significant differences amongst the treatment groups (two-way ANOVA; $p = 0.2596$) within the mussel stages. However, the weight of juvenile mussels was statistically lighter than that of adult mussels (two-way ANOVA, $p < 0.001$). There was no interactive effect between treatment groups and mussel stages on weight ($p = 0.8455$). Altogether within the juvenile cohort, 11 males, 6 females and 23 underdeveloped mussels were identified. In the adult cohort, the sex was as follow, 21 males, 7 females and 12 undetermined.

No mortalities were observed in the juvenile mussels injected with marine broth (control animals). Juvenile mussels infected with *V. mediterranei* (VM) showed 90% survival within 24h of infection and 60% survival by 72 hpc. Juvenile mussels infected with *P. swingsii* (PS) showed 60% survival within 24h, with 0% survival by 72 hpc. Juvenile mussels infected with both *V. mediterranei* and *P. swingsii* died at the fastest rate in this experiment, with 0% survival at 48 hpc (Fig. 9.2A, Log-rank test, $p < 0.001$). Adult mussels injected with marine broth showed the same response as juvenile mussels, with a 100% survival throughout the experiment. Interestingly, adult mussels injected with *V. mediterranei* also showed 100% survival at 72 hpc. When injected with *P. swingsii*, adult mussels showed 50% survival after 24h and remained at 50% for the remainder of the experiment. Infecting adult mussels with *V. mediterranei* and *P. swingsii* resulted in 40% survival after 24h, with 0% survival obtained at 72 hpc (Fig. 9.2B, Log-rank test, $p < 0.001$).

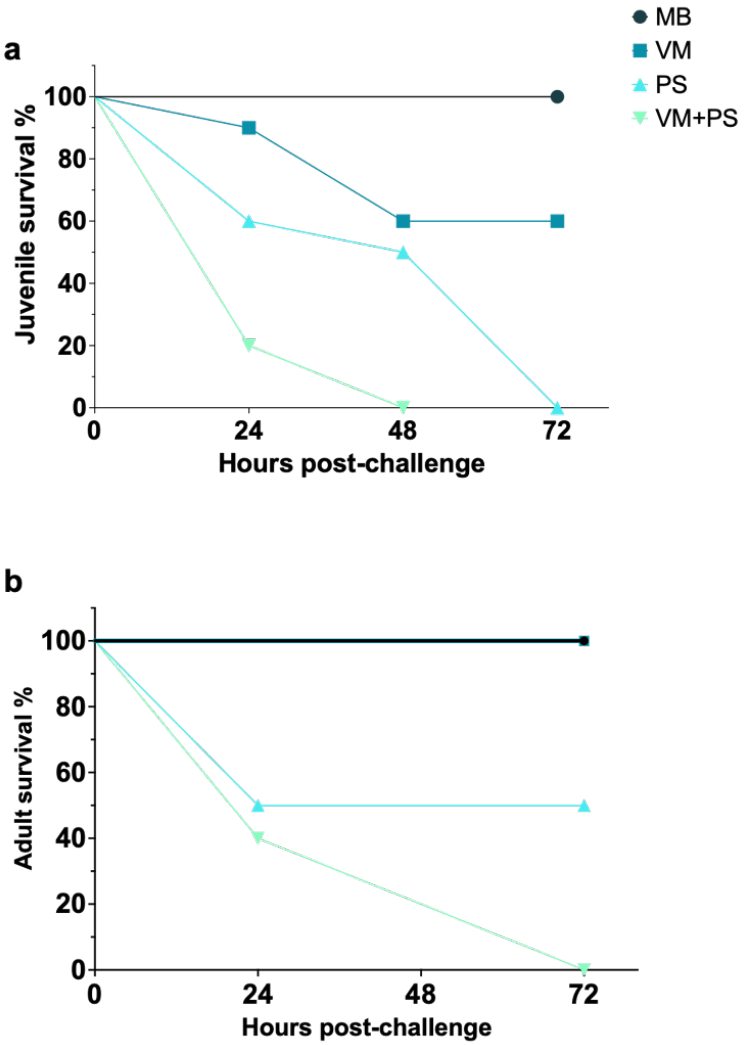

Figure 9.2. Survival plots of juvenile (A) and adult (B) mussels after a 72 h post-challenge to marine broth (MB), *V. mediterranei* (VM), *P. swingsii* (PS) and coinfection of both VM and PS (VM+PS).

The number of bacterial colony forming units (CFU) detected at 24 and 48 hpc were higher in juvenile mussels compared with adult mussels (mussel stage, $p < 0.0001$) (Fig. 9.3). However, pair-wise comparisons indicate that juvenile mussels infected with *V. mediterranei* ($p = 0.0021$) and coinfection group *V. mediterranei* + *P. swingsii* ($p = 0.03$) were statistically different when compared to mussels injected with marine broth at 24 hpc. Bacterial counts were also significantly higher in juvenile mussels infected with *P. swingsii* at 24 hpc, compared to *V. mediterranei*. No significant differences were found on CFU counts across treatment groups at 48 hpc. For juveniles, no CFU were detected in mussels injected with marine broth at 24 and 48 hpc (Fig. 9.3). On the first day (24 hpc), the highest number of CFU were detected in haemolymph collected from mussels injected with *V. mediterranei* (± 280 CFU), compared to all experimental groups (significant difference from MB control group, $p = 0.0012$). On day two (48 hpc), 73 CFU were detected in mussel haemolymph injected with *V. mediterranei* (no significant difference, $p = 0.7007$). Injection with *P. swingsii* showed 28 CFU at 24 hpc and 9

CFU at 48 hpc. This was the lowest number of CFU in all groups where bacteria were injected (no significant difference from the MB control group 24 and 48 hpc, p = 0.9758 and p = 0.9991). Co-infection of both VM and PS resulted in 197 CFU at 24 hpc (significant difference from MB control group, p = 0.0012) and 158 CFU at 48 hpc (no significant difference from MB control group, p = 0.1079). There was no interaction effect between experimental groups and time points (Treatment*Timepoint, p = 0.6849, two-way ANOVA measure).

For adult mussels, injection with marine broth resulted in haemolymph samples with no CFU at both 24 and 48 hpc (Fig. 9.3). At 24 hpc, the largest number of CFU were detected in haemolymph collected from adult mussels injected with VM (± 98 CFU) compared to all experimental groups (no significant difference from MB control group, p = 0.3507). At 48 hpc, 21 CFU were detected in mussel haemolymph injected with VM (no significant difference from the MB control group, p = 0.9826). Injection with PS in adults showed 13 CFU at 24 hpc and 5 CFU at 48 hpc. This was the lowest number of CFU in all groups where bacteria were injected (no significant difference from the MB control group 24 and 48 hpc, p = 0.9957 and p = 0.9998). Co-infection of both VM and PS resulted in 43 CFU at 24 hpc (no significant difference from MB control group, p = 0.4108) and 43 CFU at 48 hpc (no significant difference from MB control group, p = 0.8806). There was no interaction effect between experimental groups and time points (Treatment*Timepoint, p = 0.6849, two-way ANOVA).

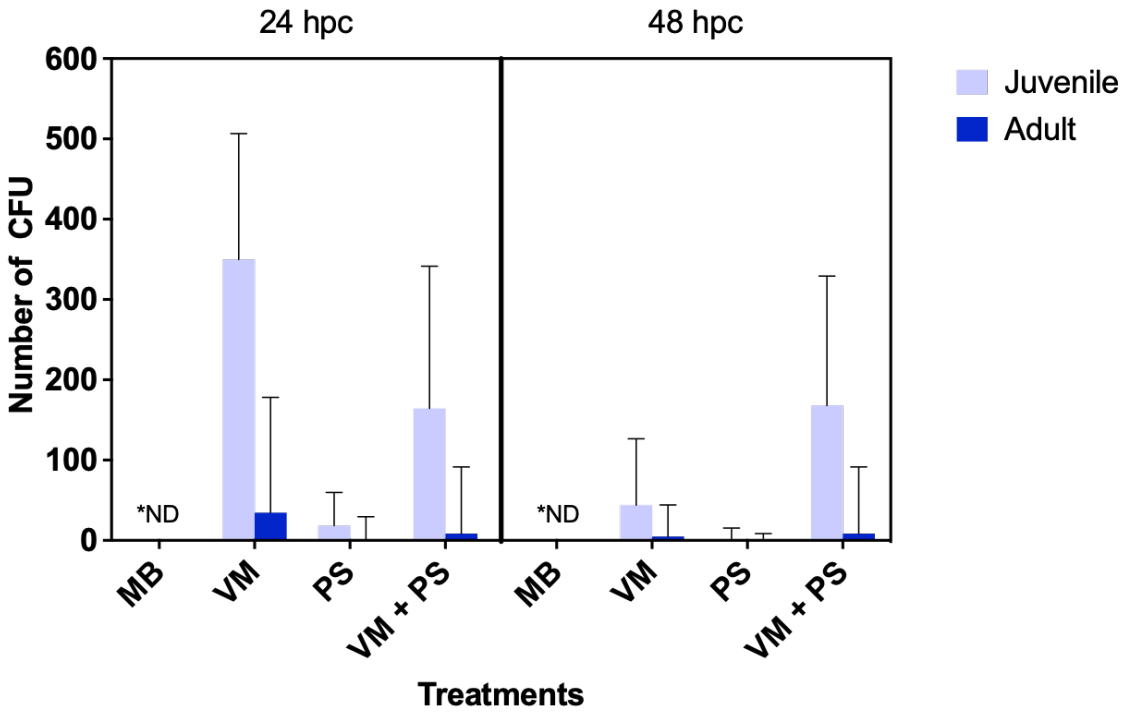

Figure 9.3. Bacterial colony counts are shown between juvenile and adult mussels across treatment groups overtime. Total replicates per mussel life stage per treatment n = 10,*ND= not detectable.

The metabolomic analyses resulted in a total of 71 features which were detected within all sampling groups and sampling times of which 48 metabolites were successfully identified. Significantly different metabolites ($p < 0.05$) as determined by two-way ANOVA can be viewed in the supplementary table. Following injection with marine broth, two-way ANOVA identified nine significantly different metabolites relating to sampling time (Fig. 9.4A). The metabolites asparagine, cysteine, dodecanoic acid, glutamic acid, glutamine, glutathione, margaric acid, trans-vaccenic, and tyrosine were higher in mussels sampled at 48 hpc compared to the mussels sampled at 24 hpc, regardless of life stage when injected with marine broth. Juvenile and adult mussels injected with *V. mediterranei* showed two metabolites (benzoic acid and citric acid) which had higher concentrations in the juveniles (compared to adults), while 21 metabolites showed significant differences due to experimental time (Fig. 9.4B). The metabolites: alanine, asparagine, aspartic acid, benzoic acid, cis-4-hydroxyproline, creatinine, cystathionine, cysteine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine displayed an increase in metabolite abundance within the 48h sampling group (compared to the 24 hpc group) following injection with *V. mediterranei*. Injection with *P. swingsii* resulted in no metabolite differences between adult and juvenile mussels sampled at 24 and 48 hpc (Fig. 9.4C). When injecting both *V. mediterranei* and *P. swingsii* into adult and juvenile mussels, 22 metabolites showed significant differences due to sampling time (Fig. 9.4D). The metabolites:

arachidonic acid, asparagine, creatinine, cystathionine, docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), eicosapentaenoic acid (EPA), glutamine, gondoic acid, hexadecanoic acid, isoleucine, leucine, lysine, margaric acid, octadecanal, ornithine, pentadecanoic acid, phenylalanine, threonine, tryptophan, tyrosine, and valine were lower in the 48 hpc sampling group (compared to the 24 hpc sampling group).

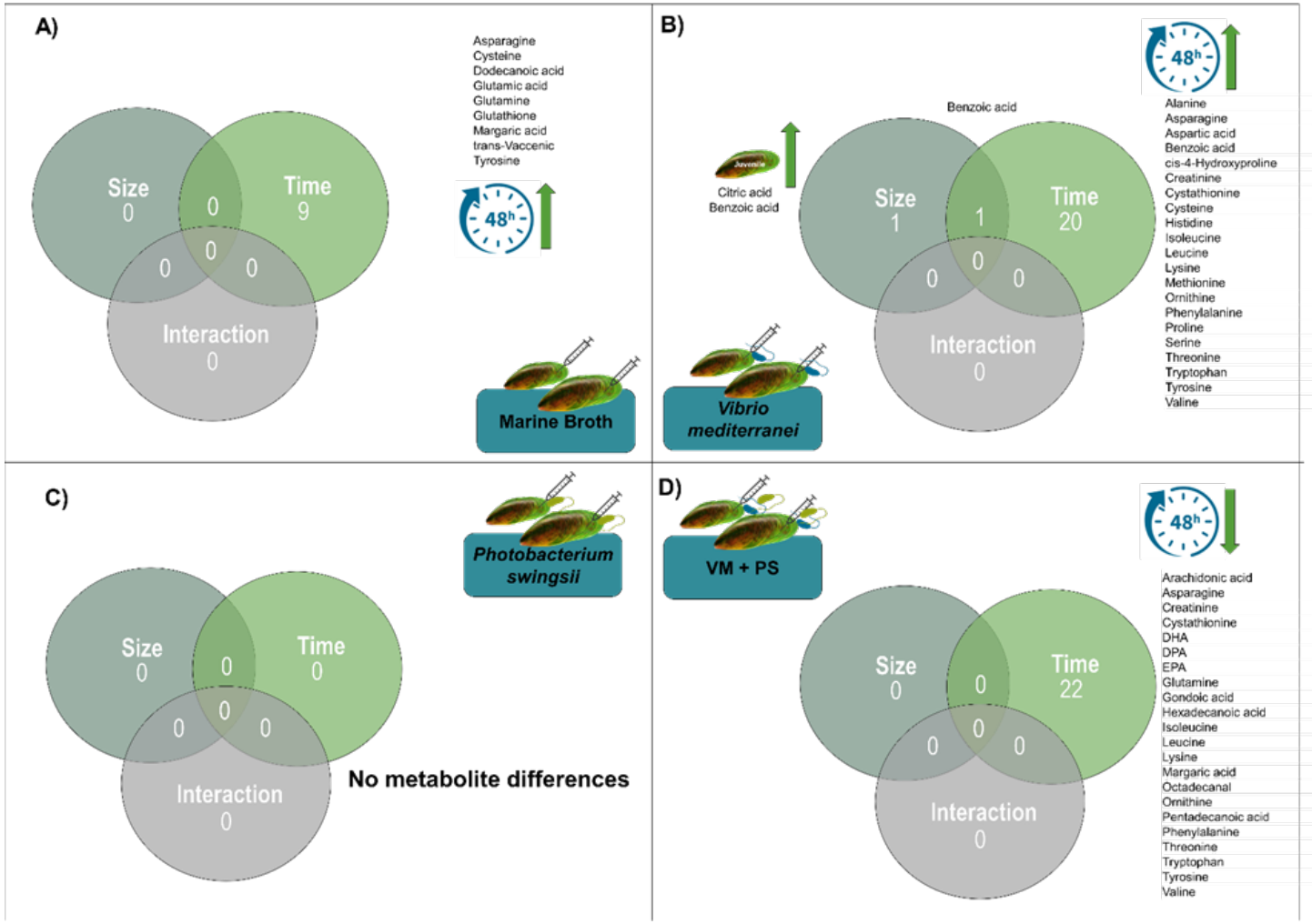

Figure 9. 4 Overview of the metabolomics results as a Venn diagram indicating the metabolite differences between adult and juvenile mussels, sampled at 24 and 48 hpc following injection with marine broth (A), *Vibrio mediterranei* (B), *Photobacterium swingsii* (C) and VM + PS (D). Metabolite differences as an increase (↑) or decrease (↓) in metabolite abundance due to affected experimental factors.

9.4 DISCUSSION AND CONCLUSIONS

Emergence of *Vibrio* and *Photobacterium* infections in bivalves depends on various factors, including the life stages of the host, diversification of pathogens and parasites in the bacterial communities, and the weakening of the host-immune system due to prolonged exposure to stressors (Destoumieux-Garzón et al., 2020). The genus *Vibrio* has been described as a disease causing agent of bivalve molluscs of various life stages (Beaz-Hidalgo et al., 2010). Typically, larvae are more susceptible to vibriosis than adults since the resistance to bacterial infection increases with age (Dubert et al., 2017). Using juvenile and adult Greenshell™ mussels as hosts, we demonstrated in the current laboratory-based study that mussels succumb more easily to co-infections compared to single infections. This was evidenced by survival, bacterial colony count, and haemolymph metabolomics data. The present study is also the first to report differences between juvenile and adult *P. canaliculus* in infection susceptibility. Indeed, results revealed that juveniles had lower survival than adults when infected with *V. mediterranei, P. swingsii,* and both pathogens. This is not surprising, as previous studies on bivalves have shown that juveniles are more vulnerable to pathogens than adults (Green et al., 2016, Albuixech-Martí et al., 2021). In juvenile mussels, the immune systems and defence mechanisms are often not fully developed compared to the adults, leaving them more susceptible to pathogens. The juvenile response can be attributed to factors such as lower haemocyte production and quantity (Canesi and Pruzzo, 2016), incomplete organ development, or a reduced capacity for immune response.

Marine broth response

The use of marine broth injection as control group in bivalve pathogen experiments is common practise to assess the putative effect of experimental handling and/or bacterial growth media (Cellura et al., 2007), as demonstrated in the current study. Here the results showed that no bacterial colonies were detected in haemolymph samples at 24 and 48 hpc from juvenile and adult mussels injected with marine broth. Additionally, no mortalities were recorded within these groups either. In a previous study on *P. canaliculus,* mortalities were seen within marine broth injection (control) group within 2-5 days of experimental time, attributed to the act of injection causing disruption to the tissue (Azizan et al., 2022). Typically, marine broth contains all the nutrients (such as minerals, peptone – source of nitrogen, vitamins and amino acids, yeast extracts – source of b-vitamins, and inorganic substances) necessary for the growth of marine bacteria (ZoBell, 1941). Yet, marine broth can also affect the metabolism of the host, as seen in the current study, where serval amino and fatty acids were increased in the haemolymph after 48 hours of injection. It has been noted that seawater has a greater salinity

than traditional culture media (Iffland-Stettner et al., 2022), resulting in a medium with high osmolarity when using something like marine broth. In the marine broth injected mussels the increased amino acids asparagine, cysteine, glutamic acid, glutamine, and tyrosine may be a physiological response to manage osmolyte levels and other regulatory processes, such as ammonia detoxification, cellular redox status, and antioxidant functions (Wu, 2009). The choice of media contributes to the metabolite profile (Daskalaki et al., 2018), making it a necessity to analyse an unspent culture medium as control within a metabolomics experiment to determine the non-biological changes that occur (Pinu and Villas-Boas, 2017). Comprehensive metabolite profiling of mussel metabolism in response to marine broth contact has not been done to date and remains a topic of interest for future studies.

Vibrio mediterranei response

Interestingly, from the metabolomics findings of the current study, only infection *with V. mediterranei* resulted in differences between juvenile and adult mussels, with an increase in benzoic acid and citric acid detected in juvenile mussels. Benzoic acid and citric acid can be used as a non-specific inhibitors of phenoloxidase (PO) activity to investigate defence mechanisms of innate immunity, as shown in a study on limpets (Quinn et al., 2020). Increases in PO activity in the haemolymph of bivalves are a response to bacterial infection, as seen with the use of *Vibrio coralliilyticus* (Van Hung et al., 2019). Although PO activity was not measured in the current study, increases of PO inhibiting metabolites were seen, suggesting an increased response to counter PO activity in the haemocytes of *P. canaliculus* in response to *V. mediterranei* infection. Strong correlations have been found between the decrease in PO activity and the occurrence of diseases in bivalve invertebrates, leading to mortalities in the host (Luna-Acosta et al., 2017), potentially as seen in the juveniles infected with *V. mediterranei* in the current investigation.

Significantly higher CFU counts were found when infecting both juveniles and adults with this species of *Vibrio*. Mussels may have had more difficulty clearing *V. mediterranei* out of their systems in the first 24 hours of infection. However, a reduction in CFU counts were seen at 48 hpc. *V. mediterranei* is known to have virulence mechanisms related to adhesion, superoxide dismutase production, and toxin production, all of which inhibit phagocytosis (Andree et al., 2021) and makes it harder for the host to eliminate. The effect of time on bacterial infection was also seen when considering the metabolite response, where all significantly different metabolites were increased at 48 hpc.

The time-related metabolite response of the *V. mediterranei* infection group significantly differed from the other infection groups. We observed multiple increased metabolites, most of

which were amino acids. When undergoing stress, such as a pathogenic infection, mussels can also oxidise amino acids to provide energy for cellular production (Wu et al., 2015) or immune response, causing a large increase in amino acid metabolites (Liu et al., 2013). Resultantly, the increased amino acids observed in the current study, can be due to protein catabolism (Muznebin et al., 2022a), in response to *V. mediterranei* infection taking place over a 48 hour timeframe. Additionally, the findings link to use of amino acids in cellular energy, as reported in clams infected with *V. harveyi*, where elevated amino acids were ascribed as a sign of energy depletion (Liu et al., 2013).

Typically, pathogens triggers an innate immune response which leads to the release of reactive oxygen species (ROS), which can be countered by antioxidant metabolites relating to the glutathione metabolic pathway (Young et al., 2017). Our study revealed increases in the metabolites cystathionine, cysteine, methionine, ornithine, and serine which supports an active glutathione pathway to partake in oxidative stress mechanisms following *V. mediterranei* infection, as previously reported in heat-stressed *Perna canaliculus* (Delorme et al., 2021b). Branched chain amino acids (BCAA) also supports the innate immunity (Nguyen et al., 2018c). Increased leucine, isoleucine, and valine additionally supports the immune system by providing energy for the biosynthesis of protective molecules, as seen after 24 hours of *V. harveyi* infection in clams (Liu et al., 2013).

Changes in proline and hydroxyproline can be associated with collagen production and stability (Inglis et al., 2016). Thus, the increased proline metabolites from *V. mediterranei* infection can indicate the degradation of muscle tissue. This is consistent with previous studies which recorded other species of *Vibrio sp*. causing collagen degradation and tissue damage in Greenshell™ mussels (Nguyen et al., 2018c). An increase in creatinine, a valuable source of carbon and nitrogen (Azizan et al., 2021), was also seen in mussels infected with *V. mediterranei*. Often, a decrease in amino acids and creatine due to the high energy demands of the mussel responding to infection are reported (Nguyen et al., 2019c). However, the increase of amino acids and creatinine over time from the current study, suggests recovery in mussels after being exposed to a pathogenic infection (Nguyen et al., 2018b). This is further supported by the significant decrease in CFU in the *V. mediterranei* injected group after 48 hours, which suggests that the mussels were able to successfully clear majority of the *V. mediterranei* after 48 hours.

It is also worth noting that the survival of juvenile mussels infected with *V. mediterranei* was higher than the survival of juveniles infected with *P. swingsii*. In the adults infected with *V. mediterranei,* no mortalities occurred. The metabolite response of the *V. mediterranei* infection

group largely differed from the other mussel infected groups, with the metabolites increasing over time. As stated above, the increase in metabolites and decrease in CFU suggest that the mussels had fought off most of the infection in the 24 hours and were recovering after 48 hours. The higher survival rate further supports this as it indicates that the mussels were able to fight off the infection successfully. This evidence suggests that the mussel's immune system was able to respond and overwhelm the *V. mediterranei* pathogenic infection after 48 hours, unlike in the other infection groups.

Photobacterium swingsii response

Infection of *P. swingsii* in both juvenile and adult mussels showed low CFU counts at 24 hpc and even lower counts by 48 hpc. Adult mussels showed higher survival to *P. swingsii* infection than juveniles. However, there were no significant metabolic differences between the age groups under this infection. There were also no significant changes in metabolites over time. This is interesting as *P. swingsii* caused 100% mortality in adults and 50% in juvenile mussels. A similar outcome, of adult mussel mortalities due to *P. swingsii* has been previously reported by Azizan et al. (2022), attributed to virulence factors produced by the bacterium. Mortalities often occur in bivalves not as a direct effect of pathogenic assault and only restricted to the host's immune system, but as a consequence of changes in other traits that might have an impact on pathogens infection processes (Labaude et al., 2017). For instance, factors such as temperature, hypoxia, and filtration rates influence food consumption in mussels, which in turn can increase the likelihood of pathogen consumption/uptake and subsequent infection (Skår and Mortensen, 2007, Lopez-Joven et al., 2011, Parisi et al., 2017).

Co-infection response

Pathogen co-infection concerning *Vibrio* sp. that affect *P. canaliculus* health and their relationship environmental stress is poorly understood and has been raised as an important knowledge gap. Physiological consequences of the first pathogen infecting the host are likely to affect the host's immune response, by either hindered or enhanced proliferation of the subsequent infections. Hence, these co-occurring pathogens can either act synergistically or antagonistically with each other (Kotob et al., 2017).

The co-infection group had the highest mortality rates, with 100% of juvenile and adult mussels dying, and this group also showed the highest CFU counts among the treatment groups. This implies that the mussel's immune system struggled to cope with the co-infection of pathogens, and we suspect that the bacteria worked in conjunction to breach the mussel's immune system. This trend has been observed in other co-infection studies in bivalves, such as oysters and fan mussels (Tall et al., 1999, Gay et al., 2004, Künili et al., 2021). In these studies, the

pathogens work together potentially in a synergistic manner; where the primary pathogen suppresses the host immune system and avoids phagocytosis, thereby causing the secondary pathogen to worsen disease severity and mussel mortality, causing the infection to spread faster (Tall et al., 1999). As *P. swingsii* and *V. mediterranei* belong to different families, they likely have different pathogenic strategies (Pruzzo et al., 2005). This would result in the pathogens attacking the mussel's immune system using two different methods, making it harder for the mussel's immune system to respond. Potentially, a rapid pathogen growth could occur, causing the pathogens to overwhelm the host, followed by death. As our understanding of the immune mechanism of mussels within the context of coinfection is limited, additional research is needed to determine the immunological mechanism underlying these pathogen interactions. Additionally, our observations on disease outcomes (i.e., mortality) showed that this parameter was sensitive to the temporal separation between pathogen exposures. This suggests, along with these data, that future research should investigate the order and timing of pathogen exposure to determine pathogen community structure in hosts and disease outcomes.

The metabolism of the co-infection group also showed a distinctly different pattern from the other infection groups, with significantly different metabolites (amino and fatty acids) decreasing over time, as previously reported in *P. canaliculus* when infected with a single pathogen (Nguyen et al., 2019c). The decrease in amino and fatty acids is believed to be caused by the high energetic demands of the mussel's immune response to the co-infection. Immune responses are known to require high energy expenditure (Ellis et al., 2014), resulting in the decreased metabolite levels observed in the current study. Co-infection of multiple pathogens likely resulted in greater stress on the mussels, creating energetic demands that depleted energy reserves and ultimately resulted in death.

Hosts and bacterial pathogens may share similar nutritional substrates and produce common metabolic products at the infection site; crosstalk between their metabolic pathways may influence infection pathogenesis (Meegha and Prasad, 2021). Considering that the metabolic response is taking place in both the host (in response to the pathogen) and in the pathogen (as it adapts and proliferates in the host environment), the metabolite results should also be considered as a consequence of the bacteria and not only the mussel, as previously demonstrated in *Vibrio sp.* infected *P. canaliculus* (Ericson et al., 2022). Metabolite profiling of *V. parahaemolyticus* fatty acids reported hexadecenoic (16:1), hexadecanoic (16:0), and octadecenoic (18:1) acids as the major fatty acids of *Vibrio* strains (Jia et al., 2014). The C16 and C18 fatty acids detected in the co-infected group of mussels in the current study, can thus be ascribed to a bacterial response, in addition to the mussel response.

Pathogenic bacteria are known to utilise compounds other than glucose as carbon sources for growth, such as fatty acids, (Ericson et al., 2022). With the reduction of fatty acids (arachidonic acid, gondoic acid, hexadecanoic acid, margaric acid, octadecanal, and pentadecanoic acid) in the current study, it can be suggested that *Vibrio sp.* infected *P. canaliculus* utilise lipid metabolites as an energy source. The importance of fatty acids (lipids) as a metabolic energy source and membrane component is prominent in the literature, with *Vibrio sp*. infections shown to affect the balance of lipid metabolism in clams (Yu et al., 2019). The current study confirms that *Vibrio sp.* affects *P. canaliculus* lipid metabolism, however the utilisation by host or pathogen is unclear, making this an important aspect for future research.

Branched chain amino acids (leucine, isoleucine, and valine), were decreased in the co-infected group of mussels suggesting a high demand of BCAAs for host energy production in response to *Vibrio* sp*.* infection, as reported previously in *P. canaliculu*s in response to *Vibrio* sp*.* DO1 infection (Nguyen et al., 2018b, Azizan et al., 2022, Ericson et al., 2022). The effect of *V. parahaemolyticus* infection on the aromatic amino acids tyrosine, phenylalanine, and tryptophan in molluscs (Lu et al., 2017) aligns with our current findings, where decreases of these amino acids suggest alteration of protein formation and synthesis of neurotransmitter derivatives.

We hypothesise that *Vibrio mediterranei* causes more destruction to mussels than *Photobacterium swingsii*. Yet, when these bacterial species are combined, the mussel response is even more profound, with mortalities occurring faster and bacterial counts remining high after 48 hours. Also, the metabolite response suggests support for immune functions and energy production in the dual infected cohort. What remains unclear is the order in which events happen, and the interplay between bacteria once inside the host. Research investigating the metabolite profiles of *Vibrio* sp. (without a host) will be beneficial in determining which aspects from the metabolite response can be related to the mussel, the bacteria, and a combination of both. Also, the effect of external factors on co-infection mechanisms will benefit from further work. For example, water temperature is an important factor for bacterial growth rates (Hoppe et al., 2002) and both *Vibrio* and *Photobacterium* have increased growth in warmer temperatures (Roquigny et al., 2021). However*, V. mediterranei* can grow at lower temperatures and is known to cause mortalities in mussels from 17°C upwards (Prado et al., 2020a). Thus, the water temperature plays an important role in the experimental design for co-infection experiments as one temperature might favour growth of one species and not the other. Future studies are needed to elucidate the potential effects of

seawater temperature on the growth of *V. mediterranei,* and *P. swingsii* used in the current experiment.

This study demonstrates that co-infections by the pathogens *P. swingsii* and *V. mediterranei* cause high mortality in Greenshell™ mussels. The results also show that juvenile mussels are more susceptible to co-infections than adult mussels and highlight complex changes in the host metabolome of Greenshell™ mussels when exposed to a co-infection. These findings provide valuable insights on the immune system of mussels, their susceptibility/resilience to pathogenic infections, and potential mechanisms of infection progression for this valuable mussel species. This study forms part of a larger research program focused on the investigation of mussel summer mortality in New Zealand.

Chapter 10: General discussion, critical assessments, concluding observations, future research and recommendations

"Our relationship with the sea is a spiritual matter. It has focused on the sea as a source of kai moana for survival."
-Darryl Crimp, New Zealand Geographic

10.1. GENERAL DISCUSSION

10.1.1 Positioning this research within the context of mussel aquaculture

Disease outbreaks can be a bottleneck for the aquaculture industry, resulting in large financial losses each year. The effect of environmental stressors along with intensive farming models have increased farm-raised mussels' susceptibility to infectious diseases (Stenton-Dozey et al., 2021). There is also a potential risk of transferring infectious diseases from aquaculture farms to consumers, particularly when the pathogens are food-borne infections (Lane et al., 2022). The New Zealand aquaculture industry is largely pathogen-free, but not immune to increasing incursions of pathogenic bacteria, such as *Photobacterium* spp. Pathogenic effects are often exacerbated by the increasing impacts of anthropogenic and climate change effects (Eggermont, 2017), with temperature changes highlighted as one of the main drivers compromising mussel health (Harvell et al., 2002, Matoo et al., 2021). Enhancement of resilience and robustness can improve adaptability to potential threats, but prior to achieving a resilience outcome, resilience mechanisms should be identified (Colditz and Hine, 2016).

The overall aims of this thesis were to **investigate the interplay of selected bacterial pathogens *Photobacterium* spp. and *Vibrio* spp. and temperature changes threatening the health status of adult, green-lipped mussels, *Perna canaliculus***, and has now been achieved and summarised in the preceding pages. In brief, the need to evaluate the immune status of Greenshell^{TM} mussels (**Chapter 2**) and explore physiological biomarkers of mussel-Vibrionaceae interactions in health and disease (**Chapter 3**) have been recognised and served as a basis for new research performed in this thesis. Before addressing the biological questions, a best practice mussel handling procedure was established, which led to discontinuation of the use of magnesium chloride ($MgCl_2$) as a relaxant agent for the rest experimental chapters in this thesis (**Chapter 4**). The identification of four bacteria from a summer mortality event, highlighted as new emerging bacterial pathogen (*Photobacterium swingsii*) for Greenshell^{TM} mussels (**Chapter 5**) sets the stage for all the challenge experiments performed in this thesis. Elucidation of the dynamics of *P. swingsii* infection was observed within mussel haemolymph and tissues overtime (**Chapter 6**). Metabolic changes within mussels in response to a laboratory-based simulated marine heatwave tracked temperature induced changes (**Chapter 7**). The interactive effects of elevated temperature and *P. swingsii* infection on mussel survival and immune responses provided the opportunity to explore summer mortality in a controlled environment (**Chapter 8**). Bacterial co-infections on juvenile and adult mussels were also explored for the first time (**Chapter 9**). By implementing a multi-omics approach, metabolic, genetic, histological, biochemical, cytological, and physiological methods were applied to achieve the aims of this thesis. This

now provides new dimensions of appreciation and understanding of Greenshell™ mussels, *Photobacterium swingsii,* temperature stress and linkage between them. The novel findings of this thesis are summarised below.

10.1.2 Research outcomes

Literature-based investigations of mussel immunity and Vibrionaceae

Despite the vast body of literature available on *Perna canaliculus*, recognised and tested immunological mechanisms are poorly understood. The literature overview given in **Chapter 2** synthesises cellular and humoral defence mechanisms within *P. canaliculus*. Additionally, *P. canaliculus* pathogens and their associated virulence factors were described, along with abiotic and biotic factors compromising mussel immunity. The most important contribution of this work included the lack of *P. canaliculus* reported studies, showcasing untouched research spaces to fully comprehend immunological functioning within this mussel species. By addressing this gap in this thesis, researcher can now better understand immunological challenges *P. canaliculus* species face and lead to the development of more efficient and sustainable aquaculture practices. This includes selecting disease-resistant strains (i.e., individuals with desirable immune traits, such as increased expression of antimicrobial peptides), optimising nutrition and feed formulations, and improving disease monitoring and management protocols. The second comprehensive review on physiological biomarkers of mussel-Vibrionaceae interactions in health and disease, outlined in **Chapter 3**, integrates literature that assessed the risk of *Vibrio sp*. pathogens for mortality in mussel species. This evaluation depicts how various mussel species respond physiologically, in terms of a biomarker response measured, when faced with a *Vibrio sp*. challenge. Further highlighted is the array of analytical tools used to investigate these responses and the need to apply multi-omics approaches when examining mussel *Vibrio sp.* responses. Throughout this thesis, a variety of assays and omics approaches were utilised, which permitted a broader range of biological responses to be captured, closer exploration of affected biological pathways, and identification of potential interactions between different types of stressors. This will facilitate more efficient rapid screening of environmental stressors in future research.

Accurate methods for experimentation

Many studies have utilised a routine method to facilitate sample collection by first anesthetising aquatic animals, to minimise handling stress (Suquet et al., 2010). Considering that metabolomics is a sensitive technique that can capture a metabolic snapshot of a biological sample at a specific time (Alfaro and Young, 2018), the effect of mussel handling will undoubtedly also be captured within the metabolome analysed. By administrating an

anaesthetic like magnesium chloride ($MgCl_2$), the physical prying of the mussel shell to obtain haemolymph sample is eliminated, yet the physiological effects of $MgCl_2$ on mussels were unclear. The experiment in **Chapter 4** provides evidence that the use $MgCl_2$ alters the haemolymph metabolome in mussels, resulting in an energy imbalance, and altered amino acids acting as neurotransmitters and osmolytes. One of the main findings was the inability to use $MgCl_2$ as a haemolymph sampling method from mussels, for subsequent experiments performed in this thesis. Ultimately the use of $MgCl_2$ within a challenge experiment inflicts bias in the results as the response measured will not only be the effect of the stressor but also the action of the anaesthetic. It remains important to include an appropriate control, such as a no-injection control to account for the action of mussel handling.

Identification and evaluation of bacteria as pathogenic agents

Pathogen identification is not a straightforward process and often, overall bacteriological profiles cannot distinguish between bacterial species. Development of metagenomics are encouraged as it allows simultaneous identification of a large number of pathogen genomes (Lupo et al., 2021). A significant discovery of this thesis is presented in **Chapter 5**, with the successful identification of isolated bacterial strains from a 2018 mussel summer mortality event. Whole genome sequences, identified the strains as *Photobacterium swingsii*; *Photobacterium rosenbergii*; *Photobacterium proteolyticum*; and *Vibrio celticus* (Azizan et al., 2022). Additionally, the multi-technique approach employed for bacterial strain typing enabled phenotypic information on the bacterial strains. By challenging Greenshell™ mussels with the four bacterial strains, it was found that *P. swingsii* was highly virulent towards adult mussels. Resultantly, *P. swingsii* was used as the main bacterial species for challenge experiments performed in this thesis. For the first time virulence factors were tested within Greenshell™ mussel bacteria. Three virulence genes, *hsp60*, *plp* and *vcpA* showed high levels of correlation with the high virulent bacterial isolates, indicating that these virulence genes might contribute to mortalities in mussel. By focusing on virulence factors produced during bacterial infections additional insight for bacterial pathogenesis was obtained.

Evaluation of Photobacterium swingsii overtime

An investigation of the association between bacterium *P. swingsii* and mussel mortality was essential, since the mere presence of an infectious agent does not necessarily indicate the presence or cause of disease (Brosnahan, 2020). In **Chapter 6**, the bacterium *P. swingsii* was further characterised via histopathological, haemocyte responses (total cell count, cell viability, bactericidal activity), bacterial proliferation overtime implemented by Greenshell™ mussels. Of considerable advancement within this chapter is the design of a *P. swingsii* specific PCR probe which enabled the quantification of bacterial specific DNA load. Considering that the

species-specific *P. swingsii* probe and protocol are now in place, they can be used to screen for the presence of *P. swingsii,* via the detection of bacterial RNA, in archival mussel samples and future mussel monitoring programs. This chapter also highlights the use of additional control treatments, utilising for the first-time heat-killed bacteria as a matching control. By using this control, researchers can determine whether the immune response observed is caused by live bacteria or if it is a general reaction to either bacterial components (e.g., cell surface molecules, secreted toxins, virulence factors, altered antigenic properties) or bacterial injection procedure. Microscopic observations added a new dimension to the adductor muscle as a site of injection. Muscle degeneration and haemocyte infiltration was still evident after 48 hours post challenge, potentially serving as added stressor to the already compromised mussel immune system. These pathologies observed can potentially serve as diagnostic markers of disease development for assessing the health status of mussels. Collectively, the significance of the findings lies in advancing our understanding of disease development, host-pathogen interactions and offering tools for the detection of *P. swingsii*. By integrating methods, such as metabolomics, transcriptomics, proteomics and with the presently utilised parameters in this thesis, researchers can obtain a more comprehensive understanding of this bacterium association to ill health of the mussels, enabling effective disease management and monitoring strategies of this pathogen in wild and farmed Greenshell™ mussel populations.

***Perna canaliculus* metabolite changes as indices of marine heatwaves**

Among the most important environmental factors, water temperature has a dramatic effect on the biological activity and metabolite rates of aquatic organisms (Rosenblum et al., 2005), and heat stress has also been identified as a crucial parameter within this thesis. The results presented in **Chapter 7**, build on previous temperature challenges performed on *P. canaliculus* for 13-months (Ericson et al., 2023c) and 8-weeks (Venter et al., 2023). The new research in this thesis showed that *P. canaliculus* has the potential to adapt to heat stress up to 24°C for five-days by activating costly defence and repair mechanisms. Ultimately, mussels exposed to a marine heatwave need additional resources to enhance energy production for organismal functioning via aerobic and anaerobic processes and upregulation of various metabolites to counter oxidative stress production. This study also demonstrates that sampling at a single time point does not accurately capture the metabolite response following a marine heatwave and that signatures of interest are time-dependant. Findings on metabolite changes during marine heatwaves provide insights into mussels' metabolic pathways and physiological adaptations under heat stress. The key metabolite biomarkers identified, and the underlying mechanisms of heat-induced physiological adaptation may offer scientists working on mussel disease a critical advantage in developing targeted interventions to support wild and farmed mussel health and survival.

Multi-factorial effects to understand complex interactions between temperature and bacterial pathogens within P. canaliculus

The interaction between pathogens and temperature has been shown to be a leading factor contributing to the increased incidence of mussel summer mortality around the world, resulting in higher rates of mortality and disease development (Wendling and Wegner, 2013, Le Roux et al., 2015). **Chapter 8** addresses the interactive effects of temperature and pathogen exposure on *P. canaliculus*. Findings from this study suggest that multiple stressors have a more severe impact on mussel survival, proposing a synergistic effect between temperature and bacteria during mortality events. However, when it came to the selected haemocyte parameters, in the presence of a high dose of bacteria, experimental sampling time had the largest effect on mussel response irrespective of the temperature. This highlights that the mussel's immune response undergoes dynamic changes over time until the bacteria are eliminated from the circulating system. Also, the results emphasise the criticality of selecting shorter sampling intervals in experimental designs to consider the temporal fluctuations in the immune response to precisely capture and comprehend the alterations in the mussel's immune system throughout a bacterial infection. Altered lipid peroxidation and total antioxidant capacity levels were recorded considering both temperature and pathogen stressors. The present study demonstrates that temperature changes affect mussel haemocytic function and lead to oxidative stress, increasing hosts susceptibility to bacterial infection and mortality.

Pathogen-coinfection experiment

Host-pathogen interactions are usually not one-to-one in aquaculture systems (Albuixech-Martí et al., 2021), due to the high prevalence and diversity of pathogens, co-infections within an individual host is often the outcome (Demann and Wegner, 2019). In **Chapter 9** the use multiple pathogens, i.e., *Vibrio mediterranei* and *Photobacterium swingsii*, compromised mussel survivability and haemolymph immune responses. Progressiveness in terms of utilising juvenile mussels was shown in this chapter, with co-infections causing higher mortality in a shorter amount of time in juvenile mussels than in adults. Arguably, juveniles are at a greater risk for pathogen infections, highlighting a whole new area of untouched *P. canaliculus* research. Metabolically, co-infections affected largely energy metabolites in both juvenile and adult mussels, suggesting a high energy expenditure and distribution of the mussel's immune response to support immune functions and protein synthesis. Additionally, different bacterial pathogens used in this study with their multitude of pathogen-specific (virulence) factors and PAMPS may influence metabolic host reactions that divert metabolic host reactions for their survival and growth, although this has not been experimentally proven (Eisenreich et al., 2013). Such interactions are likely to be explained by the observed metabolite changes in host cells following infection by a single bacterial pathogen. The inclusion of other "omics",

biochemical, and physiological studies in future research is crucial for a thorough understanding of how metabolic host targets interact with bacterial pathogens, and the impact these microbes have on the metabolism of host cells. This research contributes to the growing body of evidence on co-infections of *P. swingsii* with other infectious agents could have caused disease to manifest in these mussels.

Study as a whole

Shellfish aquaculture is a growing industry worldwide and seen as an effective way to supply to an increasing seafood demand. In New Zealand seafood, it is an important food source, with mussels collected from wild mussel beds, purchased from supermarkets, and enjoyed in restaurants. To ensure sustainability of mussel aquaculture and protection of wild populations, an important consideration is to understand the potential interactions between mussels and the environment. Subsequently, the interplay between mussels and the environment will elicit physiological responses, to maintain homeostasis and survival. By improving our knowledge on these physiological responses and mechanisms implemented by *P. canaliculus*, we are placed in a better position to control and possibly mitigate the consequences of environmental stressors.

Within this study, findings filled crucial knowledge gaps relating to mussel immunity. Both findings of cellular and humoral immune responses have now been linked to *P. canaliculus* following pathogen exposure. The functions of haemocytes to perform haemocytosis, produce reactive oxygen species, and corresponding protective mechanisms by means of antioxidant and lipid peroxidation molecules, as well as metabolites with functions relating to humoral immunity have now been described in *P. canaliculus* in response to the bacterium, *P. swingsii*. Advances have also been made when considering the virulence factors, encoded by virulence genes, of *P. swingsii*, linking this bacterium to mortality and / or disease-causing pathogen in *P. canaliculus*. Of these virulence genes, single-zinc metalloprotease (*vcpA*) gene has been recognised as toxin in disease pathogenesis; *hsp60* was hypothesised to be an infection factor and *plp* potentially contributed towards virulence of *P. swingsii*. Meaningful contributions have been made to the understanding of marine heatwaves in thermal stressed mussels which have shown to activate a significant metabolic response in several biochemical pathways related towards repair and maintenance mechanism. The combined stressor study illustrated that the rise of temperature influencing on the activity of mussels, their susceptibility to bacterial infection, immunobiological function as well as oxidative stress markers. Mussels under combination of stressors found to be significantly affected by either the sampling timepoint or pathogen treatments, without stressor interactions. It is postulated that temperature stress alone does not completely suppress the immune capabilities of *P. canaliculus*, the changes

induced by increase temperature, reduced capacity of the mussel to mount an efficient immune response overtime, thus enhancing bacterial pathogen infectivity and proliferation.

The new information in this thesis on mussel physiology, with and without the influence of temperature and pathogens provides crucial data, that can be incorporated into conservation management strategies for Greenshell^{TM} mussels in New Zealand. In this study we have learned that:

- Bacteria in the range of 10^7 and 10^9 CFU/mL results in mussel mortality. At bacterial doses lower than this, survival of mussels seems guaranteed.
- The largest number of mussel mortalities occurred within the first 48-hours of bacterial injection when mussels are kept at an ambient temperature (16°C).
- When the housing temperature exceeds 20°C and a high dose of bacteria is administrated to the mussel, mortalities still occurred by 6 days post challenge.
- The site of injection (adductor muscle) still shows signs of tissue damage 48 hours post injection.
- *P. swingsii* infection is not affected by the sex of the mussel.
- Temperature stress altered the levels of metabolite changes overtime.
- Combined stressors have a profound effect on mussel physiology overtime.
- Juvenile mussels are more at risk of bacterial infection and mortality than adult mussels.
- Two-pathogen coinfected individuals with *P. swingsii* or *Vibrio mediterranei*, more easily succumb to infection.

To this end the use of flow cytometry, bacterial plating, metabolomics, qPCR analyses, histological assessments, biochemical assays, and survival measures have proven successful to characterise, measure and track, *P. canaliculus* responses following exposure to bacterial and thermal stressors. Herein, these methods can be considered as biomarkers to monitor the influence of stressors, on a regular basis, and log findings in a database to provide evidence overtime. Only with sufficient knowledge on the status of changes and responses seen within mussels within exposure to environmental stressors can stakeholders and policy makers evaluate risks, set priorities, and make informed decisions.

One of the best ways to support the mussel industry going forward is to ensure that the industry is protected from biological harm and supported in adapting to climate change. Ultimately such improvements are likely to be achieved by selective breeding technology to improve value and resilience. Identification and enhancement of genotypes that are fittest in oxygen deprived environments, based on mussel strains that exhibit enhanced resilience to both temperature

stress and oxygen deprivation, will be essential for the sustainability of mussel stocks and their associated ecological and economical value.

10.2. CRITICAL ASSESSMENTS

Although the objectives of this study were achieved in full, the study is not without its limitations. The following is considered as study shortcomings:

- The length of the experimental exposure could have been extended to weeks to strengthen the conclusion made about how the infected mussel resistance/defence to bacterial pathogens decreased with prolonged exposure.
- Sample size of n = 10 mussels per sampling timepoint for co-infection appear to be low in this research. Despite this, variability between replicates was relatively low, and we are confident that the data obtained are representative of real differences between groups. The number of biological replications should be maximized in future studies whenever possible, if budgets and samples permit.
- Shorter sampling intervals are recommended for future as this will provide higher temporal resolution that occur rapidly within the system, which particularly crucial when studying dynamic processes such as haemocyte response that unfold quickly.
- Bacterial route of challenge (e.g., immersion vs injection) can significantly affect the response of mussels including the way the immune system responds and the subsequent infection outcomes. It is crucial to determine whether the chosen bacterial challenge route suits the animal species under study. A preliminary study may be necessary to determine the optimal bacterial challenge route for eliciting a robust and relevant immune response.
- In the same experimental design (mussel-pathogen interactions), mussels injected without any medium should be included as a control group to provide a baseline for comparison. It helps to understand the natural response of the mussels to the injection procedure itself, excluding any potential effects caused by the injection process alone.

10.3. CONCLUDING OBSERVATIONS

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

- Marie Curie -

Overall, this thesis has provided a rigorous exploration of complex biological processes within New Zealand Greenshell™ mussels (*P. canaliculus*) and deepened the understanding of their general health, disease progression, transmission mechanism and host-pathogen interactions. This thesis has enabled to the isolation, description, and characterisation of different bacterial pathogens specific to *P. canaliculus*. This thesis has also showcased that climate stress, including temperature extremes, can induce thermal stress and result in physiological disruptions of mussels. The investigation of pathogen-temperature-mussel interactions has contributed valuable insights into the potential impacts of climate change on disease dynamics, modulation of mussel cell immunity and alterations in host susceptibility during austral-summer, which also has set the first steps to unravel the pathogen interactions and host–pathogen interplay by demonstration of bacterial coinfections potential to cause disease in mussels. Consequently, the results of this thesis contributed to improve mussel health, highlighting multi-biomarker approaches and targets to monitor and assess mussel health in the laboratory, on farm and in wild populations. More generally, this study provides solid scientific support on the impacts of environmental stressors on the condition and physiology of native Greenshell™ mussels.

10.4. FUTURE RESEARCH AND RECOMMENDATIONS

Building on efforts from this thesis, the following recommendations can be viewed as new research initiatives for future studies.

Metabolite maze: The mysterious dance of molecules

Chapters 3, **6** and **8** have used metabolomics as a method to detect metabolites affected by stressors and gain deeper mechanistic insights into the response of the metabolome of *P. canaliculus*. The total number of annotated metabolites found in this thesis were 231, obtained from two different platforms (GC-MS and LC-MS tools), however, functional associations between metabolites identified are difficult to interpret since many of them are still unknown. This is a global limitation of metabolomics studies and should be recognised before aspiring

to utilise this technique. Additionally, a further challenge for the interpretation of metabolite data is to obtain a definitive understanding on biological processes since knowledge of changing metabolite levels alone is insufficient. To enhance metabolomics data interpretability and knowledge, there is a need for a significant shift towards integrating metabolomics measurements with higher-level biological information including molecular, physiological, and even population level measurement in the future. In future research, considering the integration of databases such as Reactome (https://reactome.org/what-is-reactome) and expanding the range of methods could certainly provide a more comprehensive understanding of the biological processes involved. Care should be taken when interpreting metabolomics. For example, in **Chapter 8** mussels were challenged with bacteria, making the metabolite results either a response from the mussel, the bacteria, or a combined effect of both. The use of appropriate controls and replicates can potentially account for cofounding effects of the various variables, enabling one to discriminate the metabolite response accordingly.

Bacterial virulence factors: Where are we now?

The area of bacterial virulence factors from Greenshell™ mussel pathogens has great scope for growth going forward. The use of a high-throughput method, like, time-resolved dual RNA-sequencing using tissue or haemolymph from a host-pathogen infection model, has proven to be highly successful in fish and eel studies (Tang et al., 2020, Xiong et al., 2020). By applying such an approach to mussel research, gains will be made by: (i) acquiring high-resolution dynamic transcriptome data from a prokaryotic and eukaryotic interacting system simultaneously; (ii) determining the molecular interactions between pathogens and hosts using the dual-transcriptomics data; and (iii) identify and predict critical genes associated with virulence during infection (Luo et al., 2020).

Bacterial virulence factors secreted by *P. swingsii* can be further research by investigating the transcriptome changes in response to simulation of temperature conditions during winter months (16°C) and during summer months (24°C) under laboratory conditions. Through the analysis of bacterial transcriptome data, insights may be gained into the expression levels of virulence factor genes compared to housekeeping genes. This may reveal which specific genes exhibit high expression within the bacterial cell during the temperature stress conditions.

As some virulence factors of *P. swingsii* have already been identified in **Chapter 2**, it is expected that research on the quorum-sensing system of *P. swingsii* will provide novel information on regulatory systems for controlling virulence gene expression. Many pathogenic bacteria use quorum sensing signals (QS) via cell-to-cell communication process to

coordinate the expression of bacterial virulence factors and enhance its potential to cause disease (Natrah et al., 2011, Verbrugghe et al., 2012, Defoirdt, 2014). Research into virulence factors that are involved in quorum sensing may contribute to the development of a vaccine against photobacteriosis, to the treatment of diseased mussels, and to the prevention of epidemics.

Repeated haemolymph sampling as a measure to track physiological changes in mussels

In the present study, substantial individual variability was observed over the course of a specified sampling period; however, the question of whether there is temporal variability among individuals remains unclear. For future research it is recommended to investigate the effect of repeated haemolymph sampling, from the same individual, to gain insights into the significance of haemolymph factors after exposure to stressful stimuli. This could be easily explored using a direct approach which involves longitudinal sampling of individuals before and after the challenge. Individuals exhibiting high levels of specific haemolymph components, either before or shortly after the challenge, are expected to exhibit lower pathogen burdens and enhanced survival (Ford and Paillard, 2007, Ford, 1986). It is, however, challenging to conduct repeated sampling in small organisms such as mussels as the sampling process itself may affect the parameters being evaluated. Even though repeated sampling is a common practice in human and veterinary medicine, its application in studying disease progression in individual bivalves is limited.

Role of humoral factors in molluscan defence strategies

Evaluation of immunomarker levels in this thesis mainly focused on the cellular factors, with humoral factors responsible for pathogen recognition (Allam and Raftos, 2015), lacking for *P. canaliculus.* Further studies using *P. swingsii* will benefit from an assessment of the entire immune system including humoral factors (e.g., lectins and antimicrobial peptides) (Ellis et al., 2011, Le Guernic et al., 2020), since cellular factors alone do not provide a complete picture of infection outcomes. This could be conducted by performing the multi-factor haemolymph profile as well as establishing a correlation between this profile and susceptibility to infection as seen in previous abalone study (Travers et al., 2008b).

Temperature vs. pathogens: What else to unveil on Greenshell^{TM} mussel vulnerabilities?

The present study shows that rising water temperature has a significant impact on mussels challenged with a pathogen, potentially contributing to mussel summer mortalities. Yet, other biotic and abiotic factors should also be investigated as contributors to this phenomenon. In

addition, long-term studies at aquaculture sites are needed to assess possible adaptation during continuous temperature stress. A further investigation of the synergistic effects of temperature with other environmental factors such as ocean acidification and hypoxia are warranted. Research in this area will improve our understanding of how marine bivalves respond to environmental fluctuations in general. On the other hand, elevating water temperature in mussel aquaculture offers several advantages. It helps prevent the proliferation of bacterial pathogens that are harmful to mussels since many bacterial diseases affecting mussels thrive in cooler water, so increasing water temperature can create an environment less conducive to these pathogens, enhances mussel health and growth, increases productivity, and reduces the need for antibiotics. However, temperature control must be approached cautiously to ensure environmental sustainability and avoid adverse effects on the ecosystem.

Understanding the effects of bacterial infections and temperature stress on feeding physiology, food intake, faeces production, and oxygen consumption is also an important aspect of aquaculture, but these factors are not well studied in mussels. A high temperature anomaly is also a significant threat to mussel species' recovery (Wang et al., 2015). A deeper understanding of how these anomalies affect digestive functions and whole animal physiology, as well as their interactions with diseases, is crucial for their conservation, especially since we may be able to formulate specific diets for aquaculture animals during marine heatwaves. In order to understand how to manage disease and climate change in restoration, it is essential to understand the physiological changes in other organ systems, such as reproductive potential and readiness.

Complexities of co-Infection in aquaculture systems: Research directions

To better understanding pathogenesis of complex bacterial pathogen assemblage in experimental system, co-infection, priority effects, and timing of exposure are important factors to consider. Single exposure to a pathogen and simultaneous exposure could lead to similar infection levels and disease outcomes as the immune system needs time to initiate responses (Marchetto and Power, 2018). In contrast, when the two pathogens are exposed temporally separate, it is possible to predict that the second pathogen will succeed more in infecting individuals and causing disease (Marchetto and Power, 2018). For future research, it would be interesting to investigate the impact of temporal separation between the exposure of two pathogens on infection levels, since this allows an understanding of pathogen community structure within hosts and disease outcomes.

The role of non-natural approaches in mussel research and application

The use of non-natural approaches in scientific research, such as the injection of mussels in controlled laboratory settings as seen in **Chapter 5**, **6**, **8** and **9**, offers distinct advantages for understanding specific variables and mechanisms. While these methods may not perfectly replicate natural conditions, they provide essential benefits like controlled experiments, hypothesis testing, and precise data. They are particularly valuable for identifying inoculum dose results in mussel mortality and potential biomarkers of stress or disease, which can later be validated and applied in more natural environments (Noble et al., 2017). These controlled experiments inform the development of management strategies and offer a baseline understanding of how mussels respond to stressors. To bridge the gap between laboratory findings and practical applications, researchers conduct field validation studies, integrate biomarkers into monitoring programs, and develop guidelines for mussel aquaculture and conservation. Non-natural approaches are a vital starting point, laying the foundation for a comprehensive understanding of mussel health and contributing to sustainable management practices.

References

ABDEL-AZIZ, M., EISSA, A. E., HANNA, M. & OKADA, M. A. 2013. Identifying some pathogenic *Vibrio* /*Photobacterium* species during mass mortalities of cultured Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) from some Egyptian coastal provinces. *International Journal of Veterinary Science and Medicine,* 1**,** 87-95.

ABELE, D. & PUNTARULO, S. 2004. Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 138**,** 405-415.

ACOSTA-SALMÓN, H., MARTÍNEZ-FERNÁNDEZ, E. & SOUTHGATE, P. C. 2005. Use of relaxants to obtain saibo tissue from the blacklip pearl oyster (*Pinctada margaritifer*a) and the Akoya pearl oyster (*Pinctada fucata*). *Aquaculture,* 246**,** 167-172.

ADETUNJI, A. I. & OLANIRAN, A. O. 2021. Production strategies and biotechnological relevance of microbial lipases: a review. *Brazilian Journal of Microbiology,* 52**,** 1257-1269.

ADZIGBLI, L., HAO, R., JIAO, Y., DENG, Y., DU, X., WANG, Q. & HUANG, R. 2020. Immune response of pearl oysters to stress and diseases. *Reviews in Aquaculture,* 12**,** 513-523.

AGGIO, R., VILLAS-BÔAS, S. G. & RUGGIERO, K. 2011. Metab: An R package for high-throughput analysis of metabolomics data generated by GC-MS. *Bioinformatics,* 27**,** 2316-2318.

AGUILERA-SÁEZ, L. M., ABREU, A. C., CAMACHO-RODRÍGUEZ, J., GONZÁLEZ-LÓPEZ, C. V., DEL CARMEN CERÓN-GARCÍA, M. A. & FERNÁNDEZ, I. 2019. NMR metabolomics as an effective tool to unravel the effect of light intensity and temperature on the composition of the marine microalgae *Isochrysis galbana*. *Journal of agricultural and food chemistry,* 67**,** 3879-3889.

AJADI, A., SABRI, M., ATATA, J., DAODU, O. & EMIKPE, B. 2019. Pathology and immunohistochemical evaluation of *Vibrio alginolyticus* infection in *Macrobrachium rosenbergii*. *Comparative Clinical Pathology,* 28**,** 359-368.

ALAM, M., SULTANA, M., BALAKRISH NAIR, G., SIDDIQUE, A. K., HASAN, N. A., BRADLEY SACK, R., SACK, D. A., AHMED, K. U., SADIQUE, A., WATANABE, H., GRIM, C. J., HUQ, A. & COLWELL, R. R. Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *Proceedings of the National Academy of Sciences of the United States of America*, 2007. 17801-17806.

ALAM, Y. H., KIM, R. & JANG, C. 2022. Metabolism and health impacts of dietary sugars. *Journal of lipid and atherosclerosis,* 11**,** 20.

ALBUIXECH-MARTÍ, S., CULLOTY, S. C. & LYNCH, S. A. 2021. Co-occurrence of pathogen assemblages in a keystone species the common cockle *Cerastoderma edule* on the Irish coast. *Parasitology,* 148**,** 1665-1679.

ALDRIDGE, D. C., OLLARD, I. S., BESPALAYA, Y. V., BOLOTOV, I. N., DOUDA, K., GEIST, J., HAAG, W. R., KLUNZINGER, M. W., LOPES-LIMA, M. & MLAMBO, M. C. 2023. Freshwater mussel conservation: A global horizon scan of emerging threats and opportunities. *Global Change Biology,* 29**,** 575-589.

ALFARO, A. C., NGUYEN, T. V. & MELLOW, D. 2019a. A metabolomics approach to assess the effect of storage conditions on metabolic processes of New Zealand surf clam (*Crassula aequilatera*). *Aquaculture,* 498**,** 315-321.

ALFARO, A. C., NGUYEN, T. V. & MERIEN, F. 2019b. The complex interactions of Ostreid herpesvirus 1, *Vibrio* bacteria, environment and host factors in mass mortality outbreaks of *Crassostrea gigas*. *Reviews in Aquaculture,* 11**,** 1148-1168.

ALFARO, A. C. & YOUNG, T. 2018. Showcasing metabolomic applications in aquaculture: a review. *Reviews in Aquaculture,* 10**,** 135-152.

ALIPIA, T., MAE, H. & DUNPHY, B. 2014. A non-invasive anaesthetic method for accessing the brood chamber of the Chilean flat oyster (*Ostrea chilensis*). *New Zealand Journal of Marine and Freshwater Research,* 48**,** 350-355.

ALLAM, B. & ESPINOSA, E. P. 2016. Bivalve immunity and response to infections: are we looking at the right place? *Fish & shellfish immunology,* 53**,** 4-12.

ALLAM, B., PAILLARD, C., AUFFRET, M. & FORD, S. E. 2006. Effects of the pathogenic *Vibrio tapetis* on defence factors of susceptible and non-susceptible bivalve species: II. Cellular and biochemical changes following in vivo challenge. *Fish and Shellfish Immunology,* 20**,** 384-397.

ALLAM, B., PAILLARD, C. & FORD, S. E. 2002. Pathogenicity of *Vibrio tapetis*, the etiological agent of brown ring disease in clams. *Diseases of Aquatic Organisms,* 48**,** 221-31.

ALLAM, B. & RAFTOS, D. 2015. Immune responses to infectious diseases in bivalves. *Journal of Invertebrate Pathology,* 131**,** 121-36.

AMARAL, G. R. S., DIAS, G. M., WELLINGTON-OGURI, M., CHIMETTO, L., CAMPEÃO, M. E., THOMPSON, F. L. & THOMPSON, C. C. 2014. Genotype to phenotype: Identification of diagnostic *Vibrio* phenotypes using whole genome sequences. *International Journal of Systematic and Evolutionary Microbiology,* 64**,** 357-365.

AMIN, A. K. M. R., TANAKA, M., AL-SAARI, N., FENG, G., MINO, S., OGURA, Y., HAYASHI, T., MEIRELLES, P. M., THOMPSON, F. L., GOMEZ-GIL, B., SAWABE, T. & SAWABE, T. 2017. *Thaumasiovibrio* occultus gen. nov. sp. nov. and *Thaumasiovibrio subtropicus* sp. nov. within the family *Vibrionaceae*, isolated from coral reef seawater off Ishigaki Island, Japan. *Systematic and Applied Microbiology,* 40.

ANDERSON, T. J., MORRISON, M., MACDIARMID, A., CLARK, M., ARCHINO, R. D., TRACEY, D., GORDON, D., READ, G., KETTLES, H., MORRISEY, D., WOOD, A., SMITH, A. M., PAGE, M., PAUL-BURKE, K., SCHNABEL, K., WADHWA, S. & ANDERSON, T. 2019. Review of New Zealand's Key Biogenic Habitats. *NIWA Client Report.*

ANDREE, K. B., CARRASCO, N., CARELLA, F., FURONES, D. & PRADO, P. 2021. *Vibrio mediterranei*, a potential emerging pathogen of marine fauna: investigation of pathogenicity using a bacterial challenge in *Pinna nobilis* and development of a species-specific PCR. *Journal of Applied Microbiology,* 130**,** 617-631.

ANDREWS, S. & OTHERS. 2010. *FastQC: a quality control tool for high throughput sequence data. 2010* [Online]. Available: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ [Accessed].

ANESTIS, A., LAZOU, A., PÖRTNER, H. O. & MICHAELIDIS, B. 2007. Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*.

ANESTIS, A., PÖRTNER, H. O., KARAGIANNIS, D., ANGELIDIS, P., STAIKOU, A. & MICHAELIDIS, B. 2010. Response of *Mytilus galloprovincialis* (L.) to increasing seawater temperature and to marteliosis: Metabolic and physiological parameters. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology,* 156.

AQNZ 2022. A sector overview with key facts and statistsics for 2022. *Aquaculture for New Zealand.*

ARAFA, S., SADOK, S. & ABED, A. E. 2007. Assessment of magnesium chloride as an anaesthetic for adult sea urchins (*Paracentrotus lividus*): incidence on mortality and spawning. *Aquaculture Research,* 38**,** 1673-1678.

ARMSTRONG, R., RIAZ, S., HASAN, S., IQBAL, F., RICE, T. & SYED, N. 2018. Mechanisms of anesthetic action and neurotoxicity: Lessons from molluscs. *Frontiers in Physiology,* 8**,** 1-13.

ARZUL, I., CHOLLET, B., MICHEL, J., ROBERT, M., GARCIA, C., JOLY, J.-P., FRANCOIS, C. & MIOSSEC, L. 2012. One *Perkinsus* species may hide another: characterization of *Perkinsus* species present in clam production areas of France. *Parasitology,* 139**,** 1757-1771.

ASHBY, R. 2019. *The development and implementation of genomic tools for the New Zealand Greenshell™ Mussel industry.* Doctor of Philosophy PhD thesis, University of Otago.

ASHRAFUDOULLA, M., MIZAN, M. F. R., PARK, H., BYUN, K.-H., LEE, N., PARK, S. H. & HA, S.-D. 2019. Genetic relationship, virulence factors, drug resistance profile and biofilm formation ability of Vibrio parahaemolyticus isolated from mussel. *Frontiers in microbiology,* 10**,** 513.

ASHTON, E. C., GUIST, S., ROBERTS, D. & SIGWART, J. D. 2020. Effects of environmental factors and husbandry practices on summer mortality events in the cultivated Pacific oyster *Crassostrea gigas* in the North of Ireland. *Journal of Shellfish Research,* 39**,** 13-20.

AUGUSTE, M., BALBI, T., CIACCI, C., CANONICO, B., PAPA, S., BORELLO, A., VEZZULLI, L. & CANESI, L. 2020. Shift in immune parameters after repeated exposure to nanoplastics in the marine bivalve *Mytilus*. *Frontiers in Immunology,* 11.

AUNKHAM, A., ZAHN, M., KESIREDDY, A., POTHULA, K. R., SCHULTE, A., BASLÉ, A., KLEINEKATHÖFER, U., SUGINTA, W. & VAN DEN BERG, B. 2018. Structural basis for chitin acquisition by marine *Vibrio* species. *Nature Communications,* 9**,** 220.

AVDELAS, L., AVDIC-MRAVLJE, E., BORGES MARQUES, A. C., CANO, S., CAPELLE, J. J., CARVALHO, N., COZZOLINO, M., DENNIS, J., ELLIS, T., FERNÁNDEZ POLANCO, J. M., GUILLEN, J., LASNER, T., LE BIHAN, V., LLORENTE, I., MOL, A., NICHEVA, S., NIELSEN, R., VAN OOSTENBRUGGE, H., VILLASANTE, S., VISNIC, S., ZHELEV, K. & ASCHE, F. 2021. The decline of mussel aquaculture in the European Union: causes, economic impacts and opportunities. *Reviews in Aquaculture,* 13.

AZIZAN, A., ALFARO, A. C., JARAMILLO, D., VENTER, L., YOUNG, T., FROST, E., LEE, K., VAN NGUYEN, T., KITUNDU, E., ARCHER, S. D. J., ERICSON, J. A., FOXWELL, J., QUINN, O. & RAGG, N. L. C. 2022. Pathogenicity and virulence of bacterial strains associated with summer mortality in marine mussels (*Perna canaliculus*). *FEMS Microbiology Ecology,* 98**,** 1-14.

AZIZAN, A., ALFARO, A. C., VENTER, L., JARAMILLO, D., BESTBIER, M., FOXWELL, J., BENNET, P. & YOUNG, T. 2023a. Quantification of pathogenic *Photobacterium swingsii* and characterisation of disease progression in the New Zealand Greenshell™ mussel, *Perna canaliculus*. *Journal of Invertebrate Pathology,* Under review.

AZIZAN, A., ALFARO, A. C., VENTER, L., ZHANG, J. J., ERICSON, J. A., YOUNG, T., DELORME, N. J. & RAGG, N. L. C. 2023b. Interactive effects of elevated temperature and *Photobacterium swingsii* infection on the survival and immune response of marine mussels (*Perna canaliculus*). *Science of The Total Environment,* Submitted.

AZIZAN, A., ALFARO, A. C., YOUNG, T. & VENTER, L. 2021. Beyond relaxed: magnesium chloride anaesthesia alters the circulatory metabolome of a marine mollusc (*Perna canaliculus*). *Metabolomics,* 17**,** 73.

AZIZAN, A., CARTER, J., ALFARO, A. C., VENTER, L., YOUNG, T., SHARMA, S. S. & CHEN, T. 2023c. Investigating the effect of bacterial co-infections on juvenile and adult, green-lipped mussels (*Perna canaliculus*). *Journal of World Aquaculture Society,* Accepted.

AZIZAN, A., VENTER, L. & ALFARO, A. C. 2023d. Physiological biomarkers of mussel Vibrionaceae: A review on the constraints and potentials. *Aquaculture international,* Submitted.

BAAG, S. & MANDAL, S. 2022. Combined effects of ocean warming and acidification on marine fish and shellfish: A molecule to ecosystem perspective. *Science of the Total Environment,* 802**,** 149807.

BABARRO, J. M. & DE ZWAAN, A. 2002. Influence of abiotic factors on bacterial proliferation and anoxic survival of the sea mussel *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology,* 273**,** 33-49.

BABCOCK, R. C., BUSTAMANTE, R. H., FULTON, E. A., FULTON, D. J., HAYWOOD, M. D., HOBDAY, A. J., KENYON, R., MATEAR, R. J., PLAGÁNYI, E. E. & RICHARDSON, A. J. 2019. Severe continental-scale impacts of climate change are happening now: extreme climate events impact marine habitat forming communities along 45% of Australia's coast. *Frontiers in Marine Science***,** 411.

BACHÈRE, E., ROSA, R. D., SCHMITT, P., POIRIER, A. C., MEROU, N., CHARRIÈRE, G. M. & DESTOUMIEUX-GARZON, D. 2015. The new insights into the oyster antimicrobial defense: cellular, molecular and genetic view. *Fish & shellfish immunology,* 46**,** 50-64.

BAKER-AUSTIN, C., OLIVER, J. D., ALAM, M., ALI, A., WALDOR, M. K., QADRI, F. & MARTINEZ-URTAZA, J. 2018. *Vibrio* spp. infections. *Nature Reviews Disease Primers,* 4**,** 1-19.

BAKER-AUSTIN, C., TRINANES, J., GONZALEZ-ESCALONA, N. & MARTINEZ-URTAZA, J. 2017. Non-cholera vibrios: The microbial barometer of climate change. *Trends in Microbiology,* 25.

BALBI, T., AUGUSTE, M., CORTESE, K., MONTAGNA, M., BORELLO, A., PRUZZO, C., VEZZULLI, L. & CANESI, L. 2019. Responses of *Mytilus galloprovincialis* to challenge with the emerging marine pathogen *Vibrio coralliilyticus*. *Fish and Shellfish Immunology,* 84**,** 352-360.

BALBI, T., CORTESE, K., CIACCI, C., BELLESE, G., VEZZULLI, L., PRUZZO, C. & CANESI, L. 2018. Autophagic processes in *Mytilus galloprovincialis* hemocytes: Effects of *Vibrio tapetis*. *Fish and Shellfish Immunology,* 73**,** 66-74.

BALBI, T., FABBRI, R., CORTESE, K., SMERILLI, A., CIACCI, C., GRANDE, C., VEZZULLI, L., PRUZZO, C. & CANESI, L. 2013. Interactions between *Mytilus galloprovincialis* hemocytes and the bivalve pathogens *Vibrio aestuarianus* 01/032 and *Vibrio splendidus* LGP32. *Fish and Shellfish Immunology,* 35**,** 1906-1915.

BALBI, T., FABBRI, R., MONTAGNA, M., CAMISASSI, G. & CANESI, L. 2017. Seasonal variability of different biomarkers in mussels (*Mytilus galloprovincialis*) farmed at different sites of the Gulf of La Spezia, Ligurian sea, Italy. *Marine Pollution Bulletin,* 116**,** 348-356.

BAO, M., HUO, L., WU, J., GE, D., LV, Z., CHI, C., LIAO, Z. & LIU, H. 2018. A novel biomarker for marine environmental pollution of *CAT* from *Mytilus coruscus*. *Marine Pollution Bulletin,* 127**,** 717-725.

BARBER, D., VILLASEÑOR, A. & ESCRIBESE, M. M. 2019. Metabolomics strategies to discover new biomarkers associated to severe allergic phenotypes. *Asia Pacific Allergy,* 9.

BASSIM, S., GENARD, B., GAUTHIER-CLERC, S., MORAGA, D. & TREMBLAY, R. 2015. Ontogeny of bivalve immunity: assessing the potential of next-generation sequencing techniques. *Reviews in Aquaculture,* 7**,** 197-217.

BATTISTINI, R., VARELLO, K., LISTORTI, V., ZAMBON, M., ARCANGELI, G., BOZZETTA, E., FRANCESE, D. R., ERCOLINI, C. & SERRACCA, L. 2020. Microbiological and histological analysis for the evaluation of farmed mussels (*Mytilus galloprovincialis*) health status, in coastal areas of Italy. *Pathogens,* 9**,** 395.

BAYNE, B. L., WIDDOWS, J. & THOMPSON, R. J. 1976. *Physiology II*, Cambridge University Press.

BEAZ-HIDALGO, R., BALBOA, S., ROMALDE, J. L. & FIGUERAS, M. J. 2010. Diversity and pathogenecity of Vibrio species in cultured bivalve molluscs. *Environmental microbiology reports,* 2**,** 34-43.

BEHRENS, E., RICKARD, G., ROSIER, S., WILLIAMS, J., MORGENSTERN, O. & STONE, D. 2022. Projections of future marine heatwaves for the oceans around New Zealand using New Zealand's earth system model. *Frontiers in Climate***,** 19.

BELENEVA, I. & MASLENNIKOVA, E. 2005. Hydrolytic activity of marine bacteria associated with the mussel *Mytilus trossulus*. *Mikrobiolohichnyi Zhurnal (Kiev, Ukraine: 1993),* 67**,** 3-8.

BEN CHEIKH, Y., TRAVERS, M.-A. & LE FOLL, F. 2017. Infection dynamics of a *V. splendidus* strain pathogenic to *Mytilus edulis*: In vivo and in vitro interactions with hemocytes. *Fish & Shellfish Immunology,* 70**,** 515-523.

BEN CHEIKH, Y., TRAVERS, M.-A., MORGA, B., GODFRIN, Y., RIOULT, D. & LE FOLL, F. 2016. First evidence for a *Vibrio* strain pathogenic to *Mytilus edulis* altering hemocyte immune capacities. *Developmental & Comparative Immunology,* 57**,** 107-119.

BENABDELMOUNA, A., GARCIA, C., LEDU, C., LAMY, P., MAUROUARD, E. & DÉGREMONT, L. 2018. Mortality investigation of *Mytilus edulis* and *Mytilus galloprovincialis* in France: An experimental survey under laboratory conditions. *Aquaculture,* 495**,** 831-841.

BENJAMINI, Y. & HOCHBERG, Y. 2000. On the adaptive control of the false discovery rate in multiple testing with independent statistics. *Journal of Educational and Behavioral Statistics,* 25**,** 60-83.

BETTENCOURT, R., DANDO, P., COLLINS, P., COSTA, V., ALLAM, B. & SANTOS, R. S. 2009. Innate immunity in the deep sea hydrothermal vent mussel *Bathymodiolus azoricus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 152**,** 278-289.

BETTENCOURT, R., ROCH, P., STEFANNI, S., ROSA, D., COLAÇO, A. & SANTOS, R. S. 2007. Deep sea immunity: unveiling immune constituents from the hydrothermal vent mussel *Bathymodiolus azoricus*. *Marine Environmental Research,* 64**,** 108-127.

BETTENCOURT, R., RODRIGUES, M., BARROS, I., CERQUEIRA, T., FREITAS, C., COSTA, V., PINHEIRO, M., EGAS, C. & SANTOS, R. S. 2014. Site-related differences in gene expression and bacterial densities in the mussel *Bathymodiolus azoricus* from the Menez Gwen and Lucky Strike deep-sea hydrothermal vent sites. *Fish & Shellfish Immunology,* 39**,** 343-353.

BETTI, M., CIACCI, C., LORUSSO, L. C., CANONICO, B., FALCIONI, T., GALLO, G. & CANESI, L. 2006. Effects of tumour necrosis factor α (TNFα) on *Mytilus* haemocytes: role of stress-activated mitogen-activated protein kinases (MAPKs). *Biology of the Cell,* 98**,** 233-244.

BI, Y., XUE, B., BRISCOE, D., VENNELL, R. & ZHANG, M. 2023. A new artificial intelligent approach to buoy detection for mussel farming. *Journal of the Royal Society of New Zealand,* 53**,** 27-51.

BIEL, F. M., ALLEN, F. A. & HÄSE, C. C. 2014. Autolysis in *Vibrio tubiashii* and *Vibrio coralliilyticus*. *Canadian Journal of Microbiology,* 60**,** 57-63.

BIGNELL, J. P., DODGE, M. J., FEIST, S. W., LYONS, B., MARTIN, P. D., TAYLOR, N. G. H., STONE, D., TRAVALENT, L. & STENTIFORD, G. D. 2008. Mussel histopathology: Effects of season, disease and species. *Aquatic Biology,* 2**,** 1-15.

BOMMARITO, C., WAHL, M., THIELTGES, D., PANSCH, C., ZUCCHETTA, M. & PRANOVI, F. 2022. Biotic and abiotic drivers affect parasite richness, prevalence and abundance in *Mytilus galloprovincialis* along the Northern Adriatic Sea. *Parasitology,* 149**,** 15-23.

BONDAD-REANTASO, M. G., SUBASINGHE, R. P., ARTHUR, J. R., OGAWA, K., CHINABUT, S., ADLARD, R., TAN, Z. & SHARIFF, M. 2005. Disease and health management in Asian aquaculture. *Veterinary parasitology,* 132**,** 249-272.

BOUALLEGUI, Y. 2019. Immunity in mussels: An overview of molecular components and mechanisms with a focus on the functional defenses. *Fish & Shellfish Immunology,* 89**,** 158-169.

BOUKADIDA, K., CACHOT, J., CLÉRANDEAUX, C., GOURVES, P.-Y. & BANNI, M. 2017. Early and efficient induction of antioxidant defense system in *Mytilus galloprovincialis* embryos exposed to metals and heat stress. *Ecotoxicology and environmental safety,* 138**,** 105-112.

BOX, A., CAPÓ, X., TEJADA, S., CATANESE, G., GRAU, A., DEUDERO, S., SUREDA, A. & VALENCIA, J. M. 2020. Reduced antioxidant response of the fan mussel *Pinna nobilis* related to the presence of *Haplosporidium pinnae*. *Pathogens,* 9**,** 1-14.

BOYD, E. F. 2007. The biology of vibrios. *The Lancet Infectious Diseases,* 7**,** 384.

BOYD, P. W., COLLINS, S., DUPONT, S., FABRICIUS, K., GATTUSO, J. P., HAVENHAND, J., HUTCHINS, D. A., RIEBESELL, U., RINTOUL, M. S. & VICHI, M. 2018. Experimental strategies to assess the biological ramifications of multiple drivers of global ocean change—a review. *Global change biology,* 24**,** 2239-2261.

BRENNAN, J. J. & GILMORE, T. D. 2018. Evolutionary origins of toll-like receptor signaling. *Molecular biology and evolution,* 35**,** 1576-1587.

BRIAN, J. I., OLLARD, I. S. & ALDRIDGE, D. C. 2021. Don't move a mussel? Parasite and disease risk in conservation action. *Conservation Letters,* 14**,** e12799.

BROADHURST, D., GOODACRE, R., REINKE, S. N., KULIGOWSKI, J., WILSON, I. D., LEWIS, M. R. & DUNN, W. B. 2018. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. *Metabolomics,* 14**,** 1-17.

BROEKHUIZEN, N., PLEW, D. R., PINKERTON, M. H. & GALL, M. G. 2021. Sea temperature rise over the period 2002–2020 in Pelorus Sound, New Zealand – with possible implications for the aquaculture industry. *New Zealand Journal of Marine and Freshwater Research,* 55**,** 46-64.

BROOKS, S., LYONS, B., GOODSIR, F., BIGNELL, J. & THAIN, J. 2009. Biomarker responses in mussels, an integrated approach to biological effects measurements. *Journal of Toxicology and Environmental Health - Part A: Current Issues,* 72.

BROSNAHAN, C. 2020. *Diagnostic investigation into summer mortality events of farmed Chinook salmon (Oncorhynchus tshawytscha) in New Zealand.* PhD thesis, Massey University.

BROSSET, P., COOKE, S. J., SCHULL, Q., TRENKEL, V. M., SOUDANT, P. & LEBIGRE, C. 2021. Physiological biomarkers and fisheries management. *Reviews in Fish Biology and Fisheries,* 31.

BUCKLEY, K. M., HO, E. C. H., HIBINO, T., SCHRANKEL, C. S., SCHUH, N. W., WANG, G. & RAST, J. P. 2017. IL17 factors are early regulators in the gut epithelium during inflammatory response to *Vibrio* in the sea urchin larva. *Elife,* 6**,** e23481.

BUIJS, Y., ISBRANDT, T., ZHANG, S. D., LARSEN, T. O. & GRAM, L. 2020. The antibiotic andrimid produced by *Vibrio coralliilyticus* increases expression of biosynthetic gene clusters and antibiotic production in *Photobacterium galatheae*. *Frontiers in Microbiology,* 11**,** 1-13.

BULLER, N. 2004a. *Bacteria from fish and other aquatic animals: a practical identification manual,* Wallingford, CABI.

BULLER, N. 2013. Aquatic animals; endemic and exotic bacterial pathogens. *Microbiology Australia,* 34**,** 22-24.

BULLER, N. B. 2004b. *Interpretation of biochemical identification tests and sets.,* Wallingford, CABI.

BURGE, C. A., CLOSEK, C. J., FRIEDMAN, C. S., GRONER, M. L., JENKINS, C. M., SHORE-MAGGIO, A. & WELSH, J. E. 2016a. The use of filter-feeders to manage disease in a changing world. *Integrative and Comparative Biology,* 56**,** 573-587.

BURGE, C. A., FRIEDMAN, C. S., GETCHELL, R., HOUSE, M., LAFFERTY, K. D., MYDLARZ, L. D., PRAGER, K. C., SUTHERLAND, K. P., RENAULT, T. & KIRYU, I. 2016b. Complementary approaches to diagnosing marine diseases: a union of the modern and the classic. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 371**,** 20150207.

BURGOS-ACEVES, M. A. & FAGGIO, C. 2017. An approach to the study of the immunity functions of bivalve haemocytes: physiology and molecular aspects. *Fish & Shellfish Immunology,* 67**,** 513-517.

BURTSEVA, O., KUBLANOVSKAYA, A., BAULINA, O., FEDORENKO, T., LOBAKOVA, E. & CHEKANOV, K. 2020. The strains of bioluminescent bacteria isolated from the White Sea finfishes: genera *Photobacterium*, *Aliivibrio*, *Vibrio*, *Shewanella*, and first luminous Kosakonia. *Journal of Photochemistry and Photobiology B: Biology,* 208**,** 111895.

BUTT, D., O'CONNOR, S. J., KUCHEL, R., O'CONNOR, W. A. & RAFTOS, D. A. 2008. Effects of the muscle relaxant, magnesium chloride, on the Sydney rock oyster (*Saccostrea glomerata*). *Aquaculture,* 275**,** 342-346.

CAJARAVILLE, M., DÍEZ, G., MARIGÓMEZ, I. & ANGULO, E. 1991. Consequences of keeping *Mytilus* in the laboratory as assessed by different cellular condition indices. *Helgoländer Meeresuntersuchungen,* 45**,** 445-464.

CALIFF, R. M. 2018. Biomarker definitions and their applications. *Experimental Biology and Medicine,* 243**,** 213-221.

CAMERON, A. 2002. Survey toolbox for aquatic animal diseases: a practical manual and software package.

CAMPOS, A., APRAIZ, I., DA FONSECA, R. R. & CRISTOBAL, S. 2015. Shotgun analysis of the marine mussel *Mytilus edulis* hemolymph proteome and mapping the innate immunity elements. *Proteomics,* 15**,** 4021-4029.

CAMPOS, A., TEDESCO, S., VASCONCELOS, V. & CRISTOBAL, S. 2012. Proteomic research in bivalves: Towards the identification of molecular markers of aquatic pollution. *Journal of Proteomics,* 75**,** 4346-4359.

CANESI, L., BARMO, C., FABBRI, R., CIACCI, C., VERGANI, L., ROCH, P. & GALLO, G. 2010. Effects of *Vibrio* challenge on digestive gland biomarkers and antioxidant gene expression in *Mytilus galloprovincialis*. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology,* 152**,** 399-406.

CANESI, L., BETTI, M., CIACCI, C., CITTERIO, B., PRUZZO, C. & GALLO, G. 2003. Tyrosine kinase-mediated cell signalling in the activation of *Mytilus* hemocytes: possible role of STAT-like proteins. *Biology of the Cell,* 95**,** 603-613.

CANESI, L., BETTI, M., CIACCI, C., LORUSSO, L. C., GALLO, G. & PRUZZO, C. 2005. Interactions between *Mytilus* haemocytes and different strains of *Escherichia coli* and *Vibrio cholerae* O1 El Tor: Role of kinase-mediated signalling. *Cellular Microbiology,* 7.

CANESI, L., BETTI, M., CIACCI, C., LORUSSO, L. C., PRUZZO, C. & GALLO, G. 2006. Cell signalling in the immune response of mussel hemocytes. *Invertebrate Survival Journal,* 3.

CANESI, L., BETTI, M., CIACCI, C., SCARPATO, A., CITTERIO, B., PRUZZO, C. & GALLO, G. 2002a. Signaling pathways involved in the physiological response of mussel hemocytes to bacterial challenge: The role of stress-activated p38 MAP kinases. *Developmental and Comparative Immunology,* 26.

CANESI, L., GALLO, G., GAVIOLI, M. & PRUZZO, C. 2002b. Bacteria-hemocyte interactions and phagocytosis in marine bivalves. *Microscopy Research and Technique,* 57.

CANESI, L. & PRUZZO, C. 2016. Specificity of Innate Immunity in Bivalves: A Lesson From Bacteria. *In:* BALLARIN, L. & CAMMARATA, M. (eds.) *Lessons in Immunity.* Academic Press.

CANESI, L., PRUZZO, C., TARSI, R. & GALLO, G. 2001. Surface interactions between *Escherichia coli* and hemocytes of the Mediterranean mussel *Mytilus galloprovincialis* Lam. leading to efficient bacterial clearance. *Applied and environmental microbiology,* 67**,** 464-468.

CANTON, J., NECULAI, D. & GRINSTEIN, S. 2013. Scavenger receptors in homeostasis and immunity. *Nature Reviews Immunology,* 13**,** 621-634.

CARELLA, F., ANTUOFERMO, E., FARINA, S., SALATI, F., MANDAS, D., PRADO, P., PANARESE, R., MARINO, F., FIOCCHI, E. & PRETTO, T. 2020. In the wake of the ongoing mass mortality events: co-occurrence of Mycobacterium, Haplosporidium and other pathogens in *Pinna nobilis* collected in Italy and Spain (Mediterranean Sea). *Frontiers in marine science,* 7**,** 48.

CARELLA, F., FEIST, S., BIGNELL, J. & DE VICO, G. 2015. Comparative pathology in bivalves: Aetiological agents and disease processes. *Journal of invertebrate pathology,* 131**,** 107-120.

CARRERA, M., PIÑEIRO, C. & MARTINEZ, I. 2020. Proteomic strategies to evaluate the impact of farming conditions on food quality and safety in aquaculture products. *Foods,* 9**,** 1050.

CARVER, C. E., THÉRIAULT, I. & MALLET, A. L. 2010. Infection of cultured eastern oysters *Crassostrea virginica* by the boring sponge *Cliona celata*, with emphasis on sponge life history and mitigation strategies. *Journal of Shellfish Research,* 29**,** 905-915.

CASTILLO, N., SAAVEDRA, L. M., VARGAS, C. A., GALLARDO-ESCÁRATE, C. & DÉTRÉE, C. 2017. Ocean acidification and pathogen exposure modulate the immune response of the edible mussel *Mytilus chilensis*. *Fish & Shellfish Immunology,* 70**,** 149-155.

CASTINEL, A., WEBB, S., JONES, J., PEELER, E. & FORREST, B. 2019. Disease threats to farmed green-lipped mussels *Perna canaliculus* in New Zealand: review of challenges in risk assessment and pathway analysis. *Aquaculture Environment Interactions,* 11**,** 291-304.

CAZA, F., BERNET, E., VEYRIER, F. J., BETOULLE, S. & ST-PIERRE, Y. 2020. Hemocytes released in seawater act as Trojan horses for spreading of bacterial infections in mussels. *Scientific Reports,* 10.

CELLURA, C., TOUBIANA, M., PARRINELLO, N. & ROCH, P. 2006. *HSP70* gene expression in *Mytilus galloprovincialis* hemocytes is triggered by moderate heat shock and *Vibrio anguillarum*, but not by *V. splendidus* or *Micrococcus lysodeikticus*. *Developmental and Comparative Immunology,* 30**,** 984-997.

CELLURA, C., TOUBIANA, M., PARRINELLO, N. & ROCH, P. 2007. Specific expression of antimicrobial peptide and *HSP70* genes in response to heat-shock and several bacterial challenges in mussels. *Fish and Shellfish Immunology,* 22**,** 340-350.

CHAMPELY, S., EKSTROM, C., DALGAARD, P., GILL, J., WEIBELZAHL, S., ANANDKUMAR, A., FORD, C., VOLCIC, R., DE ROSARIO, H. & DE ROSARIO, M. H. 2018. Package 'pwr'. *R package version,* 1.

CHANDHINI, S. & REJISH KUMAR, V. J. 2019. Transcriptomics in aquaculture: current status and applications. *Reviews in Aquaculture,* 11**,** 1379-1397.

CHANDURVELAN, R., MARSDEN, I. D., GAW, S. & GLOVER, C. N. 2013. Waterborne cadmium impacts immunocytotoxic and cytogenotoxic endpoints in green-lipped mussel, *Perna canaliculus*. *Aquatic toxicology,* 142**,** 283-293.

CHANDURVELAN, R., MARSDEN, I. D., GLOVER, C. N. & GAW, S. 2016. Biomarker responses of mussels exposed to earthquake disturbances. *Estuarine, Coastal and Shelf Science,* 182**,** 98-111.

CHARLES, M., BERNARD, I., VILLALBA, A., ODEN, E., BURIOLI, E. A. V., ALLAIN, G., TRANCART, S., BOUCHART, V. & HOUSSIN, M. 2020a. High mortality of mussels in northern Brittany – Evaluation of the involvement of pathogens, pathological conditions and pollutants. *Journal of Invertebrate Pathology,* 170**,** 107308.

CHARLES, M., TRANCART, S., ODEN, E. & HOUSSIN, M. 2020b. Experimental infection of *Mytilus edulis* by two *Vibrio splendidus*-related strains: Determination of pathogenicity level of strains and influence of the origin and annual cycle of mussels on their sensitivity. *Journal of Fish Diseases,* 43**,** 9-21.

CHEIKH, Y. B., TRAVERS, M.-A. & LE FOLL, F. 2017. Infection dynamics of a V. splendidus strain pathogenic to Mytilus edulis: In vivo and in vitro interactions with hemocytes. *Fish & shellfish immunology,* 70**,** 515-523.

CHELLAPACKIALAKSHMI, M. & RAVI, C. 2022. Investigation on Mollusc Lectins. *Aquatic Lectins: Immune Defense, Biological Recognition and Molecular Advancements.* Springer Nature Singapore: Springer.

CHEN, H., SOLANGI, G. S., ZHAO, C., YANG, L., GUO, J., WAN, F. & ZHOU, Z. 2019. Physiological metabolic responses of *Ophraella communa* to high temperature stress. *Frontiers in Physiology,* 10**,** 1053.

CHEN, H., WANG, M., ZHANG, H., WANG, H., ZHOU, L., ZHONG, Z., CAO, L., LIAN, C., SUN, Y. & LI, C. 2021a. microRNAs facilitate comprehensive responses of *Bathymodiolinae* mussel against symbiotic and nonsymbiotic bacteria stimulation. *Fish & Shellfish Immunology,* 119**,** 420-431.

CHEN, Y.-Q., WANG, J., LIAO, M.-L., LI, X.-X. & DONG, Y.-W. 2021b. Temperature adaptations of the thermophilic snail *Echinolittorina malaccana*: insights from metabolomic analysis. *Journal of Experimental Biology,* 224**,** jeb238659.

CHEN, Y., XU, K., LI, J., WANG, X., YE, Y. & QI, P. 2018. Molecular characterization of complement component 3 (C3) in *Mytilus coruscus* improves our understanding of bivalve complement system. *Fish & shellfish immunology,* 76**,** 41-47.

CHENG-HUA, L., JIAN-MIN, Z. & LIN-SHENG, S. 2009. A review of advances in research on marine molluscan antimicrobial peptides and their potential application in aquaculture. *Molluscan Research,* 29**,** 17.

CHONG, J., SOUFAN, O., LI, C., CARAUS, I., LI, S., BOURQUE, G., WISHART, D. S. & XIA, J. 2018. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Research,* 46**,** W486-W494.

CHONG, J., WISHART, D. S. & XIA, J. 2019. Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Current Protocols in Bioinformatics.* Wiley Online Library.

CIACCI, C., BETTI, M., CANONICO, B., CITTERIO, B., ROCH, P. & CANESI, L. 2010. Specificity of anti-*Vibrio* immune response through p38 MAPK and PKC activation in the hemocytes of the mussel *Mytilus galloprovincialis*. *Journal of Invertebrate Pathology,* 105**,** 49-55.

CIACCI, C., CITTERIO, B., BETTI, M., CANONICO, B., ROCH, P. & CANESI, L. 2009. Functional differential immune responses of *Mytilus galloprovincialis* to bacterial challenge. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology,* 153**,** 365-371.

CIACCI, C., MANTI, A., CANONICO, B., CAMPANA, R., CAMISASSI, G., BAFFONE, W. & CANESI, L. 2017. Responses of *Mytilus galloprovincialis* hemocytes to environmental strains of *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio vulnificus*. *Fish and Shellfish Immunology,* 65**,** 80-87.

COAGLIO, A. L., FERREIRA, M. A. N. D., DOS SANTOS LIMA, W. & DE JESUS PEREIRA, C. A. 2018. Identification of a phenoloxidase-and melanin-dependent defence mechanism in *Achatina fulica* infected with *Angiostrongylus vasorum*. *Parasites & vectors,* 11**,** 1-8.

COATES, C. J. & SÖDERHÄLL, K. 2021. The stress–immunity axis in shellfish. *Journal of invertebrate pathology,* 186**,** 107492.

COEN, L. D. & BISHOP, M. J. 2015. The ecology, evolution, impacts and management of host–parasite interactions of marine molluscs. *Journal of Invertebrate Pathology,* 131**,** 177-211.

COLDITZ, I. G. & HINE, B. C. 2016. Resilience in farm animals: biology, management, breeding and implications for animal welfare. *Animal Production Science,* 56**,** 1961-1983.

COOPERMAN, L. H. 1970. Plasma free fatty acid levels during general anaesthesia and operation in man. *British journal of anaesthesia,* 42**,** 131-135.

COSTA, M. M., PRADO-ALVAREZ, M., GESTAL, C., LI, H., ROCH, P., NOVOA, B. & FIGUERAS, A. 2009. Functional and molecular immune response of Mediterranean mussel (*Mytilus galloprovincialis*) haemocytes against pathogen-associated molecular patterns and bacteria. *Fish and Shellfish Immunology,* 26**,** 515-523.

COSTA, P. M., CARREIRA, S., COSTA, M. H. & CAEIRO, S. 2013. Development of histopathological indices in a commercial marine bivalve (*Ruditapes decussatus*) to determine environmental quality. *Aquatic Toxicology,* 126**,** 442-454.

COSTERTON, J. W., IRVIN, R. T. & CHENG, K. J. 1981. The bacterial glycocalyx in nature and disease. *Annual review of microbiology,* 35**,** 299-324.

COUPE, A., HOWE, L., BURROWS, E., SINE, A., PITA, A., VELATHANTHIRI, N., VALLÉE, E., HAYMAN, D., SHAPIRO, K. & ROE, W. D. 2018. First report of *Toxoplasma gondii* sporulated oocysts and *Giardia duodenalis* in commercial green-lipped mussels (*Perna canaliculus*) in New Zealand. *Parasitology Research,* 117**,** 1453-1463.

COWAN, M., PEARCE, C., FINSTON, T., MEYER, G., MARSHALL, R., EVANS, W., SUTHERLAND, T. & DE LA BASTIDE, P. 2023. Role of the *Vibrio* community, reproductive effort, and environmental parameters in intertidal Pacific oyster summer mortality in British Columbia, Canada. *Aquaculture,* 565**,** 739094.

CROCI, L., SERRATORE, P., COZZI, L., STACCHINI, A., MILANDRI, S., SUFFREDINI, E. & TOTI, L. 2001. Detection of *Vibrionaceae* in mussels and in their seawater growing area. *Letters in Applied Microbiology,* 32**,** 57-61.

CRUZ, C., CHYCKA, M., HEDDERLEY, D. & FLETCHER, G. 2016. Prevalence, characteristics and ecology of *Vibrio vulnificus* found in New Zealand shellfish. *Journal of applied microbiology,* 120**,** 1100-1107.

CULOT, A., GROSSET, N., BRUEY, Q., AUZOU, M., GIARD, J.-C., FAVARD, B., WAKATSUKI, A., BARON, S., FROUEL, S., TECHER, C. & GAUTIER, M. 2021. Isolation of *Harveyi* clade *Vibrio* spp. collected in aquaculture farms: How can the identification issue be addressed? *Journal of Microbiological Methods,* 180**,** 106106.

CZUMAJ, A., SZROK-JURGA, S., HEBANOWSKA, A., TURYN, J., SWIERCZYNSKI, J., SLEDZINSKI, T. & STELMANSKA, E. 2020. The pathophysiological role of CoA. *International Journal of Molecular Sciences,* 21**,** 9057.

DAI, Q., WANG, Z.-X., SHENG, Y.-Q., WU, Z.-W., QIU, Y., SU, P., KE, C.-H. & FENG, D.-Q. 2021. 2-Arachidonoylglycerol as an endogenous cue negatively regulates attachment of the mussel *Perna viridis*. *Frontiers in Marine Science,* 8**,** 719781.

DAI, Y.-J., HUI, K.-M., ZHANG, Y.-H., LIU, Y., WANG, Y.-Q., ZHAO, L.-J., LIN, L., CHAI, L.-Q., WEI, S. & LAN, J.-F. 2017. Three STATs are involved in the regulation of the expression of antimicrobial peptides in the triangle sail mussel, *Hyriopsis cumingii*. *Fish & Shellfish Immunology,* 63**,** 181-188.

DALZOCHIO, T., RODRIGUES, G. Z. P., PETRY, I. E., GEHLEN, G. & DA SILVA, L. B. 2016. The use of biomarkers to assess the health of aquatic ecosystems in Brazil: a review. *International Aquatic Research,* 8**,** 283–298.

DAS, A., RUSHTON, P. J. & ROHILA, J. S. 2017. Metabolomic profiling of soybeans (*Glycine max* L.) reveals the importance of sugar and nitrogen metabolism under drought and heat stress. *Plants,* 6**,** 21.

DAS, J., KUMAR, R., YADAV, S. K. & JHA, G. 2023. Nicotinic acid catabolism modulates bacterial mycophagy in *Burkholderia gladioli* strain NGJ1. *Microbiology Spectrum***,** e04457-22.

DASKALAKI, E., PILLON, N. J., KROOK, A., WHEELOCK, C. E. & CHECA, A. 2018. The influence of culture media upon observed cell secretome metabolite profiles: The balance between cell viability and data interpretability. *Analytica chimica acta,* 1037**,** 338-350.

DAVENPORT, M. 2012. Freshwater vs. Marine mussels. *Conserve Wildlife Blog* [Online]. Available from: http://www.conservewildlifenj.org/blog/2012/08/27/freshwater-vs-marine-mussels/ [Accessed 5 October 2020 2019].

DE BURGH-DAY, C. O., SPILLMAN, C. M., SMITH, G. & STEVENS, C. L. 2022. Forecasting extreme marine heat events in key aquaculture regions around New Zealand. *Journal of Southern Hemisphere Earth Systems Science,* 72**,** 58-72.

DE CARVALHO, C. C. & CARAMUJO, M. J. 2018. The various roles of fatty acids. *Molecules,* 23**,** 2583.

DE JONG, L., MOREAU, X. & THIÉRY, A. 2008. Expression of heat shock proteins as biomarker tool in aquatic invertebrates: Actual knowledge and ongoing developments for the early detection of environmental changes and ecological risks. *In:* EMMA, M. & CAMILLE, V. (eds.) *Heat-Shock Proteins: New Research.* New York: Nova Publishers.

DE LORGERIL, J., LUCASSON, A., PETTON, B., TOULZA, E., MONTAGNANI, C., CLERISSI, C., VIDAL-DUPIOL, J., CHAPARRO, C., GALINIER, R., ESCOUBAS, J. M., HAFFNER, P., DÉGREMONT, L., CHARRIÈRE, G. M., LAFONT, M., DELORT, A., VERGNES, A., CHIARELLO, M., FAURY, N., RUBIO, T., LEROY, M. A., PÉRIGNON, A., RÉGLER, D., MORGA, B., ALUNNO-BRUSCIA, M., BOUDRY, P., LE ROUX, F., DESTOUMIEUX-GARZÓN, D., GUEGUEN, Y. & MITTA, G. 2018. Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nature Communications,* 9**,** 4215.

DE O SANTOS, E., ALVES, N., DIAS, G. M., MAZOTTO, A. M., VERMELHO, A., VORA, G. J., WILSON, B., BELTRAN, V. H., BOURNE, D. G., LE ROUX, F. & THOMPSON, F. L. 2011. Genomic and proteomic analyses of the coral pathogen *Vibrio coralliilyticus* reveal a diverse virulence repertoire. *The ISME Journal,* 5**,** 1471-1483.

DE SOUZA VALENTE, C. & WAN, A. H. L. 2021. *Vibrio* and major commercially important vibriosis diseases in decapod crustaceans. *Journal of Invertebrate Pathology,* 181.

DE ZWAAN, A., DE BONT, A. T. & VERHOEVEN, A. 1982. Anaerobic energy metabolism in isolated adductor muscle of the sea mussel *Mytilus edulis* L. *Journal of comparative physiology,* 149**,** 137-143.

DECANDIA, A. L., DOBSON, A. P. & VONHOLDT, B. M. 2018. Toward an integrative molecular approach to wildlife disease. *Conservation Biology,* 32**,** 798-807.

DEFOIRDT, T. 2014. Virulence mechanisms of bacterial aquaculture pathogens and antivirulence therapy for aquaculture. *Reviews in Aquaculture,* 6**,** 100-114.

DELISLE, L., PETTON, B., BURGUIN, J. F., MORGA, B., CORPOREAU, C. & PERNET, F. 2018. Temperature modulate disease susceptibility of the Pacific oyster *Crassostrea gigas* and virulence of the Ostreid herpesvirus type 1. *Fish & Shellfish Immunology,* 80**,** 71-79.

DELORME, N., BIESSY, L., SOUTH, P., ZAMORA, L., RAGG, N. & BURRITT, D. 2020a. Stress-on-stress responses of a marine mussel, *Perna canaliculus*: food limitation reduces the ability to cope with heat stress in juveniles. *Marine Ecology Progress Series,* 644**,** 105-117.

DELORME, N. J., BURRITT, D. J., RAGG, N. L. & SOUTH, P. M. 2021a. Emersion and relative humidity modulate stress response and recovery dynamics in juvenile mussels (*Perna canaliculus*). *Metabolites,* 11**,** 580.

DELORME, N. J., FROST, E. J. & SEWELL, M. A. 2020b. Effect of acclimation on thermal limits and *hsp70* gene expression of the New Zealand sea urchin *Evechinus chloroticus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 250**,** 110806.

DELORME, N. J., VENTER, L., ROLTON, A. & ERICSON, J. A. 2021b. Integrating animal health and stress assessment tools using the green-lipped mussel *Perna canaliculus* as a case study. *Journal of Shellfish Research,* 40**,** 93-112.

DEMANN, F. & WEGNER, K. M. 2019. Infection by invasive parasites increases susceptibility of native hosts to secondary infection via modulation of cellular immunity. *Journal of Animal Ecology,* 88**,** 427-438.

DENG, Y., XU, L., CHEN, H., LIU, S., GUO, Z., CHENG, C., MA, H. & FENG, J. 2020. Prevalence, virulence genes, and antimicrobial resistance of *Vibrio* species isolated from diseased marine fish in South China. *Scientific reports,* 10**,** 14329.

DERETIC, V., SAITOH, T. & AKIRA, S. 2013. Autophagy in infection, inflammation and immunity. *Nature Reviews Immunology,* 13**,** 722-737.

DESAI, T. A. & RAO, C. V. 2010. Regulation of arabinose and xylose metabolism in *Escherichia coli*. *Applied and environmental microbiology,* 76**,** 1524-1532.

DESTOUMIEUX-GARZÓN, D., CANESI, L., OYANEDEL, D., TRAVERS, M. A., CHARRIÈRE, G. M., PRUZZO, C. & VEZZULLI, L. 2020. *Vibrio*–bivalve interactions in health and disease. *Environmental Microbiology,* 22**,** 4323–4341.

DÉTRÉE, C., LALLIER, F. H., TANGUY, A. & MARY, J. 2017. Identification and gene expression of multiple peptidoglycan recognition proteins (PGRPs) in the deep-sea mussel *Bathymodiolus azoricus*, involvement in symbiosis? *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 207**,** 1-8.

DETREE, C., NÚÑEZ-ACUÑA, G., ROBERTS, S. & GALLARDO-ESCARATE, C. 2016. Uncovering the complex transcriptome response of *Mytilus chilensis* against saxitoxin: implications of harmful algal blooms on mussel populations. *PloS one,* 11**,** e0165231.

DIMITRIADIS, V., DOMOUHTSIDOU, G. & CAJARAVILLE, M. 2004. Cytochemical and histochemical aspects of the digestive gland cells of the mussel *Mytilus galloprovincialis* (L.) in relation to function. *Journal of Molecular Histology,* 35**,** 501-509.

DIMITRIADIS, V. & KOUKOUZIKA, N. 2003. Effect of sampling procedures, transportation stress and laboratory maintenance on the structure and function of the digestive gland epithelium of the mussel *Mytilus galloprovincialis*. *Marine biology,* 142**,** 915-924.

DIRITA, V. J., PARSOT, C., JANDER, G. & MEKALANOS, J. J. Regulatory cascade controls virulence in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences*, 1991. 5403-5407.

DITTRICH, C. R., BENNETT, G. N. & SAN, K. Y. 2009. Metabolic engineering of the anaerobic central metabolic pathway in *Escherichia coli* for the simultaneous anaerobic production of isoamyl acetate and succinic acid. *Biotechnology progress,* 25**,** 1304-1309.

DOLAR, A., MAYALL, C., DROBNE, D. & KOKALJ, A. J. 2020. Modulations of immune parameters caused by bacterial and viral infections in the terrestrial crustacean *Porcellio scaber*: Implications for potential markers in environmental research. *Developmental & Comparative Immunology,* 113**,** 103789.

DOMENEGHETTI, S., VAROTTO, L., CIVETTINI, M., ROSANI, U., STAUDER, M., PRETTO, T., PEZZATI, E., ARCANGELI, G., TUROLLA, E., PALLAVICINI, A. & VENIER, P. 2014. Mortality occurrence and pathogen detection in *Crassostrea gigas* and *Mytilus galloprovincialis* close-growing in shallow waters (Goro lagoon, Italy). *Fish and Shellfish Immunology,* 41**,** 37-44.

DONAGHY, L. & VOLETY, A. K. 2011. Functional and metabolic characterization of hemocytes of the green mussel, *Perna viridis*: in vitro impacts of temperature. *Fish & shellfish immunology,* 31**,** 808-814.

DONELLI, G., VUOTTO, C. & MASTROMARINO, P. 2013. Phenotyping and genotyping are both essential to identify and classify a probiotic microorganism. *Microbial Ecology in Health & Disease,* 24**,** 20105.

DONG, H. T., NGUYEN, V. V., PHIWSAIYA, K., GANGNONNGIW, W., WITHYACHUMNARNKUL, B., RODKHUM, C. & SENAPIN, S. 2015. Concurrent infections of *Flavobacterium columnare* and *Edwardsiella ictaluri* in striped catfish, *Pangasianodon hypophthalmus* in Thailand. *Aquaculture,* 448**,** 142-150.

DONG, W., CHEN, Y., LU, W., WU, B. & QI, P. 2017. Transcriptome analysis of *Mytilus coruscus* hemocytes in response to *Vibrio alginnolyficus* infection. *Fish & Shellfish Immunology,* 70**,** 560-567.

DONG, X., YANG, Z., LIU, Z., WANG, X., YU, H., PENG, C., HOU, X., LU, W., XING, Q. & HU, J. 2022. Metabonomic analysis provides new insights into the response of Zhikong scallop (*Chlamys farreri*) to heat stress by improving energy metabolism and antioxidant capacity. *Antioxidants,* 11**,** 1084.

DONLAN, R. M. & COSTERTON, J. W. 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews,* 15**,** 167-193.

DUBERT, J., BARJA, J. L. & ROMALDE, J. L. 2017. New insights into pathogenic Vibrios affecting bivalves in hatcheries: present and future prospects. *Frontiers in Microbiology,* 8**,** 762.

DUMAS, T., BONNEFILLE, B., GOMEZ, E., BOCCARD, J., CASTRO, N. A., FENET, H. & COURANT, F. 2020. Metabolomics approach reveals disruption of metabolic pathways in the marine bivalve *Mytilus galloprovincialis* exposed to a WWTP effluent extract. *Science of the Total Environment,* 712**,** 136551.

DUNEAU, D., FERDY, J.-B., REVAH, J., KONDOLF, H., ORTIZ, G. A., LAZZARO, B. P. & BUCHON, N. 2017. Stochastic variation in the initial phase of bacterial infection predicts the probability of survival in *D. melanogaste*r. *Elife,* 6**,** e28298.

DUNPHY, B., RUGGIERO, K., ZAMORA, L. & RAGG, N. 2018. Metabolomic analysis of heat-hardening in adult green-lipped mussel (*Perna canaliculus*): A key role for succinic acid and the GABAergic synapse pathway. *Journal of thermal biology,* 74**,** 37-46.

DUNPHY, B. J., RAGG, N. L. & COLLINGS, M. G. 2013. Latitudinal comparison of thermotolerance and HSP70 production in F2 larvae of the greenshell mussel (*Perna canaliculus*). *Journal of Experimental Biology,* 216**,** 1202-1209.

DUNPHY, B. J., WATTS, E. & RAGG, N. L. C. 2015. Identifying thermally-stressed adult green-lipped mussels (*Perna canaliculus* Gmelin, 1791) via metabolomic profiling. *American Malacological Bulletin,* 33**,** 127-135.

DUPERTHUY, M., BINESSE, J., LE ROUX, F., ROMESTAND, B., CARO, A., GOT, P., GIVAUDAN, A., MAZEL, D., BACHÈRE, E. & DESTOUMIEUX-GARZÓN, D. 2010. The major outer membrane protein OmpU of *Vibrio splendidus* contributes to host antimicrobial peptide resistance and is required for virulence in the oyster *Crassostrea gigas*. *Environmental Microbiology,* 12**,** 951-963.

DWORKIN, M., FALKOW, S., ROSENBERG, E., SCHLEIFER, K.-H. & STACKEBRANDT, E. 2006. *The prokaryotes: a handbook on the biology of bacteria,* New York, Springer-Verlag.

DZIARSKI, R. & GUPTA, D. 2006. The peptidoglycan recognition proteins (PGRPs). *Genome biology,* 7**,** 232.

EGGERMONT, M. 2017. *Toolbox development to study host-pathogen interactions in the blue mussel Mytilus edulis.* Doctor of Philosophy PhD thesis, University of Ghent.

EGGERMONT, M., BOSSIER, P., PANDE, G. S. J., DELAHAUT, V., RAYHAN, A. M., GUPTA, N., ISLAM, S. S., YUMO, E., NEVEJAN, N., SORGELOOS, P., GOMEZ-GIL, B. & DEFOIRDT, T. 2017. Isolation of *Vibrionaceae* from wild blue mussel (*Mytilus edulis*) adults and their impact on blue mussel larviculture. *FEMS Microbiology Ecology,* 93**,** 1-11.

EGGERMONT, M., CORNILLIE, P., DIERICK, M., ADRIAENS, D., NEVEJAN, N., BOSSIER, P., VAN DEN BROECK, W., SORGELOOS, P., DEFOIRDT, T. & DECLERCQ, A. M. 2020. The blue mussel inside: 3D visualization and description of the vascular-related anatomy of *Mytilus edulis* to unravel hemolymph extraction. *Scientific reports,* 10**,** 6773.

EGGERMONT, M., TAMANJI, A., NEVEJAN, N., BOSSIER, P., SORGELOOS, P. & DEFOIRDT, T. 2014. Stimulation of heterotrophic bacteria associated with wild-caught blue mussel (*Mytilus edulis*) adults results in mass mortality. *Aquaculture,* 431**,** 136-138.

EISENREICH, W., HEESEMANN, J., RUDEL, T. & GOEBEL, W. 2013. Metabolic host responses to infection by intracellular bacterial pathogens. *Frontiers in cellular and infection microbiology,* 3**,** 24.

EISSA, N. & WANG, H. P. 2016. Transcriptional stress responses to environmental and husbandry stressors in aquaculture species. *Reviews in Aquaculture,* 8**,** 61-88.

ELLINGTON, W. R. 1989. Phosphocreatine represents a thermodynamic and functional improvement over other muscle phosphagens. *Journal of Experimental Biology,* 143**,** 177-194.

ELLIS, R. P., PARRY, H., SPICER, J. I., HUTCHINSON, T. H., PIPE, R. K. & WIDDICOMBE, S. 2011. Immunological function in marine invertebrates: responses to environmental perturbation. *Fish & Shellfish Immunology,* 30**,** 1209-22.

ELLIS, R. P., SPICER, J. I., BYRNE, J. J., SOMMER, U., VIANT, M. R., WHITE, D. A. & WIDDICOMBE, S. 2014. ^{1}H NMR metabolomics reveals contrasting response by male and female mussels exposed to reduced seawater pH, increased temperature, and a pathogen. *Environmental Science and Technology,* 48**,** 7044-7052.

ENCOMIO, V. G. & CHU, F. L. E. 2005. Seasonal variation of heat shock protein 70 in eastern oysters (*Crassostrea virginica*) infected with *Perkinsus marinus*(Dermo). *Journal of Shellfish Research,* 24.

ERICSON, J. A., DELORME, N. & RAGG, N. L. C. 2023a. Heat tolerance of Greenshell mussels™ (*Perna canaliculus*): collated research findings & implications for mussel farming. Cawthron Report No. 3914. 19 p. plus appendices.

ERICSON, J. A., DELORME, N. J. & RAGG, N. L. C. 2023b. Heat tolerance of Greenshell™ mussels (*Perna canaliculus*) : collated research findings and implications for mussel farming. Cawthron Institute.

ERICSON, J. A., RAGG, N. L. C. & ROLTON, A. 2021. Flow cytometric validation of a commercial kit to assess the concentration and viability of bivalve hemocytes. *Fish & Shellfish Immunology,* 119**,** 452-455.

ERICSON, J. A., VENTER, L., COPEDO, J. S., NGUYEN, V. T., ALFARO, A. C. & RAGG, N. L. C. 2023c. Chronic heat stress as a predisposing factor in summer mortality of mussels, *Perna canaliculus*. *Aquaculture,* 564**,** 738986.

ERICSON, J. A., VENTER, L., WELFORD, M. R. V., KUMANAN, K., ALFARO, A. C. & RAGG, N. L. C. 2022. Effects of seawater temperature and acute *Vibrio* sp. challenge on the haemolymph immune and metabolic responses of adult mussels (*Perna canaliculus*). *Fish and Shellfish Immunology,* 128**,** 664-675.

EYMANN, C., GÖTZE, S., BOCK, C., GUDERLEY, H., KNOLL, A. H., LANNIG, G., SOKOLOVA, I. M., ABERHAN, M. & PÖRTNER, H.-O. 2020. Thermal performance of the European flat oyster, *Ostrea edulis* (Linnaeus, 1758)—explaining ecological findings under climate change. *Marine biology,* 167**,** 1-15.

FABBRI, E., VALBONESI, P. & FRANZELLITTI, S. 2008. HSP expression in bivalves. *Invertebrate Survival Journal,* 5**,** 135-161.

FALFUSHYNSKA, H., WU, F., SOKOLOV, E. P. & SOKOLOVA, I. M. 2023. Salinity variation modulates cellular stress response to ZnO nanoparticles in a sentinel marine bivalve, the blue mussel *Mytilus sp*. *Marine Environmental Research,* 183**,** 105834.

FALFUSHYNSKA, H. I., GNATYSHYNA, L. L., OSADCHUK, O. Y., FARKAS, A., VEHOVSZKY, A., CARPENTER, D. O., GYORI, J. & STOLIAR, O. B. 2014. Diversity of the molecular responses to separate wastewater effluents in freshwater mussels. *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology,* 164**,** 51-58.

FALFUSHYNSKA, H. I., WU, F., YE, F., KASIANCHUK, N., DUTTA, J., DOBRETSOV, S. & SOKOLOVA, I. M. 2019. The effects of ZnO nanostructures of different morphology on bioenergetics and stress response biomarkers of the blue mussels *Mytilus edulis*. *Science of the Total Environment,* 694**,** 133717.

FAO 2016. The state of world fisheries and aquaculture 2016: Contributing to food security and nutrition for all. Rome.

FARMER, E. E. & MUELLER, M. J. 2013. ROS-mediated lipid peroxidation and RES-activated signaling. *Annual review of plant biology,* 64**,** 429-450.

FICHI, G., CARDETI, G., PERRUCCI, S., VANNI, A., CERSINI, A., LENZI, C., DE WOLF, T., FRONTE, B., GUARDUCCI, M. & SUSINI, F. 2015. Skin lesion-associated pathogens from *Octopus vulgaris*: first detection of *Photobacterium swingsii*, Lactococcus garvieae and betanodavirus. *Diseases of Aquatic Organisms,* 115**,** 147-156.

FIGUERAS, A., MOREIRA, R., SENDRA, M. & NOVOA, B. 2019. Genomics and immunity of the Mediterranean mussel *Mytilus galloprovincialis* in a changing environment. *Fish Shellfish Immunol,* 90**,** 440-445.

FIGUEROA, C., BUSTOS, P., TORREALBA, D., DIXON, B., SOTO, C., CONEJEROS, P. & GALLARDO, J. A. 2017. Coinfection takes its toll: Sea lice override the protective effects of vaccination against a bacterial pathogen in Atlantic salmon. *Scientific Report,* 7**,** 17817.

FISHERIES NEW ZEALAND 2021. The New Zealand Government Aquaculture Strategy. Wellington.

FISHERIES`NEW`ZEALAND 2022. The New Zealand Goverment Aquaculture Strategy: 2022 implementation plan. *Minstry for Primary Industries,* Wellington: ISBN: 978-1-99-102653-8 (online).

FLAWS, D. E. 1975. *Aspects of the biology of mussels in the Cook Strait area.*, Victoria University of Wellington.

FORD, S. E. 1986. Effect of repeated hemolymph sampling on growth, mortality, hemolymph protein and parasitism of oysters, Crassostrea virginica. *Comparative Biochemistry and Physiology Part A: Physiology,* 85**,** 465-470.

FORD, S. E., KANALEY, S. A. & LITTLEWOOD, D. T. J. 1993. Cellular responses of oysters infected with *Haplosporidium nelsoni*: Changes in circulating and tissue-infiltrating hemocytes. *Journal of Invertebrate Pathology,* 61**,** 49-57.

FORD, S. E. & PAILLARD, C. 2007. Repeated sampling of individual bivalve mollusks I: Intraindividual variability and consequences for haemolymph constituents of the Manila clam, *Ruditapes philippinarum*. *Fish and Shellfish Immunology,* 23**,** 280-291.

FOX, M., CHRISTLEY, R., LUPO, C., MOORE, H., SERVICE, M. & CAMPBELL, K. 2020. Preventing and mitigating farmed bivalve disease: a Northern Ireland case study. *Aquaculture International,* 28**,** 2397-2417.

FRAGA, C. G., OTEIZA, P. I. & GALLEANO, M. 2014. In vitro measurements and interpretation of total antioxidant capacity. *Biochimica et Biophysica Acta (BBA)-General Subjects,* 1840**,** 931-934.

FRANCO, L., ROMERO, D., GARCÍA-NAVARRO, J. A., TELES, M. & TVARIJONAVICIUTE, A. 2016. Esterase activity (EA), total oxidant status (TOS) and total antioxidant capacity (TAC) in gills of *Mytilus galloprovincialis* exposed to pollutants: Analytical validation and effects evaluation by single and mixed heavy metal exposure. *Marine Pollution Bulletin,* 102**,** 30-35.

FRANÇOIS, G., MÉLANIE, D., MARLÈNE, F. & MICHEL, F. 2015. Effects of a municipal effluent on the freshwater mussel *Elliptio complanata* following challenge with *Vibrio anguillarum*. *Journal of Environmental Sciences (China),* 37.

FREDERIKSEN, R. F., PASPALIARI, D. K., LARSEN, T., STORGAARD, B. G., LARSEN, M. H., INGMER, H., PALCIC, M. M. & LEISNER, J. J. 2013. Bacterial chitinases and chitin-binding proteins as virulence factors. *Microbiology (United Kingdom),* 159**,** 833-847.

FRIDOVICH, I. 1973. Superoxide dismutase. *Annual review of biochemistry.*

FRIZZO, R., BORTOLETTO, E., RIELLO, T., LEANZA, L., SCHIEVANO, E., VENIER, P. & MAMMI, S. 2021. NMR metabolite profiles of the bivalve mollusc *Mytilus galloprovincialis* before and after immune stimulation with *Vibrio splendidus*. *Frontiers in Molecular Biosciences,* 8.

FROELICH, B. & OLIVER, J. D. 2013. The iteractions of *Vibrio vulnificus* and the oster *Crassostrea virginica*. *Microbial Ecology,* 65**,** 807-816.

GAGNÉ, N., COCHENNEC, N., STEPHENSON, M., MCGLADDERY, S., MEYER, G. R. & BOWER, S. M. 2008. First report of a Mikrocytos-like parasite in European oysters *Ostrea edulis* from Canada after transport and quarantine in France. *Diseases of Aquatic Organisms,* 80**,** 27-35.

GALVIS, F., BARJA, J. L., LEMOS, M. L. & BALADO, M. 2021. The vibriolysin like protease VnpA and the Collagenase ColA are required for full virulence of the bivalve mollusks pathogen *Vibrio neptunius*. *Antibiotics,* 10**,** 391.

GARCÍA-GARCÍA, E., PRADO-ÁLVAREZ, M., NOVOA, B., FIGUERAS, A. & ROSALES, C. 2008. Immune responses of mussel hemocyte subpopulations are differentially regulated by enzymes of the PI 3-K, PKC, and ERK kinase families. *Developmental and Comparative Immunology,* 32**,** 637-653.

GARDUÑO, R. A., GARDUÑO, E. & HOFFMAN, P. S. 1998. Surface-associated Hsp60 chaperonin of *Legionella pneumophila* mediates invasion in a HeLa cell model. *Infection and Immunity,* 66**,** 4602-4610.

GARLOCK, T., ASCHE, F., ANDERSON, J., BJØRNDAL, T., KUMAR, G., LORENZEN, K., ROPICKI, A., SMITH, M. D. & TVETERÅS, R. 2020. A global blue revolution: Aquaculture growth across regions, species, and countries. *Reviews in Fisheries Science & Aquaculture,* 28**,** 107-116.

GARNIER, M., LABREUCHE, Y., GARCIA, C., ROBERT, M. & NICOLAS, J. L. 2007. Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. *Microb Ecol,* 53**,** 187-96.

GARNIER, M., LABREUCHE, Y. & NICOLAS, J. L. 2008. Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp. francensis subsp. nov., a pathogen of the oyster *Crassostrea gigas*. *Syst Appl Microbiol,* 31**,** 358-65.

GAY, M., RENAULT, T., PONS, A. M. & LE ROUX, F. 2004. Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas:* taxonomy and host alterations. *Disease of Aquatic Organism,* 62**,** 65-74.

GE, D., ZHANG, L., LONG, Z., CHI, C. & LIU, H. 2020. A novel biomarker for marine environmental pollution: A metallothionein from *Mytilus coruscus*. *Aquaculture Reports,* 17**,** 100364.

GEFFRÉ, A., FRIEDRICHS, K., HARR, K., CONCORDET, D., TRUMEL, C. & BRAUN, J. P. 2009. Reference values: A review. *Veterinary Clinical Pathology,* 38**,** 288-298.

GENARD, B., PERNET, F., LEMARCHAND, K., BOUDRY, P., MORAGA, D. & TREMBLAY, R. 2011. Physiological and biochemical changes associated with massive mortality events occurring in larvae of American oyster (*Crassostrea virginica*). *Aquatic Living Resources,* 24**,** 247-260.

GEORGIADES, E., FRASER, R. & JONES, B. 2016. Options to strengthen on-farm biosecurity management for commercial and non-commercial aquaculture. *Aquaculture Unit. Technical Paper No: 2016/47*.

GEORGIADES, E., KLUZA, D., BATES, T., LUBARSKY, K., BRUNTON, J., GROWCOTT, A., SMITH, T., MCDONALD, S., GOULD, B. & PARKER, N. 2020. Regulating vessel biofouling to support New Zealand's marine biosecurity system–a blue print for evidence-based decision making. *Frontiers in Marine Science,* 7**,** 390.

GEORGOULIS, I., BOCK, C., LANNIG, G., PÖRTNER, H.-O., FEIDANTSIS, K., GIANTSIS, I. A., SOKOLOVA, I. M. & MICHAELIDIS, B. 2022. Metabolic remodeling caused by heat hardening in the Mediterranean mussel *Mytilus galloprovincialis*. *Journal of Experimental Biology,* 225**,** jeb244795.

GEORGOULIS, I., FEIDANTSIS, K., GIANTSIS, I. A., KAKALE, A., BOCK, C., PÖRTNER, H. O., SOKOLOVA, I. M. & MICHAELIDIS, B. 2021. Heat hardening enhances mitochondrial potential for respiration and oxidative defence capacity in the mantle of thermally stressed *Mytilus galloprovincialis*. *Scientific reports,* 11**,** 17098.

GEPHART, J. A. & PACE, M. L. 2015. Structure and evolution of the global seafood trade network. *Environmental Research Letters,* 10**,** 125014.

GERDOL, M. 2017. Immune-related genes in gastropods and bivalves: a comparative overview. *Invertebrate Survival Journal,* 14**,** 95-111.

GERDOL, M., GOMEZ-CHIARRI, M., CASTILLO, M. G., FIGUERAS, A., FIORITO, G., MOREIRA, R., NOVOA, B., PALLAVICINI, A., PONTE, G., ROUMBEDAKIS, K., VENIER, P. & VASTA, G. R. 2018. Immunity in molluscs: Recognition and effector mechanisms, with a focus on bivalvia. *In:* COOPER, E. L. (ed.) *Advances in Comparative Immunology.* Springer, Cham.

GERDOL, M., MANFRIN, C., DE MORO, G., FIGUERAS, A., NOVOA, B., VENIER, P. & PALLAVICINI, A. 2011. The C1q domain containing proteins of the Mediterranean mussel *Mytilus galloprovincialis*: a widespread and diverse family of immune-related molecules. *Developmental & Comparative Immunology,* 35**,** 635-643.

GERDOL, M. & VENIER, P. 2015. An updated molecular basis for mussel immunity. *Fish & Shellfish Immunology,* 46**,** 17-38.

GERET, F., MANDUZIO, H., COMPANY, R., LEBOULENGER, F., BEBIANNO, M. J. & DANGER, J. M. 2004. Molecular cloning of superoxide dismutase (Cu/Zn-SOD) from aquatic molluscs. *Marine Environmental Research,* 58**,** 619-623.

GESTAL, C., ROCH, P., RENAULT, T., PALLAVICINI, A., PAILLARD, C., NOVOA, B., OUBELLA, R., VENIER, P. & FIGUERAS, A. 2008. Study of diseases and the immune system of bivalves using molecular biology and genomics. *Reviews in Fisheries Science,* 16**,** 133-156.

GHINI, V., UNGER, F. T., TENORI, L., TURANO, P., JUHL, H. & DAVID, K. A. 2015. Metabolomics profiling of pre-and post-anesthesia plasma samples of colorectal patients obtained via Ficoll separation. *Metabolomics,* 11**,** 1769-1778.

GOEL, M. K., KHANNA, P. & KISHORE, J. 2010. Understanding survival analysis: Kaplan-Meier estimate. *International journal of Ayurveda research,* 1**,** 274.

GOLDEN, C. D., KOEHN, J. Z., SHEPON, A., PASSARELLI, S., FREE, C. M., VIANA, D. F., MATTHEY, H., EURICH, J. G., GEPHART, J. A. & FLUET-CHOUINARD, E. 2021. Aquatic foods to nourish nations. *Nature,* 598**,** 315-320.

GOMEZ-GIL, B., ROQUE, A., ROTLLANT, G., PEINADO, L., ROMALDE, J. L., DOCE, A., CABANILLAS-BELTRÁN, H., CHIMETTO, L. A. & THOMPSON, F. L. 2011. *Photobacterium swingsii* sp. nov., isolated from marine organisms. *International Journal of Systematic and Evolutionary Microbiology,* 61**,** 315-319.

GOPHNA, U. & RON, E. Z. 2003. Virulence and the heat shock response. *Int J Med Microbiol,* 292**,** 453-61.

GREEN, D. S., COLGAN, T. J., THOMPSON, R. C. & CAROLAN, J. C. 2019. Exposure to microplastics reduces attachment strength and alters the haemolymph proteome of blue mussels (*Mytilus edulis*). *Environmental pollution,* 246**,** 423-434.

GREEN, T. J., VERGNES, A., MONTAGNANI, C. & DE LORGERIL, J. 2016. Distinct immune responses of juvenile and adult oysters (*Crassostrea gigas*) to viral and bacterial infections. *Veterinary research,* 47**,** 1-11.

GRIESHABER, M., HARDEWIG, I., KREUTZER, U. & PÖRTNER, H.-O. 1994. Physiological and metabolic responses to hypoxia in invertebrates. *Reviews of Physiology, Biochemistry and Pharmacology, Volume 125: Volume: 125***,** 43-147.

GRINCHENKO, A. & KUMEIKO, V. 2022. Bivalves humoral immunity: key molecules and their functions. *Russian Journal of Marine Biology,* 48**,** 399-417.

GUDKOVS, N., CUMMINS, D., JONES, B., COLLING, A., SINGANALLUR, N. & CRANE, M. 2016. Aquatic Animal Health Subprogram: Development of improved molecular diagnostic tests for *Perkinsus olseni* in Australian molluscs.

GUI, Y., ZAMORA, L. N., DUNPHY, B. & JEFFS, A. G. 2016. Understanding the ontogenetic changes in particle processing of the greenshell™ mussel, *Perna canaliculus*, in order to improve hatchery feeding practices. *Aquaculture,* 452**,** 120-127.

GULCIN, İ. 2020. Antioxidants and antioxidant methods: An updated overview. *Archives of toxicology,* 94**,** 651-715.

GUO, G., MCKENZIE, E., JONES, M., ZARATE, E., SEYMOUR, D., BAKER, P., VILLAS-BÔAS, S. & HAN, T. 2021. MassOmics: An R package of a cross-platform data processing pipeline for large-scale GC-MS untargeted metabolomics datasets. *Zenodo*.

GUREVICH, A., SAVELIEV, V., VYAHHI, N. & TESLER, G. 2013. QUAST: Quality assessment tool for genome assemblies. *Bioinformatics,* 29**,** 1072-1075.

GUTHRIE, A. G., BILKOVIC, D. M., MITCHELL, M., CHAMBERS, R., THOMPSON, J. S. & ISDELL, R. E. 2022. Ecological equivalency of living shorelines and natural marshes for fish and crustacean communities. *Ecological Engineering,* 176**,** 106511.

GUTIERREZ, A. M., BASSOLS, A., SOLER, L. & PINEIRO, M. 2020. Editorial: Factors influencing biomarker range intervals in farm animals. *Front Vet Sci,* 7**,** 587741.

HAIDER, F., FALFUSHYNSKA, H. I., TIMM, S. & SOKOLOVA, I. M. 2020. Effects of hypoxia and reoxygenation on intermediary metabolite homeostasis of marine bivalves *Mytilus edulis* and *Crassostrea gigas*. *Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology,* 242**,** 110657.

HALL, B. G., ACAR, H., NANDIPATI, A. & BARLOW, M. 2014. Growth rates made easy. *Molecular Biology and Evolution,* 31**,** 232-238.

HAN, Y., ZHOU, W., TANG, Y., SHI, W., SHAO, Y., REN, P., ZHANG, J., XIAO, G., SUN, H. & LIU, G. 2021. Microplastics aggravate the bioaccumulation of three veterinary antibiotics in the thick shell mussel *Mytilus coruscus* and induce synergistic immunotoxic effects. *Science of The Total Environment,* 770**,** 145273.

HARCET, M., PERINA, D. & PLEŠE, B. 2013. Opine dehydrogenases in marine invertebrates. *Biochemical Genetics,* 51**,** 666-676.

HARRISON, J., NELSON, K., MORCRETTE, H., MORCRETTE, C., PRESTON, J., HELMER, L., TITBALL, R. W., BUTLER, C. S. & WAGLEY, S. 2022. The increased prevalence of *Vibrio* species and the first reporting of *Vibrio jasicida* and *Vibrio rotiferianus* at UK shellfish sites. *Water Research,* 211**,** 117942.

HARVELL, C. D., MITCHELL, C. E., WARD, J. R., ALTIZER, S., DOBSON, A. P., OSTFELD, R. S. & SAMUEL, M. D. 2002. Climate warming and disease risks for terrestrial and marine biota. *Science,* 296**,** 2158-2162.

HASKÓ, G., SITKOVSKY, M. V. & SZABO, C. 2004. Immunomodulatory and neuroprotective effects of inosine. *Trends in pharmacological sciences,* 25**,** 152-157.

HAVEL, R. J. & GOLDFIEN, A. 1959. The role of the sympathetic nervous system in the metabolism of free fatty acids. *Journal of Lipid Research,* 1**,** 102-108.

HE, Z., HE, J., WANG, J., ZHANG, X., FAN, M., BUTTINO, I., QI, P., YAN, X. & LIAO, Z. 2022. Comparative transcriptomic analysis of gill and gonad from *Mytilus* under antibiotics treatment followed by different bacteria challenge. *Aquaculture,* 547**,** 737457.

HEASMAN, K. G., SCOTT, N., ERICSON, J. A., TAYLOR, D. I. & BUCK, B. H. 2020. Extending New Zealand's marine shellfish aquaculture into exposed environments – adapting to modern anthropogenic challenges. *Frontiers in Marine Science,* 7**,** 1-8.

HÉGARET, H., WIKFORS, G. H. & SOUDANT, P. 2003. Flow cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation: II. Haemocyte functions: aggregation, viability, phagocytosis, and respiratory burst. *Journal of experimental marine biology and ecology,* 293**,** 249-265.

HENDERSON, B., ALLAN, E. & COATES, A. R. 2006. Stress wars: the direct role of host and bacterial molecular chaperones in bacterial infection. *Infection and immunity,* 74**,** 3693-3706.

HENNEQUIN, C., PORCHERAY, F., WALIGORA-DUPRIET, A. J., COLLIGNON, A., BARC, M. C., BOURLIOUX, P. & KARJALAINEN, T. 2001. GroEL (Hsp60) of *Clostridium difficile* is involved in cell adherence. *Microbiology,* 147**,** 87-96.

HERNROTH, B., BADEN, S., TASSIDIS, H., HÖRNAEUS, K., GUILLEMANT, J., BERGSTRÖM LIND, S. & BERGQUIST, J. 2016. Impact of ocean acidification on antimicrobial activity in gills of the blue mussel (*Mytilus edulis*). *Fish and Shellfish Immunology,* 55**,** 452-459.

HEY, S. P., D'ANDREA, E., JUNG, E. H., TESSEMA, F., LUO, J., GYAWALI, B. & KESSELHEIM, A. S. 2019. Challenges and opportunities for biomarker validation. *Journal of Law, Medicine and Ethics,* 47**,** 357-361.

HICKMAN, R. W. & ILLINGWORTH, J. 1980. Condition cycle of the green-lipped mussel *Perna canaliculus* in New Zealand. *Marine biology.* Springer.

HO, E., KARIMI GALOUGAHI, K., LIU, C. C., BHINDI, R. & FIGTREE, G. A. 2013. Biological markers of oxidative stress: Applications to cardiovascular research and practice. *Redox Biology,* 1**,** 483-491.

HOLLAND, R. L. 2016. What makes a good biomarker? *Advances in Precision Medicine,* 1**,** 66-77.

HOOK, S. E., GALLAGHER, E. P. & BATLEY, G. E. 2014. The role of biomarkers in the assessment of aquatic ecosystem health. *Integrated Environmental Assessment and Management,* 10**,** 327-341.

HOPPE, H.-G., GOCKE, K., KOPPE, R. & BEGLER, C. 2002. Bacterial growth and primary production along a north–south transect of the Atlantic Ocean. *Nature,* 416**,** 168-171.

HOSSAIN, S., WICKRAMANAYAKE, M. V. K. S., DAHANAYAKE, P. S. & HEO, G.-J. 2020. Occurrence of virulence and extended-spectrum β-lactamase determinants in *Vibrio* spp. isolated from marketed hard-shelled mussel (*Mytilus coruscus*). *Microbial Drug Resistance,* 26**,** 391-401.

HOWELLS, J., JARAMILLO, D., BROSNAHAN, C. L., PANDE, A. & LANE, H. S. 2021. Intracellular bacteria in New Zealand shellfish are identified as *Endozoicomonas* species. *Diseases of aquatic organisms,* 143**,** 27-37.

HU, Z., XU, L., SONG, H., FENG, J., ZHOU, C., YANG, M.-J., SHI, P., LI, Y.-R., GUO, Y.-J. & LI, H.-Z. 2023. Effect of heat and hypoxia stress on mitochondrion and energy metabolism in the gill of hard clam. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 266**,** 109556.

HUANG, Y., WANG, W. & REN, Q. 2016. Identification and function of a novel C1q domain-containing (C1qDC) protein in triangle-shell pearl mussel (*Hyriopsis cumingii*). *Fish & Shellfish Immunology,* 58**,** 612-621.

HUI, T. Y., DONG, Y.-W., HAN, G.-D., LAU, S. L., CHENG, M. C., MEEPOKA, C., GANMANEE, M. & WILLIAMS, G. A. 2020. Timing metabolic depression: predicting thermal stress in extreme intertidal environments. *The American Naturalist,* 196**,** 501-511.

IBISWORLD. 2022. *Fishing and Aquaculture in New Zealand - Market Size 2008–2029* [Online]. Available: https://www.ibisworld.com/nz/market-size/fishing-aquaculture/ [Accessed 15 October 2021].

IFFLAND-STETTNER, A., OKANO, H., GRALKA, M., GUESSOUS, G., AMARNATH, K., CORDERO, O. X., HWA, T. & BONHOEFFER, S. 2022. A genome-scale metabolic model of marine heterotroph *Vibrio splendidus* sp. 1A01. *bioRxiv***,** 2022.04. 15.488298.

IKUTA, T., TAME, A., SAITO, M., AOKI, Y., NAGAI, Y., SUGIMURA, M., INOUE, K., FUJIKURA, K., OHISHI, K. & MARUYAMA, T. 2019. Identification of cells expressing two peptidoglycan recognition proteins in the gill of the vent mussel, Bathymodiolus septemdierum. *Fish & Shellfish Immunology,* 93**,** 815-822.

INGLIS, S. D., KRISTMUNDSSON, Á., FREEMAN, M. A., LEVESQUE, M. & STOKESBURY, K. 2016. Gray meat in the Atlantic sea scallop, *Placopecten magellanicus*, and the identification of a known pathogenic scallop apicomplexan. *Journal of invertebrate pathology,* 141**,** 66-75.

ISLAM, M. J., KUNZMANN, A. & SLATER, M. J. 2022a. Responses of aquaculture fish to climate change-induced extreme temperatures: A review. *Journal of the World Aquaculture Society,* 53**,** 314-366.

ISLAM, S. S. 2016. *Impact of quorum sensing on the virulence of Vibrio crassostreae and Vibrio tasmaniensis in vitro and in vivo in blue mussel larvae.* Master of science MSc thesis, Ghent University.

ISLAM, S. S., ZHANG, S., EGGERMONT, M., BRUTO, M., LE ROUX, F. & DEFOIRDT, T. 2022b. The impact of the multichannel quorum sensing systems of *Vibrio tasmaniensis* and *Vibrio crassostreae* on virulence towards blue mussel (*Mytilus edulis*) larvae. *Aquaculture,* 547**,** 737414.

IVANINA, A. V. & SOKOLOVA, I. M. 2016. Effects of intermittent hypoxia on oxidative stress and protein degradation in molluscan mitochondria. *Journal of Experimental Biology,* 219**,** 3794-3802.

JARC, E. & PETAN, T. 2019. Focus: Organelles: Lipid droplets and the management of cellular stress. *The Yale journal of biology and medicine,* 92**,** 435.

JAYARAJ, S. S., THIAGARAJAN, R., ARUMUGAM, M. & MULLAINADHAN, P. 2008. Isolation, purification and characterization of β-1, 3-glucan binding protein from the plasma of marine mussel *Perna viridis*. *Fish & Shellfish Immunology,* 24**,** 715-725.

JI, C., WU, H., WEI, L., ZHAO, J., WANG, Q. & LU, H. 2013. Responses of *Mytilus galloprovincialis* to bacterial challenges by metabolomics and proteomics. *Fish and Shellfish Immunology,* 35**,** 489-498.

JIA, J., CHEN, Y., JIANG, Y., TANG, J., YANG, L., LIANG, C., JIA, Z. & ZHAO, L. 2014. Visualized analysis of cellular fatty acid profiles of *Vibrio parahaemolyticus* strains under cold stress. *FEMS Microbiology Letters,* 357**,** 92-98.

JIANG, Q., SHI, L., KE, C., YOU, W. & ZHAO, J. 2013. Identification and characterization of *Vibrio harveyi* associated with diseased abalone *Haliotis diversicolor*. *Diseases of Aquatic Organisms,* 103**,** 133-139.

JOCKERS, M. L., THALKEN, R., L JOCKERS, M. & THALKEN, R. 2020. Introduction to dplyr. *Text Analysis with R: For Students of Literature***,** 121-132.

JOHNSON, C. N. 2013. Fitness factors in vibrios: a mini-review. *Microbial Ecology,* 65**,** 826-851.

KALOYIANNI, M., DAILIANIS, S., CHRISIKOPOULOU, E., ZANNOU, A., KOUTSOGIANNAKI, S., ALAMDARI, D., KOLIAKOS, G. & DIMITRIADIS, V. 2009. Oxidative effects of inorganic and organic contaminants on haemolymph of mussels. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 149**,** 631-639.

KARVONEN, A., JOKELA, J. & LAINE, A.-L. 2019. Importance of sequence and timing in parasite coinfections. *Trends in parasitology,* 35**,** 109-118.

KASSAMBARA, A. 2021. rstatix: pipe-friendly framework for basic statistical tests. R package version 0.7. 0. *Computer software]. https://CRAN. R-proje ct. org/packa ge= rstatix*.

KAWASAKI, T. & KAWAI, T. 2014. Toll-like receptor signaling pathways. *Frontiers in immunology,* 5**,** 461.

KESARCODI-WATSON, A., KASPAR, H., LATEGAN, M. J. & GIBSON, L. 2009a. Two pathogens of Greenshell™ mussel larvae, *Perna canaliculus* : *Vibrio splendidus* and a *V. coralliilyticus*/*neptunius* -like isolate. *Journal of Fish Diseases,* 32**,** 499-507.

KESARCODI-WATSON, A., KASPAR, H., LATEGAN, M. J. & GIBSON, L. 2012. Performance of single and multi-strain probiotics during hatchery production of Greenshell™ mussel larvae, *Perna canaliculus*. *Aquaculture,* 354**,** 56-63.

KESARCODI-WATSON, A., KASPAR, H., LATEGAN, M. J. & GIBSON, L. F. 2009b. Challenge of New Zealand Greenshell™ mussel *Perna canaliculus* larvae using two *Vibrio* pathogens: A hatchery study. *Diseases of Aquatic Organisms,* 86**,** 15-20.

KHANTAVONG, A., TUNKIJJANUKIJ, S., PATARAJINDA, S. & KANGSUWAN, A. 2009. Isolation and characterization of chitinase from marine bacteria. *Journal of Fisheries and Environment,* 33**,** 8-21.

KING, N., MCCOUBREY, D.-J. & CRESSEY, P. 2018. Risk profile: *Vibrio Parahaemolyticus* in bivalve molluscan shellfish

KING, W. L., JENKINS, C., GO, J., SIBONI, N., SEYMOUR, J. R. & LABBATE, M. 2019. Characterisation of the Pacific oyster microbiome during a summer mortality event. *Microbial Ecology,* 77**,** 502-512.

KLEIN, S. L. & FLANAGAN, K. L. 2016. Sex differences in immune responses. *Nature Reviews Immunology,* 16**,** 626-638.

KLUYTMANS, J., ZANDEE, D., ZURBURG, W. & PIETERS, H. 1980. The influence of seasonal changes on energy metabolism in Mytilus edulise (L.).—III. Anaerobic energy metabolism. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 67**,** 307-315.

KOTOB, M. H., MENANTEAU-LEDOUBLE, S., KUMAR, G., ABDELZAHER, M. & EL-MATBOULI, M. 2017. The impact of co-infections on fish: a review. *Veterinary research,* 47**,** 1-12.

KOVALCHUK, S. N., CHIKALOVETS, I. V., CHERNIKOV, O. V., MOLCHANOVA, V. I., LI, W., RASSKAZOV, V. A. & LUKYANOV, P. A. 2013. cDNA cloning and structural characterization of a lectin from the mussel *Crenomytilus grayanus* with a unique amino acid sequence and antibacterial activity. *Fish & shellfish immunology,* 35**,** 1320-1324.

KROON, F., STRETEN, C. & HARRIES, S. 2017. A protocol for identifying suitable biomarkers to assess fish health: A systematic review. *PLoS ONE,* 12**,** e0174762.

KRUKONIS, E. S. & DIRITA, V. J. 2003. From motility to virulence: sensing and responding to environmental signals in Vibrio cholerae. *Current opinion in microbiology,* 6**,** 186-190.

KUMAR, M., CHAKDAR, H., PANDIYAN, K., THAPA, S., SHAHID, M., SINGH, A., SRIVASTAVA, A. K. & SAXENA, A. K. 2022. Bacterial chitinases: genetics, engineering and applications. *World Journal of Microbiology and Biotechnology,* 38**,** 252.

KUMEIKO, V. V., SOKOLNIKOVA, Y. N., GRINCHENKO, A. V., MOKRINA, M. S. & KNIAZKINA, M. I. 2018. Immune state correlates with histopathological level and reveals molluscan health in populations of *Modiolus kurilensis* by integral health index (IHI). *Journal of Invertebrate Pathology,* 154**,** 42-57.

KÜNILI, İ. E., ERTÜRK GÜRKAN, S., AKSU, A., TURGAY, E., ÇAKIR, F., GÜRKAN, M. & ALTINAĞAÇ, U. 2021. Mass mortality in endangered fan mussels *Pinna nobilis* (Linnaeus 1758) caused by co-infection of *Haplosporidium pinnae* and multiple *Vibrio* infection in Çanakkale Strait, Turkey. *Biomarkers,* 26**,** 450-461.

KWAN, T. N. & BOLCH, C. J. S. 2015. Genetic diversity of culturable *Vibrio* in an Australian blue mussel *Mytilus galloprovincialis* hatchery. *Diseases of Aquatic Organisms,* 116**,** 37-46.

LABAUDE, S., MORET, Y., CÉZILLY, F., REULAND, C. & RIGAUD, T. 2017. Variation in the immune state of Gammarus pulex (*Crustacea, Amphipoda*) according to temperature: Are extreme temperatures a stress? *Developmental & Comparative Immunology,* 76**,** 25-33.

LABELLA, A. M., ARAHAL, D. R., CASTRO, D., LEMOS, M. L. & BORREGO, J. J. 2017. Revisiting the genus Photobacterium: taxonomy, ecology and pathogenesis. *Int Microbiol,* 20**,** 1-10.

LABREUCHE, Y., LAMBERT, C., SOUDANT, P., BOULO, V., HUVET, A. & NICOLAS, J.-L. 2006. Cellular and molecular hemocyte responses of the Pacific oyster, *Crassostrea gigas,* following bacterial infection with *Vibrio aestuarianus* strain 01/32. *Microbes and Infection,* 8**,** 2715-2724.

LACROIX, C., COQUILLÉ, V., GUYOMARCH, J., AUFFRET, M. & MORAGA, D. 2014. A selection of reference genes and early-warning mRNA biomarkers for environmental monitoring using Mytilus spp. as sentinel species. *Marine Pollution Bulletin.*

LAITANO, M. V. & FERNÁNDEZ-GIMENEZ, A. V. 2016. Are mussels always the best bioindicators? Comparative study on biochemical responses of three marine invertebrate species to chronic port pollution. *Bulletin of Environmental Contamination and Toxicology,* 97.

LAITH, A. A., ROS-AMIRA, M. K., SHEIKH, H. I., EFFENDY, A. W. M. & NAJIAH, M. 2021. Histopathological and immunological changes in green mussel, *Perna viridis*, challenged with *Vibrio alginolyticus*. *Fish and Shellfish Immunology,* 118**,** 169-179.

LAM, O., WHEELER, J. & TANG, C. M. 2014. Thermal control of virulence factors in bacteria: A hot topic. *Virulence,* 5**,** 852-862.

LANE, H. S., BROSNAHAN, C. L. & POULIN, R. 2020. Aquatic disease in New Zealand: synthesis and future directions. *New Zealand Journal of Marine and Freshwater Research***,** 1-42.

LANE, H. S., BROSNAHAN, C. L. & POULIN, R. 2022. Aquatic disease in New Zealand: synthesis and future directions. *New Zealand Journal of Marine and Freshwater Research,* 56 1-42.

LASSALLE, G., DE MONTAUDOUIN, X., SOUDANT, P. & PAILLARD, C. 2007. Parasite co-infection of two sympatric bivalves, the Manila clam (*Ruditapes philippinarum*) and the cockle (*Cerastoderma edule*) along a latitudinal gradient. *Aquatic Living Resources,* 20**,** 33-42.

LATTOS, A., BITCHAVA, K., GIANTSIS, I. A., THEODOROU, J. A., BATARGIAS, C. & MICHAELIDIS, B. 2021a. The implication of vibrio bacteria in the winter mortalities of the critically endangered *Pinna nobilis*. *Microorganisms,* 9**,** 1-15.

LATTOS, A., FEIDANTSIS, K., GEORGOULIS, I., GIANTSIS, I. A., KARAGIANNIS, D., THEODOROU, J. A., STAIKOU, A. & MICHAELIDIS, B. 2021b. Pathophysiological responses of *Pinna nobilis* individuals enlightens the etiology of mass mortality situation in the mediterranean populations. *Cells,* 10**,** 2838.

LATTOS, A., GIANTSIS, I. A., KARAGIANNIS, D. & MICHAELIDIS, B. 2020. First detection of the invasive Haplosporidian and Mycobacteria parasites hosting the endangered bivalve *Pinna nobilis* in Thermaikos Gulf, North Greece. *Marine environmental research,* 155**,** 104889.

LAW, C., BARR, N., GALL, M., CUMMINGS, V., CURRIE, K., MURDOCH, J., HALLIDAY, J., FROST, E., STEVENS, C. & PLEW, D. 2020. Futureproofing the green-lipped mussel aquaculture industry against ocean acidification. *National Science challenges - Sustainable seas.* Wellington, New Zealand: National Institute for Water and Atmospheric Research and the University of Otago.

LE GUERNIC, A., GEFFARD, A., LE FOLL, F. & LADEIRO, M. P. 2020. Comparison of viability and phagocytic responses of hemocytes withdrawn from the bivalves *Mytilus edulis* and *Dreissena polymorpha*, and exposed to human parasitic protozoa. *International journal for parasitology,* 50**,** 75-83.

LE ROUX, F. & BLOKESCH, M. 2018. Eco-evolutionary dynamics linked to horizontal gene transfer in Vibrios. *Annual Review of Microbiology,* 72.

LE ROUX, F., GAY, M., LAMBERT, C., NICOLAS, J., GOUY, M. & BERTHE, F. 2004. Phylogenetic study and identification of *Vibrio splendidus*-related strains based on *gyrB* gene sequences. *Diseases of Aquatic Organisms,* 58**,** 143-150.

LE ROUX, F., GOUBET, A., THOMPSON, F. L., FAURY, N., GAY, M., SWINGS, J. & SAULNIER, D. 2005. *Vibrio gigantis* sp. nov., isolated from the haemolymph of cultured oysters (*Crassostrea gigas*). *International Journal of Systematic and Evolutionary Microbiology,* 55**,** 2251-2255.

LE ROUX, F., WEGNER, K. M., BAKER-AUSTIN, C., VEZZULLI, L., OSORIO, C. R., AMARO, C., RITCHIE, J. M., DEFOIRDT, T., DESTOUMIEUX-GARZÓN, D., BLOKESCH, M., MAZEL, D., JACQ, A., CAVA, F., GRAM, L., WENDLING, C. C., STRAUCH, E., KIRSCHNER, A. & HUEHN, S. 2015. The emergence of *Vibrio* pathogens in Europe: Ecology, evolution and pathogenesis (Paris, 11-12 March 2015). *Frontiers in Microbiology,* 6.

LEGENDRE, F., MACLEAN, A., APPANNA, V. & APPANNA, V. 2020. Biochemical pathways to α-ketoglutarate, a multi-faceted metabolite. *World Journal of Microbiology and Biotechnology,* 36**,** 1-11.

LELLIS, W. A., PLERHOPLES, T. A. & LELLIS, K. A. 2000. Evaluation of potential anesthetics for the freshwater mussel *Elliptio complanata*. *Journal of Shellfish Research,* 19**,** 983-990.

LEONARD, J. A., COPE, W. G., BARNHART, M. C. & BRINGOLF, R. B. 2014. Metabolomic, behavioral, and reproductive effects of the synthetic estrogen 17 α-ethinylestradiol on the unionid mussel *Lampsilis fasciola*. *Aquatic Toxicology,* 150**,** 103-116.

LEONI, G., DE POLI, A., MARDIROSSIAN, M., GAMBATO, S., FLORIAN, F., VENIER, P., WILSON, D. N., TOSSI, A., PALLAVICINI, A. & GERDOL, M. 2017. Myticalins: a novel multigenic family of linear, cationic antimicrobial peptides from marine mussels (*Mytilus* spp.). *Marine Drugs,* 15**,** 261.

LESSER, M. P. 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. *Annu. Rev. Physiol.,* 68**,** 253-278.

LEULIER, F. & LEMAITRE, B. 2008. Toll-like receptors—taking an evolutionary approach. *Nature Reviews Genetics,* 9**,** 165-178.

LEWBART, G. A. & MOSLEY, C. 2012. Clinical Anesthesia and Analgesia in Invertebrates. *Journal of Exotic Pet Medicine,* 21**,** 59-70.

LI, H., PARISI, M. G., TOUBIANA, M., CAMMARATA, M. & ROCH, P. 2008. Lysozyme gene expression and hemocyte behaviour in the Mediterranean mussel, *Mytilus galloprovincialis* , after injection of various bacteria or temperature stresses. *Fish and Shellfish Immunology,* 25**,** 143-152.

LI, J., NI, X. D., LIU, Y. J. & LU, C. P. 2011. Detection of three virulence genes *alt* , *ahp* and *aerA* in *Aeromonas hydrophila* and their relationship with actual virulence to zebrafish. *Journal of Applied Microbiology,* 110**,** 823-830.

LI, L., CHEN, M. & STOREY, K. B. 2019a. Metabolic response of longitudinal muscles to acute hypoxia in sea cucumber *Apostichopus japonicus* (Selenka): A metabolome integrated analysis. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics,* 29**,** 235-244.

LI, M., PENNER, G. B., HERNANDEZ-SANABRIA, E., OBA, M. & GUAN, L. L. 2009a. Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. *Journal of Applied Microbiology,* 107**,** 1924-1934.

LI, R., ZHANG, R., ZHANG, L., ZOU, J., XING, Q., DOU, H., HU, X., ZHANG, L., WANG, R. & BAO, Z. 2015. Characterizations and expression analyses of *NF-κB* and *Rel* genes in the Yesso scallop (*Patinopecten yessoensis*) suggest specific response patterns against Gram-negative infection in bivalves. *Fish & shellfish immunology,* 44**,** 611-621.

LI, S., ALFARO, A. C., NGUYEN, T. V., YOUNG, T. & LULIJWA, R. 2020. An integrated omics approach to investigate summer mortality of New Zealand Greenshell™ mussels. *Metabolomics,* 16**,** 1-16.

LI, W., RAOULT, D. & FOURNIER, P. E. 2009b. Bacterial strain typing in the genomic era. *FEMS Microbiology Reviews,* 33**,** 892-916.

LI, X., SHI, C., YANG, B., LI, Q. & LIU, S. 2023. High temperature aggravates mortalities of the Pacific oyster (*Crassostrea gigas*) infected with *Vibrio*: A perspective from homeostasis of digestive microbiota and immune response. *Aquaculture,* 568**,** 739309.

LI, Y.-F., LIU, Y.-Z., CHEN, Y.-W., CHEN, K., BATISTA, F. M., CARDOSO, J. C., CHEN, Y.-R., PENG, L.-H., ZHANG, Y. & ZHU, Y.-T. 2019b. Two toll-like receptors identified in the mantle of *Mytilus coruscus* are abundant in haemocytes. *Fish & Shellfish Immunology,* 90**,** 134-140.

LI, Y., BENKENDORFF, K., QIN, J. G. & LI, X. 2012. Exploring correlations between spawning mediated response and summer mortality in Pacific oyster *Crassostrea gigas*. *In:* JIAN G., Q. (ed.) *Oysters: Physiology, Ecological Distribution and Mortality.* New York: Nova Science Publishers.

LI, Y., SONG, X., WANG, W., WANG, L., YI, Q., JIANG, S., JIA, Z., DU, X., QIU, L. & SONG, L. 2017. The hematopoiesis in gill and its role in the immune response of Pacific oyster *Crassostrea gigas* against secondary challenge with *Vibrio splendidus*. *Developmental & Comparative Immunology,* 71**,** 59-69.

LIANG, Y., WU, Y., LI, J., PENG, R., JIANG, M., JIANG, X., CHEN, S. & LIN, J. 2022. Effects of ammonia toxicity on the histopathology, detoxification, oxidative stress, and immune response of the cuttlefish *Sepia pharaonis* and the mitigation of γ-aminobutyric acid. *Ecotoxicology and Environmental Safety,* 232**,** 113256.

LIANG, Z.-W., LI, S.-Y., ZHANG, X.-L., CHEN, C.-Y., SUN, W.-J., GU, Z.-Q., HUANG, J., HE, J.-Y., QI, P.-Z. & GUO, B.-Y. 2023. Morphological change and differential proteomics analysis of gill in *Mytilus coruscus* under starvation. *Frontiers in Physiology,* 14**,** 426.

LIAO, Z., WANG, X. C., LIU, H. H., FAN, M. H., SUN, J. J. & SHEN, W. 2013. Molecular characterization of a novel antimicrobial peptide from *Mytilus coruscus*. *Fish and Shellfish Immunology,* 34**,** 610-616.

LIAO, Z., YANG, Z., WANG, Y., HE, J., HE, Z., ZHANG, X., BUTTINO, I., QI, P., FAN, M. & GUO, B. 2022. Molecular characterization of peptidoglycan recognition proteins from *Mytilus coruscus*. *Fish & Shellfish Immunology,* 131**,** 612-623.

LIU, C. H. & CHEN, J. C. 2004. Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus*. *Fish Shellfish Immunol,* 16**,** 321-34.

LIU, H., HE, J., ZHAO, R., CHI, C. & BAO, Y. 2015. A novel biomarker for marine environmental pollution of pi-class glutathione S-transferase from *Mytilus coruscus*. *Ecotoxicology and Environmental Safety,* 118**,** 47-54.

LIU, H., WU, J., XU, M. & HE, J. 2016. A novel biomarker for marine environmental pollution of HSP90 from *Mytilus coruscus*. *Marine Pollution Bulletin,* 111**,** 428-434.

LIU, H. H., HE, J. Y., CHI, C. F. & SHAO, J. 2014a. Differential *HSP70* expression in *Mytilus coruscus* under various stressors. *Gene,* 543**,** 166-173.

LIU, X., JI, C., ZHAO, J., WANG, Q., LI, F. & WU, H. 2014b. Metabolic profiling of the tissue-specific responses in mussel *Mytilus galloprovincialis* towards *Vibrio harveyi* challenge. *Fish and Shellfish Immunology,* 39**,** 372-377.

LIU, X., SUN, H., WANG, Y., MA, M. & ZHANG, Y. 2014c. Gender-specific metabolic responses in hepatopancreas of mussel *Mytilus galloprovincialis* challenged by *Vibrio harveyi*. *Fish and Shellfish Immunology,* 40**,** 407-413.

LIU, X., ZHAO, J., WU, H. & WANG, Q. 2013. Metabolomic analysis revealed the differential responses in two pedigrees of clam *Ruditapes philippinarum* towards *Vibrio harveyi* challenge. *Fish & shellfish immunology,* 35**,** 1969-1975.

LIU, Y., ZHAN, X., CATALANO, S. R., QIN, J., HAN, J. & LI, X. 2022. Investigation on redox status and gene expression related to larval cryopreservation in the Pacific oyster *Crassostrea gigas*. *Fisheries Science,* 88**,** 377-386.

LOPEZ-JOVEN, C., DE BLAS, I., RUIZ-ZARZUELA, I., FURONES, M. & ROQUE, A. 2011. Experimental uptake and retention of pathogenic and nonpathogenic *Vibrio parahaemolyticus* in two

species of clams: *Ruditapes decussatus* and *Ruditapes philippinarum*. *Journal of applied microbiology,* 111**,** 197-208.

LORI, S., ROVERE, G. D., EZZAT, L., SMITS, M., FERRARESSO, S. S., BABBUCCI, M., MARIN, M. G., MASIERO, L., FABRELLO, J., GARRO, E., CARRARO, L., CARDAZZO, B., PATARNELLO, T., MATOZZO, V., BARGELLONI, L. & MILAN, M. 2020. The effects of glyphosate and AMPA on the mediterranean mussel *Mytilus galloprovincialis* and its microbiota. *Environmental Research,* 182**,** 108984.

LOZANO-LEÓN, A., OSORIO, C. R., NUÑEZ, S., MARTÍNEZ-URTAZA, J. & MAGARIÑOS, B. 2003. Occurrence of *Photobacterium damselae* subsp. *damselae* in bivavlve molluscs from Northwest Spain. *Bulletin of the European Association of Fish Pathologists,* 23**,** 40-44.

LU, J., SHI, Y., CAI, S. & FENG, J. 2017. Metabolic responses of *Haliotis diversicolor* to *Vibrio parahaemolyticus* infection. *Fish & Shellfish Immunology,* 60**,** 265-274.

LUNA-ACOSTA, A., BREITWIESER, M., RENAULT, T. & THOMAS-GUYON, H. 2017. Recent findings on phenoloxidases in bivalves. *Marine Pollution Bulletin,* 122**,** 5-16.

LUO, G., SUN, Y., HUANG, L., SU, Y., ZHAO, L., QIN, Y., XU, X. & YAN, Q. 2020. Time-resolved dual RNA-seq of tissue uncovers *Pseudomonas plecoglossicida* key virulence genes in host-pathogen interaction with *Epinephelus coioides*. *Environmental Microbiology,* 22**,** 677-693.

LUPO, C., BOUGEARD, S., LE BIHAN, V., BLIN, J. L., ALLAIN, G., AZEMA, P., BENOIT, F., BECHEMIN, C., BERNARD, I. & BLACHIER, P. 2021. Mortality of marine mussels *Mytilus edulis* and *M. galloprovincialis*: systematic literature review of risk factors and recommendations for future research. *Reviews in Aquaculture,* 13**,** 504-536.

MACARA, G. R. The climate and weather of New Zealand. Niwa Science and Technology Series, 2018. 1-64.

MACHADO, H., GIUBERGIA, S., MATEIU, R. V. & GRAM, L. 2015. *Photobacterium galatheae* sp. nov, a bioactive bacterium isolated from a mussel in the Solomon Sea. *International Journal of Systematic and Evolutionary Microbiology,* 65**,** 4503-4507.

MACKAY, I. M., ARDEN, K. E. & NITSCHE, A. 2002. Real-time PCR in virology. *Nucleic acids research,* 30**,** 1292-1305.

MACKENZIE, A. L. 2014. The risk to New Zealand shellfish aquaculture from paralytic shellfish poisoning (PSP) toxins. *New Zealand Journal of Marine and Freshwater Research,* 48**,** 430-465.

MACKENZIE, C. L., LYNCH, S. A., CULLOTY, S. C. & MALHAM, S. K. 2014. Future oceanic warming and acidification alter immune response and disease status in a commercial shellfish species, *Mytilus edulis* L. *PLoS ONE,* 9**,** e9971.

MAGARINOS, B., ROMALDE, J., BARJA, J. & TORANZO, A. 1994. Evidence of a dormant but infective state of the fish pathogen Pasteurella piscicida in seawater and sediment. *Applied and environmental microbiology,* 60**,** 180-186.

MAH, T. F. C. & O'TOOLE, G. A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology,* 9**,** 34-39.

MALEKI, F., KHOSRAVI, A., NASSER, A., TAGHINEJAD, H. & AZIZIAN, M. 2016. Bacterial heat shock protein activity. *Journal of clinical and diagnostic research: JCDR,* 10**,** BE01.

MALHAM, S. K., COTTER, E., O'KEEFFE, S., LYNCH, S., CULLOTY, S. C., KING, J. W., LATCHFORD, J. W. & BEAUMONT, A. R. 2009. Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the Irish Sea: The influence of temperature and nutrients on health and survival. *Aquaculture,* 287**,** 128-138.

MARCHETTO, K. M. & POWER, A. G. 2018. Coinfection timing drives host population dynamics through changes in virulence. *The American Naturalist,* 191**,** 173-183.

MARIA, V. L., AMORIM, M. J., BEBIANNO, M. J. & DONDERO, F. 2016. Transcriptomic effects of the non-steroidal anti-inflammatory drug Ibuprofen in the marine bivalve *Mytilus galloprovincialis* Lam. *Marine environmental research,* 119**,** 31-39.

MARQUES, A., OLLEVIER, F., VERSTRAETE, W., SORGELOOS, P. & BOSSIER, P. 2006. Gnotobiotically grown aquatic animals: Opportunities to investigate host-microbe interactions. *Journal of Applied Microbiology,* 100**,** 903-918.

MARTCHEVA, M. & PILYUGIN, S. S. 2006. The role of coinfection in multidisease dynamics. *SIAM Journal on Applied Mathematics,* 66**,** 843-871.

MARTINS, E., FIGUERAS, A., NOVOA, B., SANTOS, R. S., MOREIRA, R. & BETTENCOURT, R. 2014. Comparative study of immune responses in the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* and the shallow-water mussel *Mytilus galloprovincialis* challenged with *Vibrio* bacteria. *Fish and Shellfish Immunology,* 40**,** 485-499.

MARTINS, E., SANTOS, R. S. & BETTENCOURT, R. 2015. *Vibrio diabolicus* challenge in *Bathymodiolus azoricus* populations from Menez Gwen and Lucky Strike hydrothermal vent sites. *Fish and Shellfish Immunology,* 47**,** 962-97.

MARTINS, E., SANTOS, R. S. & BETTENCOURT, R. 2017a. Deep sea mussel *Bathymodiolus azoricus* exposure to *Vibrio diabolicus* induces the expression of apoptotic genes. *EC Microbiology,* 10**,** 96-109.

MARTINS, I., GOULART, J., MARTINS, E., MORALES-ROMAN, R., MARIN, S., RIOU, V., COLACO, A. & BETTENCOURT, R. 2017b. Physiological impacts of acute Cu exposure on deep-sea vent mussel *Bathymodiolus azoricus* under a deep-sea mining activity scenario. *Aquatic toxicology,* 193**,** 40-49.

MATANZA, X. M. & OSORIO, C. R. 2018. Transcriptome changes in response to temperature in the fish pathogen *Photobacterium damselae* subsp. *damselae* : Clues to understand the emergence of disease outbreaks at increased seawater temperatures. *PLOS ONE,* 13**,** e0210118.

MATEO, D. R., SIAH, A., ARAYA, M. T., BERTHE, F. C. J., JOHNSON, G. R. & GREENWOOD, S. J. 2009. Differential in vivo response of soft-shell clam hemocytes against two strains of *Vibrio splendidus*: Changes in cell structure, numbers and adherence. *Journal of Invertebrate Pathology,* 102**,** 50-56.

MATOO, O. B., IVANINA, A. V., ULLSTAD, C., BENIASH, E. & SOKOLOVA, I. M. 2013. Interactive effects of elevated temperature and CO2 levels on metabolism and oxidative stress in two common marine bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 164**,** 545-553.

MATOO, O. B., LANNIG, G., BOCK, C. & SOKOLOVA, I. M. 2021. Temperature but not ocean acidification affects energy metabolism and enzyme activities in the blue mussel, *Mytilus edulis*. *Ecology and evolution,* 11**,** 3366-3379.

MATOZZO, V., ERCOLINI, C., SERRACCA, L., BATTISTINI, R., ROSSINI, I., GRANATO, G., QUAGLIERI, E., PEROLO, A., FINOS, L., ARCANGELI, G., BERTOTTO, D., RADAELLI, G., CHOLLET, B., ARZUL, I. & QUAGLIO, F. 2018. Assessing the health status of farmed mussels (*Mytilus galloprovincialis*) through histological, microbiological and biomarker analyses. *Journal of Invertebrate Pathology,* 153**,** 165-179.

MCCARTER, L. L. 2001. Polar flagellar motility of the *Vibrionaceae*. *Microbiology and Molecular Biology Reviews,* 65**,** 445-462.

MCFALL-NGAI, M., HADFIELD, M. G., BOSCH, T. C. G., CAREY, H. V., DOMAZET-LOŠO, T., DOUGLAS, A. E., DUBILIER, N., EBERL, G., FUKAMI, T., GILBERT, S. F., HENTSCHEL, U., KING, N., KJELLEBERG, S., KNOLL, A. H., KREMER, N., MAZMANIAN, S. K., METCALF, J. L., NEALSON, K., PIERCE, N. E., RAWLS, J. F., REID, A., RUBY, E. G., RUMPHO, M., SANDERS, J. G., TAUTZ, D.

& WERNEGREEN, J. J. Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences* 2013.

MCMAHON, R. F. & BOGAN, A. E. 2001. Ecology and Classification of North American Freshwater Invertebrates. *Ecology and classification of North American freshwater invertebrates*, 315-399.

MEEGHA, P. & PRASAD, T. 2021. Metabolomics: a promising tool to study disease biomarkers and host-pathogen interactions. *In:* HAMEED, S. & FATIMA, Z. (eds.) *Integrated Omics Approaches to Infectious Diseases.* Singapore: Springer.

MEISTER, A. 1988. Glutathione metabolism and its selective modification. *Journal of Biological Chemistry,* 263, 17205-17208.

MENG, J., ZHU, Q., ZHANG, L., LI, C., LI, L., SHE, Z., HUANG, B. & ZHANG, G. 2013. Genome and transcriptome analyses provide insight into the euryhaline adaptation mechanism of *Crassostrea gigas*. *PLoS One,* 8, e58563.

MICCOLI, A., SARACENI, P. R. & SCAPIGLIATI, G. 2019. Vaccines and immune protection of principal Mediterranean marine fish species. *Fish and Shellfish Immunology,* 94, 800-809.

MICHAEL, K., MAAS, E., MACKAY, J., HULSTON, D., MCVEAGH, M. & FORMAN, J. 2015. New qPCR (quantitative polymerase chain reaction) and haematological sampling methods for monitoring Bonamia exitiosa infections and oyster (Ostrea chilensis) mortality in the Foveaux Strait fishery (OYU 5). *New Zealand Fisheries Assessment Report,* 39.

MILLER, M. R., ABSHIRINI, M., WOLBER, F. M., TUTERANGIWHIU, T. R. & KRUGER, M. C. 2023. Greenshell Mussel Products: A Comprehensive Review of Sustainability, Traditional Use, and Efficacy. *Sustainability,* 15, 3912.

MITTA, G., VANDENBULCKE, F. & ROCH, P. 2000. Original involvement of antimicrobial peptides in mussel innate immunity. *FEBS letters,* 486, 185-190.

MOI, I. M., ROSLAN, N. N., LEOW, A. T. C., ALI, M. S. M., RAHMAN, R. N. Z. R. A., RAHIMPOUR, A. & SABRI, S. 2017. The biology and the importance of *Photobacterium* species. *Applied Microbiology and Biotechnology,* 101, 4371-4385.

MOORE, D. & HEILWECK, M. 2022. Aquaculture: Prehistoric to traditional to modern. *Aquaculture: Ocean blue carbon meets UN-SDGS.* Springer.

MOORE, M. N., ICARUS ALLEN, J. & MCVEIGH, A. 2006. Environmental prognostics: An integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. *Marine Environmental Research,* 61, 278-304.

MOORE, R. E., KIRWAN, J., DOHERTY, M. K. & WHITFIELD, P. D. 2007. Biomarker discovery in animal health and disease: The spplication of post-genomic technologies. *Biomarker Insights,* 2, 185-196.

MOREIRA, R., PEREIRO, P., CANCHAYA, C., POSADA, D., FIGUERAS, A. & NOVOA, B. 2015. RNA-Seq in *Mytilus galloprovincialis*: Comparative transcriptomics and expression profiles among different tissues. *BMC Genomics,* 16, 728.

MORLEY, N. J. 2010. Interactive effects of infectious diseases and pollution in aquatic molluscs. *Aquatic Toxicology,* 96, 27-36.

MORTON, J. E. & MILLER, M. 1973. *The New Zealand Sea Shore,* London, Collins.

MOSCHINO, V., MENEGHETTI, F. & DA ROS, L. 2010. Use of biomarkers to assess the welfare of the edible clam, *Ruditapes philippinarum*: may it be a tool for proving areas of origin? *Aquaculture international,* 18, 327-337.

MOUSSA, M., CAUVIN, E., LE PIOUFFLE, A., LUCAS, O., BIDAULT, A., PAILLARD, C., BENOIT, F., THUILLIER, B., TREILLES, M., TRAVERS, M. A. & GARCIA, C. 2021. A MALDI-TOF MS database for fast identification of *Vibrio* spp. potentially pathogenic to marine mollusks. *Applied Microbiology and Biotechnology,* 105**,** 2527-2539.

MUNN, C. B. 2015. The role of vibrios in diseases of corals. *Microbiology Spectrum,* 3**,** 1-12.

MUZNEBIN, F., ALFARO, A. C., VENTER, L. & YOUNG, T. 2022a. Acute thermal stress and endotoxin exposure modulate metabolism and immunity in marine mussels (*Perna canaliculus*). *Journal of Thermal Biology,* 110**,** 103327.

MUZNEBIN, F., ALFARO, A. C. & WEBB, S. C. 2022b. *Perkinsus olseni* and other parasites and abnormal tissue structures in New Zealand Greenshell™ mussels (*Perna canaliculus*) across different seasons. *Aquaculture International***,** 1-36.

MUZNEBIN, F., ALFARO, A. C., WEBB, S. C. & MERIEN, F. 2022c. Characterization of mussel (*Perna canaliculus*) haemocytes and their phagocytic activity across seasons. *Aquaculture Research,* 53**,** 4288-4303.

MYDLARZ, L. D., JONES, L. E. & HARVELL, C. D. 2006. Innate immunity, environmental drivers, and disease ecology of marine and freshwater invertebrates. *Annu. Rev. Ecol. Evol. Syst.,* 37**,** 251-288.

NAJWAA, M. N., DANIELB, A. M. D., AMIN, K. & EFFENDYA, A. 2015. Detection of virulence genes in *Vibrio alginolyticus* isolated from green mussel, *Perna viridis*. *J. Teknol,* 77**,** 19-23.

NAKA, H. & CROSA, J. H. 2011. Genetic determinants of virulence in the marine fish pathogen *Vibrio anguillarum*. *Fish Pathology,* 46**,** 1-10.

NAMBA, K., KABAYASHI, M., AIDA, S., UEMATSU, K., YOSHIDA, M., KONDO, Y. & MIYATA, Y. 1995. Persistent relaxation of the adductor muscle of oyster *Crassostrea gigas* induced by magnesium ion. *Fisheries science,* 61**,** 241-244.

NAMIESNIK, J., MONCHEVA, S., PARK, Y.-S., HAM, K.-S., HEO, B.-G., TASHMA, Z., KATRICH, E. & GORINSTEIN, S. 2008. Concentration of bioactive compounds in mussels *Mytilus galloprovincialis* as an indicator of pollution. *Chemosphere,* 73**,** 938-944.

NATRAH, F. M. I., RUWANDEEPIKA, H. A. D., PAWAR, S., KARUNASAGAR, I., SORGELOOS, P., BOSSIER, P. & DEFOIRDT, T. 2011. Regulation of virulence factors by quorum sensing in *Vibrio harveyi*. *Veterinary Microbiology,* 154**,** 124-129.

NAYLOR, R. L., HARDY, R. W., BUSCHMANN, A. H., BUSH, S. R., CAO, L., KLINGER, D. H., LITTLE, D. C., LUBCHENCO, J., SHUMWAY, S. E. & TROELL, M. 2021. A 20-year retrospective review of global aquaculture. *Nature,* 591**,** 551-563.

NEILSON, M. 2019. New Zealand seas in 2018 hottest since records began, dire warning for marine life. *New Zealand Herald.*

NEWTON, M. & WEBB, S. 2019. Summer mortality of green-lipped mussels 2017-18: A survey of New Zealand Mussel farmers. Cawthron Report No. 3258. *Cawthron Report* Nelson: Cawthron Institute.

NEWTON, T. & COPE, W. 2006. *Biomarker responses of unionid mussels to environmental contaminants*, CRC Press.

NGUYEN, T. V. & ALFARO, A. C. 2019. Targeted metabolomics to investigate antimicrobial activity of itaconic acid in marine molluscs. *Metabolomics,* 15**,** 97.

NGUYEN, T. V. & ALFARO, A. C. 2020a. Applications of omics to investigate responses of bivalve haemocytes to pathogen infections and environmental stress. *Aquaculture,* 518**,** 734488.

NGUYEN, T. V. & ALFARO, A. C. 2020b. Metabolomics investigation of summer mortality in New Zealand Greenshell™ mussels (*Perna canaliculus*). *Fish & Shellfish Immunology,* 106**,** 783-791.

NGUYEN, T. V., ALFARO, A. C., MERIEN, F., LULIJWA, R. & YOUNG, T. 2018a. Copper-induced immunomodulation in mussel (*Perna canaliculus*) haemocytes. *Metallomics,* 10**,** 965-978.

NGUYEN, T. V., ALFARO, A. C., MERIEN, F. & YOUNG, T. 2019a. In vitro study of apoptosis in mussel *(Perna canaliculus*) haemocytes induced by lipopolysaccharide. *Aquaculture,* 503**,** 8-15.

NGUYEN, T. V., ALFARO, A. C., MERIEN, F., YOUNG, T. & GRANDIOSA, R. 2018b. Metabolic and immunological responses of male and female new Zealand Greenshell™ mussels (*Perna canaliculus*) infected with *Vibrio* sp. *Journal of Invertebrate Pathology,* 157**,** 80-89.

NGUYEN, T. V., ALFARO, A. C., YOUNG, T., GREEN, S., ZARATE, E. & MERIEN, F. 2019b. Itaconic acid inhibits growth of a pathogenic marine *Vibrio* strain: A metabolomics approach. *Scientific Reports,* 9**,** 5937.

NGUYEN, T. V., ALFARO, A. C., YOUNG, T. & MERIEN, F. 2019c. Tissue-specific immune responses to Vibrio sp. infection in mussels (*Perna canaliculus*): A metabolomics approach. *Aquaculture,* 500**,** 118-125.

NGUYEN, T. V., ALFARO, A. C., YOUNG, T., RAVI, S. & MERIEN, F. 2018c. Metabolomics study of immune responses of New Zealand Greenshell™ mussels (*Perna canaliculus*) infected with pathogenic *Vibrio* sp. *Marine Biotechnology,* 20**,** 396-409.

NGUYEN, T. V., RAGG, N. L., ALFARO, A. C. & ZAMORA, L. N. 2020. Physiological stress associated with mechanical harvesting and transport of cultured mussels (*Perna canaliculus*): A metabolomics approach. *Aquaculture,* 529**,** 735657.

NIU, C., YU, D., WANG, Y., REN, H., JIN, Y., ZHOU, W., LI, B., CHENG, Y., YUE, J., GAO, Z. & LIANG, L. 2013. Common and pathogen-specific virulence factors are different in function and structure. *Virulence,* 4**,** 473-482.

NOBLE, T., COMAN, G., COWLEY, J., WADE, N., SELLARS, M. & JERRY, D. 2017. Comparison of methods for uniformly challenging Black Tiger shrimp (*Penaeus monodon*) with gill-associated virus. *Aquaculture,* 473**,** 191-196.

NOE, J. T. & MITCHELL, R. A. 2019. Tricarboxylic acid cycle metabolites in the control of macrophage activation and effector phenotypes. *Journal of leukocyte biology,* 106**,** 359-367.

NUSS, A. M., BECKSTETTE, M., PIMENOVA, M., SCHMÜHL, C., OPITZ, W., PISANO, F., HEROVEN, A. K. & DERSCH, P. 2017. Tissue dual RNA-seq allows fast discovery of infection-specific functions and riboregulators shaping host–pathogen transcriptomes. *Proceedings of the National Academy of Sciences,* 114**,** E791-E800.

O'BOYLE, N., HOUEIX, B., KILCOYNE, M., JOSHI, L. & BOYD, A. 2013. The MSHA pilus of *Vibrio parahaemolyticus* has lectin functionality and enables TTSS-mediated pathogenicity. *International Journal of Medical Microbiology,* 303**,** 563-573.

OH, R., LEE, M. J., KIM, Y.-O., NAM, B.-H., KONG, H. J., KIM, J.-W., PARK, J.-Y., SEO, J.-K. & KIM, D.-G. 2020. Myticusin-beta, antimicrobial peptide from the marine bivalve, *Mytilus coruscus*. *Fish & Shellfish Immunology,* 99**,** 342-352.

OKUDA, J., NAKAI, T., CHANG, P. S., OH, T., NISHINO, T., KOITABASHI, T. & NISHIBUCHI, M. 2001. The toxR gene of *Vibrio* (*Listonella*) *anguillarum* controls expression of the major outer membrane proteins but not virulence in a natural host model. *Infection and immunity,* 69**,** 6091-6101.

OLIVER, C., SÁNCHEZ, P., VALENZUELA, K., HERNÁNDEZ, M., PONTIGO, J. P., RAUCH, M. C., GARDUÑO, R. A., AVENDAÑO-HERRERA, R. & YÁÑEZ, A. J. 2020. Subcellular location of

Piscirickettsia salmonis heat shock protein 60 (Hsp60) chaperone by using immunogold labeling and proteomic analysis. *Microorganisms,* 8**,** 117.

OLIVER, E. C. J., BENTHUYSEN, J. A., BINDOFF, N. L., HOBDAY, A. J., HOLBROOK, N. J., MUNDY, C. N. & PERKINS-KIRKPATRICK, S. E. 2017. The unprecedented 2015/16 Tasman Sea marine heatwave. *Nature Communications,* 8**,** 16101

OLIVER, L. M. & FISHER, W. S. 1999. Appraisal of prospective bivalve immunomarkers. *Biomarkers,* 4**,** 510-530.

ORDÁS, M. C., ALBAIGÉS, J., BAYONA, J., ORDAS, A. & FIGUERAS, A. 2007. Assessment of in vivo effects of the Prestige fuel oil spill on the Mediterranean mussel immune system. *Archives of Environmental Contamination and Toxicology,* 52**,** 200-206.

OTTAVIANI, D., SANTARELLI, S., BACCHIOCCHI, S., MASINI, L., GHITTINO, C. & BACCHIOCCHI, I. 2005. Presence of pathogenic *Vibrio parahaemolyticus strains* in mussels from the Adriatic Sea, Italy. *Food microbiology,* 22**,** 585-590.

OUBELLA, R., MAES, P., ALLAM, B., PAILLARD, C. & AUFFRET, M. 1996. Selective induction of hemocytic response in *Ruditapes philippinarum* (Bivalvia) by different species of *Vibrio*(Bacteria). *Aquatic Living Resources,* 9**,** 137-143.

OWESON, C. & HERNROTH, B. 2009. A comparative study on the influence of manganese on the bactericidal response of marine invertebrates. *Fish & Shellfish Immunology,* 27**,** 500-507.

PAILLARD, C., LE ROUX, F. & BORREGO, J. J. 2004. Bacterial disease in marine bivalves, a review of recent studies: Trends and evolution. *Aquatic Living Resources,* 17**,** 477 - 498.

PALAMAE, S., MITTAL, A., YINGKAJORN, M., SAETANG, J., BUATONG, J., TYAGI, A., SINGH, P. & BENJAKUL, S. 2022. *Vibrio parahaemolyticus* Isolates from asian green mussel: Molecular characteristics, virulence and their inhibition by chitooligosaccharide-Tea polyphenol conjugates. *Foods,* 11**,** 4048.

PAREEK, V., PEDLEY, A. M. & BENKOVIC, S. J. 2021. Human de novo purine biosynthesis. *Critical reviews in biochemistry and molecular biology,* 56**,** 1-16.

PARISI, M.-G., LI, H., JOUVET, L. B., DYRYNDA, E. A., PARRINELLO, N., CAMMARATA, M. & ROCH, P. 2008a. Differential involvement of mussel hemocyte sub-populations in the clearance of bacteria. *Fish & Shellfish Immunology,* 25**,** 834-840.

PARISI, M. G., LI, H., JOUVET, L. B. P., DYRYNDA, E. A., PARRINELLO, N., CAMMARATA, M. & ROCH, P. 2008b. Differential involvement of mussel hemocyte sub-populations in the clearance of bacteria. *Fish and Shellfish Immunology,* 25**,** 834-840.

PARISI, M. G., MAISANO, M., CAPPELLO, T., OLIVA, S., MAUCERI, A., TOUBIANA, M. & CAMMARATA, M. 2019. Responses of marine mussel *Mytilus galloprovincialis* (Bivalvia: Mytilidae) after infection with the pathogen *Vibrio splendidus*. *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology,* 221**,** 1-9.

PARISI, M. G., MAURO, M., SARÀ, G. & CAMMARATA, M. 2017. Temperature increases, hypoxia, and changes in food availability affect immunological biomarkers in the marine mussel *Mytilus galloprovincialis*. *Journal of Comparative Physiology B,* 187**,** 1117-1126.

PARRY, H. E. & PIPE, R. K. 2004. Interactive effects of temperature and copper on immunocompetence and disease susceptibility in mussels (*Mytilus edulis*). *Aquatic Toxicology,* 69**,** 311-325.

PATTI, G. J., YANES, O. & SIUZDAK, G. 2012. Metabolomics: the apogee of the omics trilogy. *Nature Reviews Molecular Cell Biology,* 13**,** 263-269.

PEART, R. 2019. Farming the Sea. *Environmental Defence Society.* Auckland.

PETTON, B., DESTOUMIEUX-GARZÓN, D., PERNET, F., TOULZA, E., DE LORGERIL, J., DEGREMONT, L. & MITTA, G. 2021. The Pacific oyster mortality syndrome, a polymicrobial and multifactorial disease: state of knowledge and future directions. *Frontiers in Immunology,* 12**,** 630343.

PHILIPP, E. E., KRAEMER, L., MELZNER, F., POUSTKA, A. J., THIEME, S., FINDEISEN, U., SCHREIBER, S. & ROSENSTIEL, P. 2012. Massively parallel RNA sequencing identifies a complex immune gene repertoire in the lophotrochozoan *Mytilus edulis*. *PloS one,* 7**,** e33091.

PICOT, S., FAURY, N., ARZUL, I., CHOLLET, B., RENAULT, T. & MORGA, B. 2020. Identification of the autophagy pathway in a mollusk bivalve, *Crassostrea gigas*. *Autophagy,* 16**,** 2017-2035.

PIESZ, J. L., SCRO, A. K., CORBETT, R., LUNDGREN, K. M., SMOLOWITZ, R. & GOMEZ-CHIARRI, M. 2022. Development of a multiplex qPCR for the quantification of three protozoan parasites of the eastern oyster *Crassostrea virginica*. *Diseases of Aquatic Organisms,* 151**,** 111-121.

PINHEIRO, J., BATES, D., DEBROY, S., SARKAR, D., HEISTERKAMP, S., VAN WILLIGEN, B. & MAINTAINER, R. 2017. Package 'nlme'. *Linear and nonlinear mixed effects models, version,* 3**,** 274.

PINU, F. R., GOLDANSAZ, S. A. & JAINE, J. 2019. Translational metabolomics: Current challenges and future opportunities. *Metabolites.*

PINU, F. R. & VILLAS-BOAS, S. G. 2017. Extracellular microbial metabolomics: The state of the art. *Metabolites,* 7**,** 43.

PISARENKO, O. I., STUDNEVA, I. M., SHULZHENKO, V. S. & KAPELKO, V. I. 1990. Relations between the energy state of the myocardium and release of some products of anaerobic metabolism during underperfusion. *Pflügers Archiv European Journal of Physiology,* 55**,** 114-125.

PODRABSKY, J. E. & SOMERO, G. N. 2004. Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*. *Journal of Experimental Biology,* 207**,** 2237-2254.

POTASMAN, I., PAZ, A. & ODEH, M. 2002. Infectious outbreaks associated with bivalve shellfish consumption: A worldwide perspective. *Clinical Infectious Diseases,* 35**,** 921-928.

POWELL, J. A., RAGG, N. L. C. & DUNPHY, B. J. 2017. Phenotypic biomarkers in selectively-bred families of the Greenshell™ mussel (*Perna canaliculus*): Anaerobic enzyme and shell gape behaviour as biomarkers of prolonged emersion tolerance. *Aquaculture,* 479**,** 601-608.

PRADO, P., CARRASCO, N., CATANESE, G., GRAU, A., CABANES, P., CARELLA, F., GARCÍA-MARCH, J. R., TENA, J., ROQUE, A. & BERTOMEU, E. 2020a. Presence of *Vibrio mediterranei* associated to major mortality in stabled individuals of *Pinna nobilis* L. *Aquaculture,* 519**,** 734899.

PRADO, P., CARRASCO, N., CATANESE, G., GRAU, A., CABANES, P., CARELLA, F., GARCÍA-MARCH, J. R., TENA, J., ROQUE, A., BERTOMEU, E., GRAS, N., CAIOLA, N., FURONES, M. D. & ANDREE, K. B. 2020b. Presence of *Vibrio mediterranei* associated to major mortality in stabled individuals of *Pinna nobilis* L. *Aquaculture,* 519**,** 734899.

PRADO, S., DUBERT, J., DA COSTA, F., MARTÍNEZ-PATIÑO, D. & BARJA, J. L. 2014. Vibrios in hatchery cultures of the razor clam, *Solen marginatus* (Pulteney). *Journal of Fish Diseases,* 37**,** 209-217.

PRAYITNO, S. B. & LATCHFORD, J. W. 1995. Experimental infections of crustaceans with luminous bacteria related to *Photobacterium* and *Vibrio*: Effect of salinity and pH on infectiosity. *Aquaculture,* 132**,** 105-112.

PROCTOR, L. M. & GUNSALUS, R. P. 2000. Anaerobic respiratory growth of *Vibrio harveyi* , *Vibrio fischeri*and *Photobacterium leiognathi* with trimethylamine N-oxide, nitrate and fumarate: Ecological implications. *Environmental Microbiology,* 2**,** 399-406.

PROVENZANO, D. & KLOSE, K. E. 2000. Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes Vibrio cholerae bile resistance, virulence factor expression, and intestinal colonization. *Proceedings of the National Academy of Sciences,* 97**,** 10220-10224.

PRUZZO, C., GALLO, G. & CANESI, L. 2005. Persistence of vibrios in marine bivalves: The role of interactions with haemolymph components. *Environmental Microbiology,* 7**,** 761-772.

PUCHNICK-LEGAT, A., LEGAT, J. F. A., GOMES, C. H. A. D. M., SUHNEL, S. & MELO, C. M. R. 2015. Anesthesia in oysters of the genus *Crassostrea* cultured in Brazil. *Boletim do Instituto de Pesca,* 41**,** 785-793.

PUDGERD, A., KRUANGKUM, T., SRITUNYALUCKSANA, K., VANICHVIRIYAKIT, R., IMSONPANG, S. & CHOTWIWATTHANAKUN, C. 2021. Immunopathogenesis of hematopoietic tissues in response to *Vibrio parahaemolyticus* (VPAHPND) infection in *Macrobrachium rosenbergii*. *Fish & Shellfish Immunology,* 110**,** 10-22.

PUERTO, M., CAMPOS, A., PRIETO, A., CAMEÁN, A., DE ALMEIDA, A. M., COELHO, A. V. & VASCONCELOS, V. 2011. Differential protein expression in two bivalve species; *Mytilus galloprovincialis* and *Corbicula fluminea*; exposed to *Cylindrospermopsis raciborskii* cells. *Aquatic toxicology,* 101**,** 109-116.

PUGLIESE, C., MAZZA, R., ANDREWS, P. L. R., CERRA, M. C., FIORITO, G. & GATTUSO, A. 2016. Effect of different formulations of magnesium chloride used as anesthetic agents on the performance of the isolated heart of *Octopus vulgaris*. *Frontiers in Physiology,* 7**,** 1-13.

PUSPITA, M. & HUTABARAT, J. Effects of temperature in the phenoloxidase activity of *Perna viridis* challenged with *Vibrio harveyi*. Gadjah Mada University in 2011 (National proceeding), 2015. 1-10.

QIN, C. L., HUANG, W., ZHOU, S. Q., WANG, X. C., LIU, H. H., FAN, M. H., WANG, R. X., GAO, P. & LIAO, Z. 2014. Characterization of a novel antimicrobial peptide with chiting-biding domain from *Mytilus coruscus*. *Fish and Shellfish Immunology,* 41**,** 362-370.

QUINN, E. A., MALKIN, S. H., ROWLEY, A. F. & COATES, C. J. 2020. Laccase and catecholoxidase activities contribute to innate immunity in slipper limpets, *Crepidula fornicata*. *Developmental & Comparative Immunology,* 110**,** 103724.

QUINN, E. A., MALKIN, S. H., THOMAS, J. E., POOLE, R., DAVIES, C. E., ROWLEY, A. F. & COATES, C. J. 2022. Invasive slipper limpets (*Crepidula fornicata*) act like a sink, rather than source, of *Vibrio* spp. *Biological Invasions,* 24**,** 3647-3659.

RABINOWITZ, J. D. & ENERBÄCK, S. 2020. Lactate: the ugly duckling of energy metabolism. *Nature Metabolism,* 2**,** 566-571.

RAGG, N. L., GALE, S. L., LE, D. V., HAWES, N. A., BURRITT, D. J., YOUNG, T., ERICSON, J. A., HILTON, Z., WATTS, E. & BERRY, J. 2019. The effects of aragonite saturation state on hatchery-reared larvae of the Greenshell mussel *Perna canaliculus*. *Journal of Shellfish Research,* 38**,** 779-793.

RAHMAN, M., HENDERSON, S., MILLER-EZZY, P., LI, X. & QIN, J. 2019. Immune response to temperature stress in three bivalve species: Pacific oyster *Crassostrea gigas*, Mediterranean mussel *Mytilus galloprovincialis* and mud cockle *Katelysia rhytiphora*. *Fish & shellfish immunology,* 86**,** 868-874.

RAM, K. & WICKHAM, H. 2018. wesanderson: a Wes Anderson palette generator. *R package version 0.3,* 6**,** 2018.

RATNAWATI, S. E., KUULIALA, L., WALGRAEVE, C., DEMEESTERE, K., RAGAERT, P. & DEVLIEGHERE, F. 2023. The effect of high oxygen modified atmospheres on the quality degradation of packed live blue mussels (*Mytilus edulis*). *LWT,* 177**,** 114537.

RAUTA, P. R., SAMANTA, M., DASH, H. R., NAYAK, B. & DAS, S. 2014. Toll-like receptors (TLRs) in aquatic animals: signaling pathways, expressions and immune responses. *Immunology letters,* 158**,** 14-24.

REICHLEY, S. R., WARE, C., GREENWAY, T. E., WISE, D. J. & GRIFFIN, M. J. 2015. Real-time polymerase chain reaction assays for the detection and quantification of *Edwardsiella tarda*, *Edwardsiella piscicida*, and *Edwardsiella piscicida*-like species in catfish tissues and pond water. *Journal of Veterinary Diagnostic Investigation,* 27**,** 130-9.

REN, J. S., RAGG, N. L. C., CUMMINGS, V. J. & ZHANG, J. 2020. Ocean acidification and dynamic energy budget models: Parameterisation and simulations for the green-lipped mussel. *Ecological Modelling,* 426**,** 109069.

REN, Q., SUN, R. R., ZHAO, X. F. & WANG, J. X. 2009. A selenium-dependent glutathione peroxidase (*Se-GPx*) and two glutathione S-transferases (*GSTs*) from Chinese shrimp (*Fenneropenaeus chinensis*). *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology,* 149**,** 613-623.

REN, Q., ZHONG, X., YIN, S. W., HAO, F. Y., HUI, K. M., ZHANG, Z., ZHANG, C. Y., YU, X. Q. & WANG, W. 2013. The first Toll receptor from the triangle-shell pearl mussel *Hyriopsis cumingii*. *Fish and Shellfish Immunology,* 34**,** 1287-1293.

REN, W., RAJENDRAN, R., ZHAO, Y., TAN, B., WU, G., BAZER, F. W., ZHU, G., PENG, Y., HUANG, X., DENG, J. & YIN, Y. 2018. Amino acids as mediators of metabolic cross talk between host and pathogen. *Frontiers in Immunology,* 9**,** 1-13.

RENAULT, T. 2009. Trends and perspectives in preventing and controlling infectious diseases in molluscs. *Aquaculture research progress. Nova Science Publishers, Lancaster***,** 99-126.

RENAULT, T. 2015. Immunotoxicological effects of environmental contaminants on marine bivalves. *Fish & shellfish immunology,* 46**,** 88-93.

RENWRANTZ, L., SIEGMUND, E. & WOLDMANN, M. 2013. Variations in hemocyte counts in the mussel, *Mytilus edulis*: Similar reaction patterns occur in disappearance and return of molluscan hemocytes and vertebrate leukocytes. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 164**,** 629-637.

RESHEF, L., RON, E. & ROSENBERG, E. 2008. Genome analysis of the coral bleaching pathogen *Vibrio shiloi*. *Archives of microbiology,* 190**,** 185-194.

REVERCHON, S. & NASSER, W. 2013. Dickeya ecology, environment sensing and regulation of virulence programme. *Environmental microbiology reports,* 5**,** 622-636.

REVERTER, M., TAPISSIER-BONTEMPS, N., SARTER, S., SASAL, P. & CARUSO, D. 2021. Moving towards more sustainable aquaculture practices: a meta-analysis on the potential of plant-enriched diets to improve fish growth, immunity and disease resistance. *Reviews in Aquaculture,* 13**,** 537-555.

REY-CAMPOS, M., MOREIRA, R., GERDOL, M., PALLAVICINI, A., NOVOA, B. & FIGUERAS, A. 2019a. Immune tolerance in *Mytilus galloprovincialis* hemocytes after repeated contact with *Vibrio splendidus*. *Frontiers in Immunology,* 10**,** 1-15.

REY-CAMPOS, M., MOREIRA, R., VALENZUELA-MUÑOZ, V., GALLARDO-ESCÁRATE, C., NOVOA, B. & FIGUERAS, A. 2019b. High individual variability in the transcriptomic response of Mediterranean mussels to *Vibrio* reveals the involvement of myticins in tissue injury. *Scientific Reports,* 9**,** 1-15.

REYDEN, C. A., DELORME, N. J., SOUTH, P. M. & AGUIRRE, J. D. 2023. Impacts of seeding density on the oxidative stress response of the Greenshell™ mussel, *Perna canaliculus*. *Aquaculture International***,** 1-17.

RICHARD, J. C., CAMPBELL, L. J., LEIS, E. M., AGBALOG, R. E., DUNN, C. D., WALLER, D. L., KNOWLES, S., PUTNAM, J. G. & GOLDBERG, T. L. 2021. Mussel mass mortality and the microbiome: Evidence for shifts in the bacterial microbiome of a declining freshwater bivalve. *Microorganisms,* 9**,** 1976.

RÍOS-CASTRO, R., ARANGUREN, R., ROMERO, A., BANCHI, E., PALLAVICINI, A., NOVOA, B. & FIGUERAS, A. 2022. Assessment of the environmental distribution of the protozoan parasite *Perkinsus olseni* by next-generation sequencing, qPCR and histopathology allows the identification of alternative bivalve hosts. *Aquaculture,* 552**,** 737984.

RIPLEY, B., VENABLES, B., BATES, D. M., HORNIK, K., GEBHARDT, A., FIRTH, D. & RIPLEY, M. B. 2013. Package 'mass'. *Cran r,* 538**,** 113-120.

RITZENHOFEN, L., BUER, A. L., GYRAITE, G., DAHLKE, S., KLEMMSTEIN, A. & SCHERNEWSKI, G. 2021. Blue mussel (*Mytilus* spp.) cultivation in mesohaline eutrophied inner coastal waters: Mitigation potential, threats and cost effectiveness. *PeerJ,* 9**,** e11247.

RIVERA-INGRAHAM, G. A., ROCCHETTA, I., MEYER, S. & ABELE, D. 2013. Oxygen radical formation in anoxic transgression and anoxia-reoxygenation: Foe or phantom? Experiments with a hypoxia tolerant bivalve. *Marine Environmental Research.*

ROLTON, A. & RAGG, N. L. C. 2020. Green-lipped mussel (*Perna canaliculus*) hemocytes: A flow cytometric study of sampling effects, sub-populations and immune-related functions. *Fish and Shellfish Immunology,* 103**,** 181-189.

ROMALDE, J. L., DIEGUEZ, A. L., LASA, A. & BALBOA, S. 2014. New *Vibrio* species associated to molluscan microbiota: a review. *Frontiers in Microbiology,* 4**,** 1-11.

ROMERO, A., COSTA, M., FORN-CUNI, G., BALSEIRO, P., CHAMORRO, R., DIOS, S., FIGUERAS, A. & NOVOA, B. 2014. Occurrence, seasonality and infectivity of *Vibrio* strains in natural populations of mussels *Mytilus galloprovincialis*. *Dis Aquat Organ,* 108**,** 149-63.

ROMERO, A., NOVOA, B. & FIGUERAS, A. 2015. The complexity of apoptotic cell death in mollusks: an update. *Fish & Shellfish Immunology,* 46**,** 79-87.

ROMERO, A., NOVOA, B. & FIGUERAS, A. 2022. Genomic and transcriptomic identification of the cathepsin superfamily in the Mediterranean mussel *Mytilus galloprovincialis*. *Developmental & Comparative Immunology,* 127**,** 104286.

RONCARATI, D. & SCARLATO, V. 2017. Regulation of heat-shock genes in bacteria: from signal sensing to gene expression output. *FEMS microbiology reviews,* 41**,** 549-574.

ROQUIGNY, R., MOUGIN, J., LE BRIS, C., BONNIN-JUSSERAND, M., DOYEN, P. & GRARD, T. 2021. Characterization of the marine aquaculture microbiome: A seasonal survey in a seabass farm. *Aquaculture,* 531**,** 735987.

ROSANI, U., VAROTTO, L., ROSSI, A., ROCH, P., NOVOA, B., FIGUERAS, A., PALLAVICINI, A. & VENIER, P. 2011. Massively parallel amplicon sequencing reveals isotype-specific variability of antimicrobial peptide transcripts in *Mytilus galloprovincialis*. *PLoS ONE,* 6**,** e26680.

ROSENBLUM, E., VIANT, M., BRAID, B., MOORE, J., FRIEDMAN, C. & TJEERDEMA, R. 2005. Characterizing the metabolic actions of natural stresses in the California red abalone, *Haliotis rufescens* using 1 H NMR metabolomics. *Metabolomics,* 1**,** 199-209.

ROZNERE, I., WATTERS, G. T., WOLFE, B. A. & DALY, M. 2017. Effects of relocation on metabolic profiles of freshwater mussels: Metabolomics as a tool for improving conservation techniques. *Aquatic Conservation: Marine and Freshwater Ecosystems,* 27**,** 919-926.

RUBIOLO, J. A., BOTANA, L. M. & MARTÍNEZ, P. 2019. Insights into mussel microbiome. *Microbial Communities in Aquaculture Ecosystems.* Cham: Springer International Publishing.

RUIZ, Y., SUÁREZ, P., ALONSO, A., LONGO, E. & SAN JUAN, F. 2013. Mutagenicity test using *Vibrio harveyi* in the assesment of water quality from mussel farms. *Water Research,* 47.

RUWANDEEPIKA, H. A. D., DEFOIRDT, T., BHOWMICK, P. P., KARUNASAGAR, I., KARUNASAGAR, I. & BOSSIER, P. 2011. In vitro and in vivo expression of virulence genes in *Vibrio* isolates belonging to the *Harveyi* clade in relation to their virulence towards gnotobiotic brine shrimp *Artemia franciscana*. *Environmental Microbiology,* 13**,** 506-517.

RUWANDEEPIKA, H. A. D., SANJEEWA PRASAD JAYAWEERA, T., PABAN BHOWMICK, P., KARUNASAGAR, I., BOSSIER, P. & DEFOIRDT, T. 2012. Pathogenesis, virulence factors and virulence regulation of vibrios belonging to the *Harveyi* clade. *Reviews in Aquaculture,* 4.

RYAN, P. B., BURKE, T. A., COHEN HUBAL, E. A., CURA, J. J. & MCKONE, T. E. 2007. Using biomarkers to inform cumulative risk assessment. *Environmental Health Perspectives,* 115.

SABIO, G. & DAVIS, R. J. TNF and MAP kinase signalling pathways. *Seminars in immunology*, 2014. 237-245.

SACO, A., PANEBIANCO, A., BLANCO, S., NOVOA, B., DIZ, A. P. & FIGUERAS, A. 2021. Integration of transcriptomics and proteomics improves the characterization of the role of mussel gills in a bacterial waterborne infection. *Frontiers in Marine Science,* 8**,** 735309.

SACO, A., REY-CAMPOS, M., NOVOA, B. & FIGUERAS, A. 2020. Transcriptomic response of mussel gills after a *Vibrio splendidus* infection demonstrates their role in the immune response. *Frontiers in Immunology,* 11**,** 1-18.

SAID, R. M. & NASSAR, S. E. 2022. Mortality, energy reserves, and oxidative stress responses of three native freshwater mussels to temperature as an indicator of potential impacts of climate change: A laboratory experimental approach. *Journal of Thermal Biology,* 104**,** 103154.

SALWAY, J. G. 2004. *Metabolism at a glance*, John Wiley & Sons.

SAMAIN, J. F. 2011. Review and perspectives of physiological mechanisms underlying genetically-based resistance of the Pacific oyster *Crassostrea gigas* to summer mortality. *Aquatic Living Resources,* 24**,** 227–236.

SAN MARTIN, V. A., VASQUEZ LAVÍN, F., PONCE OLIVA, R. D., LERDÓN, X. P., RIVERA, A., SARRAMALERA, L. & GELCICH, S. 2020. Exploring the adaptive capacity of the mussel mariculture industry in Chile. *Aquaculture,* 519**,** 734856.

SANTOS, A., HAUSER-DAVIS, R. A., SANTOS, M. J. S. & DE SIMONE, S. G. 2017. Potentially toxic filamentous fungi associated to the economically important *Nodipecten nodosus* (Linnaeus, 1758) scallop farmed in southeastern Rio de Janeiro, Brazil. *Marine Pollution Bulletin,* 115**,** 75-79.

SAUVE, A. A. 2008. NAD+ and vitamin B3: from metabolism to therapies. *Journal of Pharmacology and Experimental Therapeutics,* 324**,** 883-893.

SAWABE, T., OGURA, Y., MATSUMURA, Y., FENG, G., AMIN, A. R., MINO, S., NAKAGAWA, S., SAWABE, T., KUMAR, R., FUKUI, Y., SATOMI, M., MATSUSHIMA, R., THOMPSON, F. L., GOMEZ-GIL, B., CHRISTEN, R., MARUYAMA, F., KUROKAWA, K. & HAYASHI, T. 2013. Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. *Frontiers in Microbiology,* 4**,** 414.

SCHRIMPE-RUTLEDGE, A. C., CODREANU, S. G., SHERROD, S. D. & MCLEAN, J. A. 2016. Untargeted Metabolomics Strategies-Challenges and Emerging Directions. *Journal of the American Society for Mass Spectrometry.* 2016/09/13 ed.

SCHUBERT, M., LINDGREEN, S. & ORLANDO, L. 2016. AdapterRemoval v2: rapid adapter trimming, identification, and read merging. *BMC Research Notes,* 9**,** 88.

SCHYMANSKI, E. L., JEON, J., GULDE, R., FENNER, K., RUFF, M., SINGER, H. P. & HOLLENDER, J. 2014. Identifying small molecules via high resolution mass spectrometry: Communicating confidence. *Environmental Science & Technology,* 48**,** 2097-2098.

SEGARRA, A., PÉPIN, J. F., ARZUL, I., MORGA, B., FAURY, N. & RENAULT, T. 2010. Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Research,* 153.

SEIBOTH, B. & METZ, B. 2011. Fungal arabinan and L-arabinose metabolism. *Applied Microbiology and Biotechnology,* 89**,** 1665-1673.

SENDRA, M., SACO, A., REY-CAMPOS, M., NOVOA, B. & FIGUERAS, A. 2020. Immune-responsive gene 1 (*IRG1*) and dimethyl itaconate are involved in the mussel immune response. *Fish & Shellfish Immunology,* 106**,** 645-655.

SHANG, Y., WANG, X., SHI, Y., HUANG, W., SOKOLOVA, I., CHANG, X., CHEN, D., WEI, S., KHAN, F. U. & HU, M. 2023. Ocean acidificationf affects the bioenergetics of marine mussels as revealed by high-coverage quantitative metabolomics. *Science of The Total Environment,* 858**,** 160090.

SHEN, S. S., QU, X. Y., ZHANG, W. Z., LI, J. & LV, Z. Y. 2019. Infection against infection: Parasite antagonism against parasites, viruses and bacteria. *Infectious Diseases of Poverty,* 8**,** 1-12.

SHI, L. Y., LIANG, S., LUO, X., KE, C. H. & ZHAO, J. 2017. Microbial community of Pacific abalone (*Haliotis discus hannai*) juveniles during a disease outbreak in South China. *Aquaculture Research,* 48**,** 1080-1088.

SHIELDS, J. L., BARNES, P. & HEATH, D. D. 2008. Growth and survival differences among native, introduced and hybrid blue mussels (*Mytilus* spp.): genotype, environment and interaction effects. *Marine Biology,* 154**,** 919-928.

SHINODA, S. & MIYOSHI, S. 2011. Proteases produced by vibrios. *Biocontrol Science,* 16**,** 1-11.

SIEGLE, M. R., TAYLOR, E. B. & O'CONNOR, M. I. 2018. Prior heat accumulation reduces survival during subsequent experimental heat waves. *Journal of Experimental Marine Biology and Ecology,* 501**,** 109-117.

SIGEL, A., SIGEL, H. & K O SIGEL, R. 2015. Metallothioneins and related chelators. *In:* SIGEL, H., SIGEL, A. & SIGEL, R. K. O. (eds.) *Metallothioneins and related chelators.* De Gruyter.

SILVA, D. C., NETO, J. M., NUNES, C., GONÇALVES, F. J., COIMBRA, M. A., MARQUES, J. C. & GONÇALVES, A. M. 2021. Assessment of seasonal and spatial variations in the nutritional content of six edible marine bivalve species by the response of a set of integrated biomarkers. *Ecological Indicators,* 124**,** 107378.

SILVA, I. P., DE SOUZA CARNEIRO, C., SARAIVA, M. A. F., DE OLIVEIRA, T. A. S., DE SOUSA, O. V. & EVANGELISTA-BARRETO, N. S. 2018. Antimicrobial resistance and potential virulence of *Vibrio parahaemolyticus* isolated from water and bivalve mollusks from Bahia, Brazil. *Marine Pollution Bulletin,* 131**,** 757-762.

SKÅR, C. K. & MORTENSEN, S. 2007. Fate of infectious salmon anaemia virus (ISAV) in experimentally challenged blue mussels *Mytilus edulis*. *Diseases of aquatic organisms,* 74**,** 1-6.

SKEIN, L., ROBINSON, T. B. & ALEXANDER, M. E. 2018. Impacts of mussel invasions on the prey preference of two native predators. *Behavioral Ecology,* 29**,** 353-359.

SMART, K. F., AGGIO, R. B. M., VAN HOUTTE, J. R. & VILLAS-BÔAS, S. G. 2010. Analytical platform for metabolome analysis of microbial cells using methyl chloroformate derivatization followed by gas chromatography-mass spectrometry. *Nature Protocols,* 5**,** 1709-1729.

SMITH, K. E., BURROWS, M. T., HOBDAY, A. J., KING, N. G., MOORE, P. J., SEN GUPTA, A., THOMSEN, M. S., WERNBERG, T. & SMALE, D. A. 2023. Biological impacts of marine heatwaves. *Annual review of marine science,* 15**,** 119-145.

SOKOLOVA, I., EVANS, S. & HUGHES, F. 2004. Cadmium-induced apoptosis in oyster hemocytes involves disturbance of cellular energy balance but no mitochondrial permeability transition. *Journal of Experimental Biology,* 207**,** 3369-3380.

SOKOLOVA, I. M. 2009. Apoptosis in molluscan immune defense. *Invertebrate Survival Journal,* 6**,** 49-58.

SOKOLOVA, I. M., FREDERICH, M., BAGWE, R., LANNIG, G. & SUKHOTIN, A. A. 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine environmental research,* 79**,** 1-15.

SOLIMAN, T. & INGLIS, G. J. 2018. Forecasting the economic impacts of two biofouling invaders on aquaculture production of green-lipped mussels *Perna canaliculus* in New Zealand. *Aquaculture Environment Interactions,* 10**,** 1-12.

SONG, L., WANG, L., QIU, L. & ZHANG, H. 2010. Bivalve immunity. *Advance Experimental Medical Biology,* 708**,** 44-65.

SONG, M., ZHAO, J., WEN, H.-S., LI, Y., LI, J.-F., LI, L.-M. & TAO, Y.-X. 2019. The impact of acute thermal stress on the metabolome of the black rockfish *(Sebastes schlegelii)*. *PloS one,* 14**,** e0217133.

SOON, T. K. & ZHENG, H. 2020. Climate change and bivalve mass mortality in temperate regions. *Reviews of environmental contamination and toxicology volume 251***,** 109-129.

SOTO, E., BOWLES, K., FERNANDEZ, D. & HAWKE, J. P. 2010. Development of a real-time PCR assay for identification and quantification of the fish pathogen *Francisella noatunensis* subsp. *orientalis*. *Dis Aquat Organ,* 89**,** 199-207.

SOUTH, P. M., DELORME, N. J., SKELTON, B. M., FLOERL, O. & JEFFS, A. G. 2022. The loss of seed mussels in longline aquaculture. *Reviews in Aquaculture,* 14**,** 440-455.

SOUZA, C. P., ALMEIDA, B. C., COLWELL, R. R. & RIVERA, I. N. 2011. The importance of chitin in the marine environment. *Marine biotechnology,* 13**,** 823-830.

SRINIVASAN, R., CAREY-SMITH, T. & MACARA, G. 2021. Trend analysis on frequency of New Zealand climate extremes. *Weather and Climate,* 41**,** 18-29.

STABILI, L., ACQUAVIVA, M. I. & CAVALLO, R. A. 2005. *Mytilus galloprovincialis* filter feeding on the bacterial community in a Mediterranean coastal area (Northern Ionian Sea, Italy). *Water Research,* 39.

STAMATAKIS, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics,* 30**,** 1312-1313.

STENTIFORD, G. D., VIANT, M. R., WARD, D. G., JOHNSON, P. J., MARTIN, A., WENBIN, W., COOPER, H. J., LYONS, B. P. & FEIST, S. W. 2005. Liver tumors in wild flatfish: A histopathological, proteomic, and metabolomic study. *OMICS A Journal of Integrative Biology.*

STENTON-DOZEY, J. M., HEATH, P., REN, J. S. & ZAMORA, L. N. 2021. New Zealand aquaculture industry: research, opportunities and constraints for integrative multitrophic farming. *New Zealand Journal of Marine and Freshwater Research,* 55**,** 265-285.

STEVENS, C. L., O'CALLAGHAN, J. M., CHISWELL, S. M. & HADFIELD, M. G. 2021. Physical oceanography of New Zealand/Aotearoa shelf seas–a review. *New Zealand Journal of Marine and Freshwater Research,* 55**,** 6-45.

STEVENS, C. L., SPILLMAN, C. M., BEHRENS, E., BROEKHUIZEN, N., HOLLAND, P., MATTHEWS, Y., NOLL, B., O'CALLAGHAN, J. M., RAMPAL, N. & SMITH, R. O. 2022. Horizon scan on the benefits of ocean seasonal forecasting in a future of increasing marine heatwaves for Aotearoa New Zealand. *Frontiers in Climate*, 92.

SU, J. H., CHANG, M. C., LEE, Y. S., TSENG, I. C. & CHUANG, Y. C. 2004. Cloning and characterization of the lipase and lipase activator protein from *Vibrio vulnificus* CKM-1. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression,* 1678, 7-13.

SUGUMAR, G., NAKAI, T., HIRATA, Y., MATSUBARA, D. & MUROGA, K. 1998. *Vibrio splendidus* biovar II as the causative agent of bacillary necrosis of Japanese oyster *Crassostrea gigas* larvae. *Diseases of Aquatic Organisms,* 33, 111-118.

SUMNER, L. W., AMBERG, A., BARRETT, D., BEALE, M. H., BEGER, R., DAYKIN, C. A., FAN, T. W.-M., FIEHN, O., GOODACRE, R. & GRIFFIN, J. L. 2007a. Proposed minimum reporting standards for chemical analysis: chemical analysis working group (CAWG) metabolomics standards initiative (MSI). *Metabolomics,* 3, 211-221.

SUMNER, L. W., AMBERG, A., BARRETT, D., BEALE, M. H., BEGER, R., DAYKIN, C. A., FAN, T. W.-M., FIEHN, O., GOODACRE, R., GRIFFIN, J. L., HANKEMEIER, T., HARDY, N., HARNLY, J., HIGASHI, R., KOPKA, J., LANE, A. N., LINDON, J. C., MARRIOTT, P., NICHOLLS, A. W., REILY, M. D., THADEN, J. J. & VIANT, M. R. 2007b. Proposed minimum reporting standards for chemical analysis. *Metabolomics,* 3, 211-221.

SUNILA, I. & LABANCA, J. 2003. Apoptosis in the pathogenesis of infectious diseases of the eastern oyster Crassostrea virginica. *Diseases of aquatic organisms,* 56, 163-170.

SUPLICY, F. M. 2020. A review of the multiple benefits of mussel farming. *Reviews in Aquaculture,* 12, 204-223.

SUQUET, M., ARAYA, R. G., LEBRUN, L., QUEAU, I., MINGANT, C. & ROBERT, R. 2010. Anaesthesia and gonad sampling in the European flat oyster (*Ostrea edulis*). *Aquaculture,* 308, 196-198.

SURES, B., NACHEV, M., SELBACH, C. & MARCOGLIESE, D. J. 2017. Parasite responses to pollution: what we know and where we go in 'Environmental Parasitology'. *Parasites and Vectors,* 10, 1-19.

SUSI, H., BARRÈS, B., VALE, P. F. & LAINE, A.-L. 2015. Co-infection alters population dynamics of infectious disease. *Nature Communications,* 6, 5975.

SUSSMAN, M., MIEOG, J. C., DOYLE, J., VICTOR, S., WILLIS, B. L. & BOURNE, D. G. 2009. Vibrio zinc-metalloprotease causes photoinactivation of coral endosymbionts and coral tissue lesions. *PLoS ONE,* 4, e4511.

SUTTON, D. & GARRICK, R. 1993. Bacterial disease of cultured giant darn *Tridacna gigas* larvae. *Diseases of Aquatic Organisms,* 16, 47-53.

SYMONDS, J. E., CLARKE, S. M., KING, N., WALKER, S. P., BLANCHARD, B., SUTHERLAND, D., ROBERTS, R., PREECE, M. A., TATE, M., BUXTON, P. & DODDS, K. G. 2019. Developing successful breeding programs for New Zealand aquaculture: A perspective on progress and future genomic opportunities. *Frontiers in Genetics,* 10, 1-7.

TAGLIAFERRO, M., ROCHA, C., MARQUES, J. C. & GONÇALVES, A. M. 2022. Assessment of metal exposure (uranium and copper) in fatty acids and carbohydrate profiles of *Calamoceras marsupus* larvae (Trichoptera) and *Alnus glutinosa* leaf litter. *Science of The Total Environment,* 836, 155613.

TAKEMURA, A. F., CHIEN, D. M. & POLZ, M. F. 2014. Associations and dynamics of Vibrionaceae in the environment, from the genus to the population level. *Frontiers in Microbiology,* 5, 1-26.

TALL, B., LA PEYRE, J., BIER, J., MILIOTIS, M., HANES, D., KOTHARY, M., SHAH, D. & FAISAL, M. 1999. *Perkinsus marinus* extracellular protease modulates survival of *Vibrio vulnificus* in eastern oyster (*Crassostrea virginica*) hemocytes. *Applied and environmental microbiology,* 65**,** 4261-4263.

TANG, Y., XIN, G., ZHAO, L.-M., HUANG, L.-X., QIN, Y.-X., SU, Y.-Q., ZHENG, W.-Q., WU, B., LIN, N. & YAN, Q.-P. 2020. Novel insights into host-pathogen interactions of large yellow croakers (*Larimichthys crocea*) and pathogenic bacterium *Pseudomonas plecoglossicida* using time-resolved dual RNA-seq of infected spleens. *Zoological Research,* 41**,** 314.

TANGUY, A., BIERNE, N., SAAVEDRA, C., PINA, B., BACHÈRE, E., KUBE, M., BAZIN, E., BONHOMME, F., BOUDRY, P. & BOULO, V. 2008. Increasing genomic information in bivalves through new EST collections in four species: development of new genetic markers for environmental studies and genome evolution. *Gene,* 408**,** 27-36.

TANGUY, M., GAUTHIER-CLERC, S., PELLERIN, J., DANGER, J. M. & SIAH, A. 2018. The immune response of *Mytilus edulis* hemocytes exposed to *Vibrio splendidus* LGP32 strain: A transcriptomic attempt at identifying molecular actors. *Fish and Shellfish Immunology,* 74**,** 268-280.

TANGUY, M., MCKENNA, P., GAUTHIER-CLERC, S., PELLERIN, J., DANGER, J. M. & SIAH, A. 2013a. Functional and molecular responses in *Mytilus edulis* hemocytes exposed to bacteria, *Vibrio splendidus*. *Developmental and Comparative Immunology,* 39**,** 419-429.

TANGUY, M., MCKENNA, P., GAUTHIER-CLERC, S., PELLERIN, J., DANGER, J. M. & SIAH, A. 2013b. Sequence analysis of a normalized cDNA library of *Mytilus edulis* hemocytes exposed to *Vibrio splendidus* LGP32 strain. *Results in Immunology,* 3**,** 40-50.

TAO, Y., YANG, Z.-Y., ZHANG, X. & WU, H.-J. 2014. Molecular cloning and mRNA expression of the peptidoglycan recognition protein gene HcPGRP1 and its isoform HcPGRP1a from the freshwater mussel Hyriopsis cumingi. *Genetics and Molecular Biology,* 37**,** 508-517.

TAYLOR, A. M., MAHER, W. A. & UBRIHIEN, R. P. 2017. Mortality, condition index and cellular responses of *Anadara trapezia* to combined salinity and temperature stress. *Journal of experimental marine biology and ecology,* 497**,** 172-179.

THOMPSON, C. C., VICENTE, A. C. P., SOUZA, R. C., VASCONCELOS, A. T. R., VESTH, T., ALVES, N., USSERY, D. W., IIDA, T. & THOMPSON, F. L. 2009. Genomic taxonomy of vibrios. *BMC Evolutionary Biology,* 9**,** 258.

THOMPSON, F. L., GEVERS, D., THOMPSON, C. C., DAWYNDT, P., NASER, S., HOSTE, B., MUNN, C. B. & SWINGS, J. 2005a. Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Applied and Environmental Microbiology,* 71**,** 5107-5115.

THOMPSON, F. L., THOMPSON, C. C., NASER, S., HOSTE, B., VANDEMEULEBROECKE, K., MUNN, C., BOURNE, D. & SWINGS, J. 2005b. *Photobacterium rosenbergii* sp. nov. and *Enterovibrio coralii* sp. nov., vibrios associated with coral bleaching. *International Journal of Systematic and Evolutionary Microbiology,* 55**,** 913-917.

THOMPSON, F. L. S., J. 2006. Taxonomy of the Vibrios. *In:* THOMPSON, F. L., AUSTIN, B., & SWINGS, J. (eds.) *The Biology of Vibrios.* American Society for Microbiology.

THOMPSON, J., RANDA, M. A., MARCELINO, L. A., TOMITA-MITCHELL, A., LIM, E. & POLZ, M. F. 2004. Diversity and dynamics of a North Atlantic coastal *Vibrio* community. *Applied and Environmental Microbiology,* 70**,** 4103-4110.

TIAN, Y., LIU, J. & PAN, L. 2020. The mechanism of Mitogen-Activated Protein Kinases to mediate apoptosis and immunotoxicity induced by Benzo [a] pyrene on hemocytes of scallop *Chlamys farreri* in vitro. *Fish & shellfish immunology,* 102**,** 64-72.

TOEWS, S., BEVERLEY-BURTON, M. & LAWRIMORE, T. 1993. Helminth and protist parasites of zebra mussels, *Dreissena polymorpha* (Pallas, 1771), in the Great Lakes region of southwestern Ontario, with comments on associated bacteria. *Canadian Journal of Zoology,* 71**,** 1763-1766.

TOMANEK, L. & ZUZOW, M. J. 2010. The proteomic response of the mussel congeners *Mytilus galloprovincialis* and *M. trossulus* to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. *Journal of Experimental Biology,* 213**,** 3559-3574.

TOUBIANA, M., GERDOL, M., ROSANI, U., PALLAVICINI, A., VENIER, P. & ROCH, P. 2013. Toll-like receptors and MyD88 adaptors in *Mytilus*: complete cds and gene expression levels. *Developmental & Comparative Immunology,* 40**,** 158-166.

TOUBIANA, M., ROSANI, U., GIAMBELLUCA, S., CAMMARATA, M., GERDOL, M., PALLAVICINI, A., VENIER, P. & ROCH, P. 2014. Toll signal transduction pathway in bivalves: complete cds of intermediate elements and related gene transcription levels in hemocytes of immune stimulated *Mytilus galloprovincialis*. *Developmental & Comparative Immunology,* 45**,** 300-312.

TRACY, A. M., WEIL, E. & HARVELL, C. D. 2018. Octocoral co-infection as a balance between host immunity and host environment. *Oecologia,* 186**,** 743-753.

TRAVERS, M.-A., BARBOU, A., LE GOÏC, N., HUCHETTE, S., PAILLARD, C. & KOKEN, M. 2008a. Construction of a stable GFP-tagged *Vibrio harveyi* strain for bacterial dynamics analysis of abalone infection. *FEMS microbiology letters,* 289**,** 34-40.

TRAVERS, M.-A., BOETTCHER MILLER, K., ROQUE, A. & FRIEDMAN, C. S. 2015. Bacterial diseases in marine bivalves. *Journal of Invertebrate Pathology,* 131**,** 11-31.

TRAVERS, M.-A., LE GOÏC, N., HUCHETTE, S., KOKEN, M. & PAILLARD, C. 2008b. Summer immune depression associated with increased susceptibility of the European abalone, *Haliotis tuberculata* to *Vibrio harveyi* infection. *Fish & shellfish immunology,* 25**,** 800-808.

TRAVERS, M.-A., MERSNI ACHOUR, R., HAFFNER, P., TOURBIEZ, D., CASSONE, A.-L., MORGA, B., DOGHRI, I., GARCIA, C., RENAULT, T., FRUITIER-ARNAUDIN, I. & SAULNIER, D. 2014. First description of French *V. tubiashii* strains pathogenic to mollusk: I. Characterization of isolates and detection during mortality events. *Journal of Invertebrate Pathology,* 123**,** 38-48.

TRESNAKOVA, N., FAMULARI, S., ZICARELLI, G., IMPELLITTERI, F., PAGANO, M., PRESTI, G., FILICE, M., CAFERRO, A., GULOTTA, E., SALVATORE, G., SANDOVA, M., VAZZANA, I., IMBROGNO, S., CAPILLO, G., SAVOCA, S., VELISEK, J. & FAGGIO, C. 2023. Multi-characteristic toxicity of enantioselective chiral fungicide tebuconazole to a model organism Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819 (Bivalve: Mytilidae). *Sci Total Environ,* 862**,** 160874.

TRIVEDI, D. K., HOLLYWOOD, K. A. & GOODACRE, R. 2017. Metabolomics for the masses: The future of metabolomics in a personalized world. *New Horizons in Translational Medicine,* 3**,** 294-305.

TRUEBANO, M., BURNS, G., THORNE, M. A., HILLYARD, G., PECK, L. S., SKIBINSKI, D. O. & CLARK, M. S. 2010. Transcriptional response to heat stress in the Antarctic bivalve *Laternula elliptica*. *Journal of Experimental Marine Biology and Ecology,* 391**,** 65-72.

TUGIZIMANA, F., MHLONGO, M., PIATER, L. & DUBERY, I. 2018. Metabolomics in plant priming research: The way forward? *International Journal of Molecular Sciences,* 19**,** 1759.

URBANCZYK, H., AST, J. C. & DUNLAP, P. V. 2011. Phylogeny, genomics, and symbiosis of *Photobacterium*. *FEMS Microbiology Reviews,* 35**,** 324-342.

USHEVA, L., VASCHENKO, M. & DURKINA, V. 2006. Histopathology of the digestive gland of the bivalve mollusk *Crenomytilus grayanus* (Dunker, 1853) from southwestern Peter the Great Bay, Sea of Japan. *Russian Journal of Marine Biology,* 32**,** 166-172.

USHIJIMA, B., MEYER, J. L., THOMPSON, S., PITTS, K., MARUSICH, M. F., TITTL, J., WEATHERUP, E., REU, J., WETZELL, R. & AEBY, G. S. 2020. Disease diagnostics and potential coinfections by *Vibrio coralliilyticus* during an ongoing coral disease outbreak in Florida. *Frontiers in Microbiology,* 11**,** 569354.

VAKILY, J. M. 1989. *The biology and culture of mussels of the genus Perna*, WorldFish.

VAN BAALEN, M. & SABELIS, M. W. 1995. The dynamics of multiple infection and the evolution of virulence. *American Naturalist,* 146**,** 881-910.

VAN BELKUM, A., TASSIOS, P. T., DIJKSHOORN, L., HAEGGMAN, S., COOKSON, B., FRY, N. K., FUSSING, V., GREEN, J., FEIL, E., GERNER-SMIDT, P., BRISSE, S. & STRUELENS, M. 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clinical Microbiology and Infection,* 13**,** 1-46.

VAN DE BRAAK, C., BOTTERBLOM, M., TAVERNE, N. V., VAN MUISWINKEL, W., ROMBOUT, J. & VAN DER KNAAP, W. 2002. The roles of haemocytes and the lymphoid organ in the clearance of injected *Vibrio* bacteria in *Penaeus monodon* shrimp. *Fish & shellfish immunology,* 13**,** 293-309.

VAN DEN BERG, R. A., HOEFSLOOT, H. C., WESTERHUIS, J. A., SMILDE, A. K. & VAN DER WERF, M. J. 2006. Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC genomics,* 7**,** 142.

VAN DER OOST, R., BEYER, J. & VERMEULEN, N. P. E. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environmental Toxicology and Pharmacology,* 13**,** 57-149.

VAN HUNG, N., DE SCHRYVER, P., DUNG, N. V., NEVEJAN, N. & BOSSIER, P. 2019. *Ralstonia eutropha*, containing high poly-β-hydroxybutyrate levels, regulates the immune response in mussel larvae challenged with *Vibrio coralliilyticus*. *Fish and Shellfish Immunology,* 84**,** 196-203.

VAN THOAI, N., HUC, C., PHO, D. B. & OLOMUCKI, A. 1969. Octopine déshydrogénase purification et propriétés catalytiques. *Biochimica et Biophysica Acta (BBA)-Enzymology,* 191**,** 46-57.

VÁZQUEZ-DORADO, S., SANJUAN, A., COMESAÑA, Á. S. & DE CARLOS, A. 2011. Identification of octopine dehydrogenase from *Mytilus galloprovincialis*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 160**,** 94-103.

VÁZQUEZ, J. A., DOCASAL, S., MIRÓN, J., GONZÁLEZ, M. P. & MURADO, M. 2006. Proteases production by two *Vibrio* species on residuals marine media. *Journal of Industrial Microbiology and Biotechnology,* 33**,** 661-668.

VENIER, P., DE PITTÀ, C., BERNANTE, F., VAROTTO, L., DE NARDI, B., BOVO, G., ROCH, P., NOVOA, B., FIGUERAS, A. & PALLAVICINI, A. 2009. MytiBase: a knowledgebase of mussel (*M. galloprovincialis*) transcribed sequences. *BMC genomics,* 10**,** 1-16.

VENIER, P., DOMENEGHETTI, S., SHARMA, N., PALLAVICINI, A. & GERDOL, M. 2016. Immune-related signaling in mussel and bivalves. *Lessons in Immunity.* Elsevier.

VENIER, P., VAROTTO, L., ROSANI, U., MILLINO, C., CELEGATO, B., BERNANTE, F., LANFRANCHI, G., NOVOA, B., ROCH, P., FIGUERAS, A. & PALLAVICINI, A. 2011. Insights into the innate immunity of the Mediterranean mussel *Mytilus galloprovincialis*. *BMC Genomics,* 12.

VENTER, L., ALFARO, A. C., RAGG, N., DELORME, N. & ERICSON, J. A. 2023. The effect of simulated marine heatwaves on green-lipped mussels, *Perna canaliculus*: A near-natural experimental approach. *Journal of Thermal Biology,* Under review.

VENTER, L., ALFARO, A. C., VAN NGUYEN, T. & LINDEQUE, J. Z. 2022. Metabolite profiling of abalone (*Haliotis iris*) energy metabolism: a Chatham Islands case study. *Metabolomics,* 18**,** 52.

VENTER, L., LOOTS, D. T., MIENIE, L. J., JANSEN VAN RENSBURG, P. J., MASON, S., VOSLOO, A. & LINDEQUE, J. Z. 2018a. Uncovering the metabolic response of abalone (*Haliotis midae*) to environmental hypoxia through metabolomics. *Metabolomics,* 14**,** 1-12.

VENTER, L., LOOTS, D. T., VOSLOO, A., JANSEN VAN RENSBURG, P. & LINDEQUE, J. Z. 2018b. Abalone growth and associated aspects: now from a metabolic perspective. *Reviews in Aquaculture,* 10**,** 451-473.

VENTER, L., MIENIE, L. J., VOSLOO, A., LOOTS, D. T., JANSEN VAN RENSBURG, P. & LINDEQUE, J. Z. 2019. Effect of proline-enriched abalone feed on selected metabolite levels of slow-growing adult *Haliotis midae*. *Aquaculture Research,* 50**,** 1057-1067.

VENTER, L., YOUNG, T., ALFARO, A. C. & LINDEQUE, J. Z. 2021. Establishing sampling confidence parameters: Effect of sampling and transport conditions on haemocyte and metabolite profiles of Greenshell mussels. *Aquaculture,* 538**,** 736538.

VERBRUGGHE, E., BOYEN, F., GAASTRA, W., BEKHUIS, L., LEYMAN, B., VAN PARYS, A., HAESEBROUCK, F. & PASMANS, F. 2012. The complex interplay between stress and bacterial infections in animals. *Veterinary microbiology,* 155**,** 115-127.

VERLECAR, X., JENA, K. & CHAINY, G. 2007. Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature. *Chemico-biological interactions,* 167**,** 219-226.

VEZZULLI, L., COLWELL, R. R. & PRUZZO, C. 2013. Ocean warming and spread of pathogenic vibrios in the aquatic environment. *Microbial Ecology,* 65**,** 817-825.

VEZZULLI, L., STAGNARO, L., GRANDE, C., TASSISTRO, G., CANESI, L. & PRUZZO, C. 2018. Comparative 16SrDNA gene-based microbiota profiles of the Pacific Oyster (*Crassostrea gigas)* and the Mediterranean Mussel (*Mytilus galloprovincialis*) from a shellfish farm (Ligurian Sea, Italy). *Microbial Ecology,* 75**,** 495-504.

VIDAL-LIÑÁN, L., BELLAS, J., SALGUEIRO-GONZÁLEZ, N., MUNIATEGUI, S. & BEIRAS, R. 2015. Bioaccumulation of 4-nonylphenol and effects on biomarkers, acetylcholinesterase, glutathione-S-transferase and glutathione peroxidase, in *Mytilus galloprovincialis* mussel gills. *Environmental Pollution,* 200**,** 133-139.

WALLER, D. L. & COPE, W. G. 2019. The status of mussel health assessment and a path forward. *Freshwater Mollusk Biology and Conservation,* 22**,** 26.

WAN, Y., LIU, C. & MA, Q. 2019a. Structural analysis of a *Vibrio* phospholipase reveals an unusual Ser–His–chloride catalytic triad. *Journal of Biological Chemistry,* 294**,** 11391-11401.

WAN, Y., LIU, C. & MA, Q. 2019b. Structural analysis of a *Vibrio phospholipase* reveals an unusual Ser–His–chloride catalytic triad. *Journal of Biological Chemistry,* 294**,** 11391-11401.

WANG, C., ZHAO, J., MU, C., WANG, Q., WU, H. & WANG, C. 2013a. cDNA cloning and mRNA expression of four glutathione S-transferase (*GST*) genes from *Mytilus galloprovincialis*. *Fish and Shellfish Immunology,* 34**,** 697-703.

WANG, D., LOOR, A., BELS, L. D., STAPPEN, G. V., BROECK, W. V. D. & NEVEJAN, N. 2021a. Dynamic immune response to vibriosis in Pacific oyster *Crassostrea gigas* larvae during the infection process as supported by accurate positioning of GFP-tagged Vibrio strains. *Microorganisms,* 9**,** 1523.

WANG, D., MBEWE, N., DE BELS, L., COUCK, L., VAN STAPPEN, G., VAN DEN BROECK, W. & NEVEJAN, N. 2021b. Pathogenesis of experimental vibriosis in blue mussel (*Mytilus edulis*) larvae based on accurate positioning of GFP-tagged *Vibrio* strains and histopathological and ultrastructural changes of the host. *Aquaculture,* 535**,** e736347.

WANG, J. 2018. Neutrophils in tissue injury and repair. *Cell and tissue research,* 371**,** 531-539.

WANG, J., DONG, B., YU, Z.-X. & YAO, C.-L. 2018a. The impact of acute thermal stress on green mussel *Perna viridis*: oxidative damage and responses. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 222**,** 7-15.

WANG, L., QIU, L., ZHOU, Z. & SONG, L. 2013b. Research progress on the mollusc immunity in China. *Developmental & Comparative Immunology,* 39**,** 2-10.

WANG, L., SONG, X. & SONG, L. 2018b. The oyster immunity. *Developmental & Comparative Immunology,* 80**,** 99-118.

WANG, M., SU, X., LI, Y., JUN, Z. & LI, T. 2010. Cloning and expression of the *Mn-SOD* gene from *Phascolosoma esculenta*. *Fish and Shellfish Immunology,* 29.

WANG, Q., YUAN, Z., WU, H., LIU, F. & ZHAO, J. 2013c. Molecular characterization of a manganese superoxide dismutase and copper/zinc superoxide dismutase from the mussel *Mytilus galloprovincialis*. *Fish and Shellfish Immunology,* 34.

WANG, W., CUI, J., MA, H., LU, W. & HUANG, J. 2021c. Targeting pyrimidine metabolism in the era of precision cancer medicine. *Frontiers in Oncology,* 11**,** 684961.

WANG, Y., HU, M., SHIN, P. K. S. & CHEUNG, S. G. 2011. Immune responses to combined effect of hypoxia and high temperature in the green-lipped mussel *Perna viridis*. *Marine Pollution Bulletin,* 63**,** 201-208.

WANG, Y., LI, L., HU, M. & LU, W. 2015. Physiological energetics of the thick shell mussel *Mytilus coruscus* exposed to seawater acidification and thermal stress. *Science of the Total Environment,* 514**,** 261-272.

WEBB, S. & DUNCAN, J. 2019. New Zealand shellfish health monitoring 2007 to 2017: insights and projections. Cawthron Report No. 2568. *Cawthron Report*

WEBB, S., GAW, S., MARSDEN, I. & MCRAE, N. 2020. Biomarker responses in New Zealand green-lipped mussels *Perna canaliculus* exposed to microplastics and triclosan. *Ecotoxicology and Environmental Safety,* 201**,** 110871.

WEI, H., WANG, H., TANG, L., MU, C., YE, C., CHEN, L. & WANG, C. 2019. High-throughput sequencing reveals the core gut microbiota of the mud crab (*Scylla paramamosain*) in different coastal regions of southern China. *BMC Genomics,* 20**,** 829.

WEIHRAUCH, D. & ALLEN, G. J. P. 2018. Ammonia excretion in aquatic invertebrates: New insights and questions. *Journal of Experimental Biology,* 221**,** 1-12.

WENDLING, C. C. & WEGNER, K. M. 2013. Relative contribution of reproductive investment, thermal stress and *Vibrio* infection to summer mortality phenomena in Pacific oysters. *Aquaculture,* 412**,** 88-96.

WENNING, R. J. & DI GIULIO, R. T. 1988. The effects of paraquat on microsomal oxygen reduction and antioxidant defenses in ribbed mussels (*Geukensia demissa*) and wedge clams (*Rangia cuneata*). *Marine environmental research,* 24**,** 301-305.

WESTERMANN, A. J., GORSKI, S. A. & VOGEL, J. 2012. Dual RNA-seq of pathogen and host. *Nature Reviews Microbiology,* 10**,** 618-630.

WICKHAM, H., CHANG, W. & WICKHAM, M. H. 2016. Package 'ggplot2'. *Create elegant data visualisations using the grammar of graphics. Version,* 2**,** 1-189.

WICKHAM, H. & WICKHAM, M. H. 2017. Package tidyverse. *Easily Install and Load the 'Tidyverse*.

WINLOW, W., POLESE, G., MOGHADAM, H.-F., AHMED, I. A. & DI COSMO, A. 2018. Sense and insensibility – An appraisal of the effects of clinical anesthetics on gastropod and cephalopod molluscs as a step to improved welfare of cephalopods. *Frontiers in Physiology,* 9**,** 1-24.

WONG, H.-C., PENG, P.-Y., LAN, S.-L., CHEN, Y.-C., LU, K.-H., SHEN, C.-T. & LAN, S.-F. 2002. Effects of heat shock on the thermotolerance, protein composition, and toxin production of *Vibrio parahaemolyticus*. *Journal of Food Protection,* 65**,** 499-507.

WORLD HEALTH, O. & INTERNATIONAL PROGRAMME ON CHEMICAL, S. Biomarkers and risk assessment : concepts and principles / published under the joint sponsorship of the United Nations environment Programme, the International Labour Organisation, and the World Health Organization. 1993 1993 Geneva. World Health Organization.

WU, F., LU, W., SHANG, Y., KONG, H., LI, L., SUI, Y., HU, M. & WANG, Y. 2016. Combined effects of seawater acidification and high temperature on hemocyte parameters in the thick shell mussel *Mytilus coruscus*. *Fish and Shellfish Immunology,* 56**,** 554-562.

WU, F., TANG, K., YUAN, M., SHI, X., SHAKEELA, Q. & ZHANG, X. H. 2015. Studies on bacterial pathogens isolated from diseased torafugu (*Takifugu rubripes*) cultured in marine industrial recirculation aquaculture system in Shandong Province, China. *Aquaculture Research,* 46**,** 736-744.

WU, G. 2009. Amino acids: metabolism, functions, and nutrition. *Amino acids,* 37**,** 1-17.

WU, H., JI, C., WEI, L., ZHAO, J. & LU, H. 2013. Proteomic and metabolomic responses in hepatopancreas of *Mytilus galloprovincialis* challenged by *Micrococcus luteus* and *Vibrio anguillarum*. *Journal of Proteomics,* 94**,** 54-67.

WU, H. & WANG, W. X. 2011. Tissue-specific toxicological effects of cadmium in green mussels (*Perna viridis*): Nuclear magnetic resonance-based metabolomics study. *Environmental Toxicology and Chemistry,* 30**,** 806-812.

WU, J., BAO, M., GE, D., HUO, L., LV, Z., CHI, C., LIAO, Z. & LIU, H. 2017. The expression of superoxide dismutase in *Mytilus coruscus* under various stressors. *Fish and Shellfish Immunology,* 70**,** 361-371.

WU, S., CHEN, W., LU, S., ZHANG, H. & YIN, L. 2022. Metabolic engineering of shikimic acid biosynthesis pathway for the production of shikimic acid and its branched products in microorganisms: Advances and prospects. *Molecules,* 27**,** 4779.

XIE, S. W., LI, Y. T., ZHOU, W. W., TIAN, L. X., LI, Y. M., ZENG, S. L. & LIU, Y. J. 2017. Effect of γ-aminobutyric acid supplementation on growth performance, endocrine hormone and stress tolerance of juvenile Pacific white shrimp, *Litopenaeus vannamei*, fed low fishmeal diet. *Aquaculture Nutrition,* 23**,** 54-62.

XIONG, F., XIONG, J., WU, Y. F., CAO, L., HUANG, W. S. & CHANG, M. X. 2020. Time-resolved RNA-seq provided a new understanding of intestinal immune response of European eel (*Anguilla anguilla*) following infection with *Aeromonas hydrophila*. *Fish & Shellfish Immunology,* 105**,** 297-309.

XU, F., GAO, T. & LIU, X. 2020a. Metabolomics adaptation of juvenile pacific abalone *Haliotis discus hannai* to heat stress. *Scientific Reports,* 10**,** 1-11.

XU, K., ZHANG, Z., XU, Z., TANG, Z., LIU, L., LU, Z. & QI, P. 2019. A novel invertebrate toll-like receptor is involved in TLR mediated signal pathway of thick shell mussel *Mytilus coruscus*. *Developmental & Comparative Immunology,* 97**,** 11-19.

XU, M.-Y., WANG, P., SUN, Y.-J., YANG, L. & WU, Y.-J. 2020b. Identification of metabolite biomarkers in serum of rats exposed to chlorpyrifos and cadmium. *Scientific Reports.*

XU, M., WU, J., GE, D., WU, C., CHI, C., LV, Z., LIAO, Z. & LIU, H. 2018. A novel toll-like receptor from *Mytilus coruscus* is induced in response to stress. *Fish and Shellfish Immunology,* 78**,** 331-337.

YANG, H., XU, Z., GUO, B., ZHANG, X., LIAO, Z., QI, P. & YAN, X. 2021. Integrated analysis of miRNAome and transcriptome reveals miRNA-mRNA network regulation in *Vibrio alginolyticus* infected thick shell mussel *Mytilus coruscus*. *Molecular Immunology,* 132**,** 217-226.

YANG, J., HE, J., LIU, L., HE, M., ZHANG, X., BUTTINO, I., GUO, B., YAN, X. & LIAO, Z. 2022. Expression profiles of antimicrobial peptides in *Mytilus coruscus*. *Aquaculture,* 548**,** 737709.

YANG, Z., LI, J., LI, Y., WU, H. & WANG, X. 2013. Molecular cloning and functional characterization of a short peptidoglycan recognition protein (HcPGRPS1) from the freshwater mussel, *Hyriopsis cumingi*. *Molecular Immunology,* 56**,** 729-738.

YAO, C.-L. & SOMERO, G. N. 2012. The impact of acute temperature stress on hemocytes of invasive and native mussels (*Mytilus galloprovincialis* and *Mytilus californianus*): DNA damage, membrane integrity, apoptosis and signaling pathways. *Journal of Experimental Biology,* 215**,** 4267-4277.

YE, Y., GAO, J., JIAO, R., LI, H., WU, Q., ZHANG, J. & ZHONG, X. 2015. The membrane proteins involved in virulence of *Cronobacter sakazakii* virulent G362 and attenuated L3101 isolates. *Frontiers in microbiology,* 6**,** 1238.

YILDIZ, F. H. & VISICK, K. L. 2009. *Vibrio* biofilms: so much the same yet so different. *Trends in Microbiology,* 17**,** 109-118.

YOON, K., MIN, K., JUNG, Y., KWON, K., LEE, J. & OH, S. 2008. A model of the effect of temperature on the growth of pathogenic and nonpathogenic *Vibrio parahaemolyticus* isolated from oysters in Korea. *Food microbiology,* 25**,** 635-641.

YOUNG, T. & ALFARO, A. C. 2018. Metabolomic strategies for aquaculture research: a primer. *Reviews in Aquaculture,* 10**,** 26-56.

YOUNG, T., ALFARO, A. C. & VILLAS-BÔAS, S. 2015. Identification of candidate biomarkers for quality assessment of hatchery-reared mussel larvae via GC/MS-based metabolomics. *New Zealand Journal of Marine and Freshwater Research,* 49**,** 87-95.

YOUNG, T., ALFARO, A. C. & VILLAS-BÔAS, S. G. 2016. Metabolic profiling of mussel larvae: effect of handling and culture conditions. *Aquaculture International,* 24**,** 843-856.

YOUNG, T., KESARCODI-WATSON, A., ALFARO, A. C., MERIEN, F., NGUYEN, T. V., MAE, H., LE, D. V. & VILLAS-BÔAS, S. 2017. Differential expression of novel metabolic and immunological biomarkers in oysters challenged with a virulent strain of OsHV-1. *Developmental & Comparative Immunology,* 73**,** 229-245.

YOUNG, T., WALKER, S. P., ALFARO, A. C., FLETCHER, L. M., MURRAY, J. S., LULIJWA, R. & SYMONDS, J. 2019. Impact of acute handling stress, anaesthesia, and euthanasia on fish plasma biochemistry: implications for veterinary screening and metabolomic sampling. *Fish Physiology and Biochemistry,* 45**,** 1485-1494.

YU, J., WANG, H., YUE, X. & LIU, B. 2019. Dynamic immune and metabolism response of clam *Meretrix petechialis* to Vibrio challenge revealed by a time series of transcriptome analysis. *Fish & shellfish immunology,* 94**,** 17-26.

ZANNELLA, C., MOSCA, F., MARIANI, F., FRANCI, G., FOLLIERO, V., GALDIERO, M., TISCAR, P. G. & GALDIERO, M. 2017. Microbial diseases of bivalve mollusks: infections, immunology and antimicrobial defense. *Marine drugs,* 15**,** 182.

ZARE JEDDI, M., HOPF, N. B., VIEGAS, S., PRICE, A. B., PAINI, A., VAN THRIEL, C., BENFENATI, E., NDAW, S., BESSEMS, J., BEHNISCH, P. A., LENG, G., DUCA, R. C., VERHAGEN, H., CUBADDA, F., BRENNAN, L., ALI, I., DAVID, A., MUSTIELES, V., FERNANDEZ, M. F., LOURO, H. & PASANEN-KASE, R. 2021. Towards a systematic use of effect biomarkers in population and occupational biomonitoring. *Environment International,* 146**,** 106257.

ZENG, Z., WANG, Y., ANWAR, M., HU, Z., WANG, C., LOU, S. & LI, H. 2022. Molecular cloning and expression analysis of mytilin-like antimicrobial peptides from Asian green mussel *Perna viridis*. *Fish & Shellfish Immunology,* 121**,** 239-244.

ZHA, H., JEFFS, A., DONG, Y. & LEWIS, G. 2018. Potential virulence factors of bacteria associated with tail fan necrosis in the spiny lobster,*Jasus edwardsii*. *Journal of Fish Diseases,* 41**,** 817-828.

ZHAI, S., YANG, B., ZHANG, F., LI, Q. & LIU, S. 2021. Estimation of genetic parameters for resistance to *Vibrio alginolyticus* infection in the Pacific oyster (*Crassostrea gigas*). *Aquaculture,* 538.

ZHANG, C., HE, J., WANG, X., YANG, Y., HUANG, Q., QIAO, F., SHI, Q., QIN, J. & CHEN, L. 2022. Gamma-aminobutyric acid enhances hypoxia tolerance of juvenile Chinese mitten crab (*Eriocheir sinensis*) by regulating respiratory metabolism and alleviating neural excitotoxicity. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 260**,** 109409.

ZHANG, H. W., HUANG, Y., MAN, X., WANG, Y., HUI, K. M., YIN, S. W. & ZHANG, X. W. 2017. HcToll3 was involved in anti-*Vibrio* defense in freshwater pearl mussel, *Hyriopsis cumingii*. *Fish and Shellfish Immunology,* 63.

ZHANG, S., YANG, Q., EGGERMONT, M. & DEFOIRDT, T. 2023a. Quorum-sensing interference in vibrios. *Reviews in Aquaculture***,** 1-15.

ZHANG, X. H. & AUSTIN, B. 2005. Haemolysins in *Vibrio* species. *Journal of Applied Microbiology,* 98**,** 1011-1019.

ZHANG, Y.-Y., YU, S.-E., WANG, W.-L., YANG, L.-E., LU, Q.-Q., XIE, C.-T., LIU, T. & DONG, Y.-W. 2023b. Temperature sensitivity of marine macroalgae for aquaculture in China. *Aquaculture***,** 739262.

ZHANG, Y., HU, L., OSEI-ADJEI, G., ZHANG, Y., YANG, W., YIN, Z., LU, R., SHENG, X., YANG, R., HUANG, X. & ZHOU, D. 2018. Autoregulation of ToxR and its regulatory actions on major virulence gene loci in *Vibrio parahaemolyticus*. *Frontiers in Cellular and Infection Microbiology,* 8**,** 1-12.

ZHANG, Z., ZHOU, C., FAN, K., ZHANG, L., LIU, Y. & LIU, P.-F. 2021. Metabolomics analysis of the effects of temperature on the growth and development of juvenile European seabass (*Dicentrarchus labrax*). *Science of The Total Environment,* 769**,** 145155.

ZHAO, J., LI, C., CHEN, A., LI, L., SU, X. & LI, T. 2010a. Molecular characterization of a novel big defensin from clam *Venerupis philippinarum*. *PLoS ONE,* 5**,** e13480.

ZHAO, J., QIU, L., NING, X., CHEN, A., WU, H. & LI, C. 2010b. Cloning and characterization of an invertebrate type lysozyme from *Venerupis philippinarum*. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology,* 156**,** 56-60.

ZHAO, X., SUN, S., SHI, W., SUN, X., ZHANG, Y., ZHU, L., SUI, Q., XIA, B., QU, K. & CHEN, B. 2021. Mussel byssal attachment weakened by anthropogenic noise. *Frontiers in Marine Science,* 8**,** 1957.

ZHOU, C. 2012. General anesthesia mediated by effects on ion channels. *World Journal of Critical Care Medicine,* 1**,** 80.

ZHU, J., MILLER, M. B., VANCE, R. E., DZIEJMAN, M., BASSLER, B. L. & MEKALANOS, J. J. 2002. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences,* 99**,** 3129-3134.

ZHUKOVA, N. V. 2019. Fatty acids of marine mollusks: Impact of diet, bacterial symbiosis and biosynthetic potential. *Biomolecules,* 9**,** 857.

ZILBERBERG, C., SERENO, D., LIMA, G., CUSTÓDIO, M. R. & LÔBO-HAJDU, G. 2011. Effect of mussel's gender and size on a stress response biomarker. *Water, Air, & Soil Pollution,* 217**,** 317-320.

ZOBELL, C. E. 1941. Studies on marine bacteria, I: the cultural requirements of heterotrophic aerobes. *Journal of Marine Research,* 4**,** 42-75.

ZURBURG, W. & DE ZWAAN, A. 1981. The role of amino acids in anaerobiosis and osmoregulation in bivalves. *Journal of Experimental Zoology,* 215**,** 315-325.

Appendices

SUPPLEMENTARY MATERIAL FROM CHAPTER 3

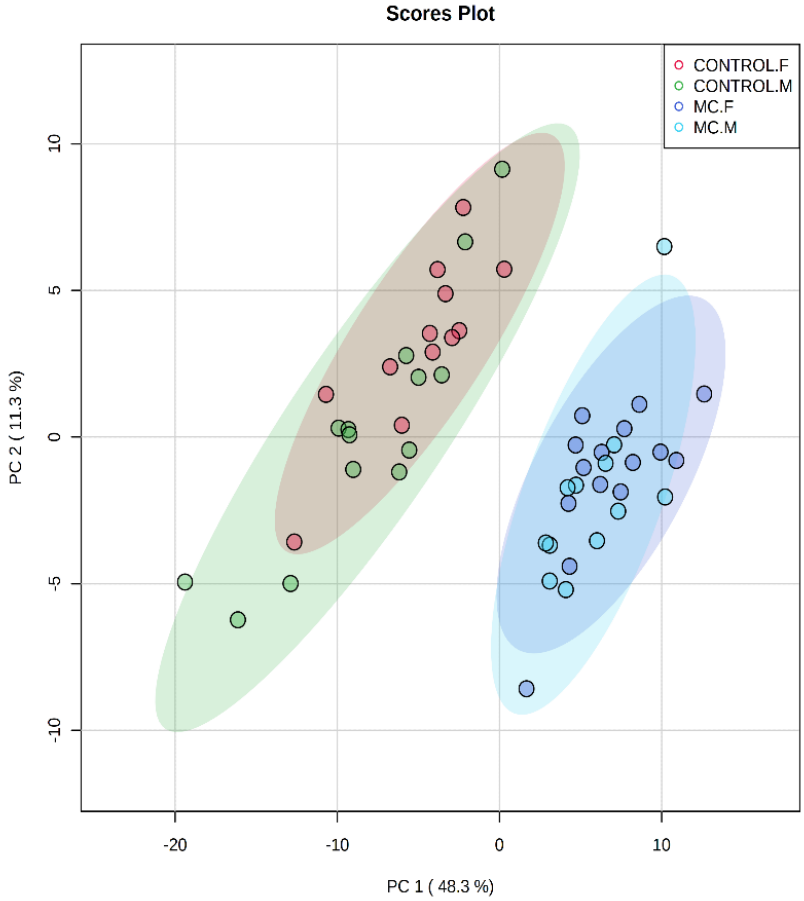

Figure S1. Overview of mussel haemolymph metabolites between male and female mussels collected from control and $MgCl_2$ (MC) treated specimens as a PCA score plot.

SUPPLEMENTARY MATERIAL FROM CHAPTER 4

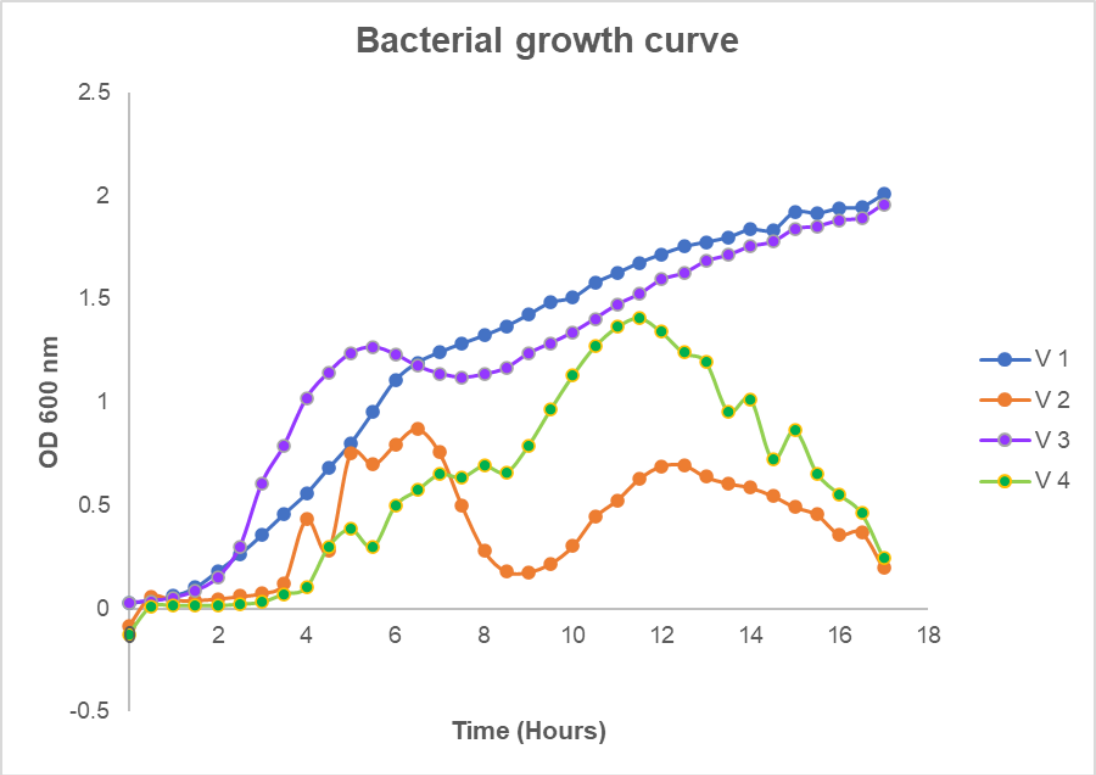

Figure S1: Growth curve analysis of four bacteria isolated in this study in marine broth at 22°C (average ± standard deviation of 3 replicates).

Table S1. qPCR Primers used for identification and genotyping of the four bacterial isolates

Primer target	Primer type	Sequence (5′–3′)
elf	**Forward**	ATGGACCTGGAAGTAGTGCC
elf	**Reverse**	CGAACCAAAGAAACAACGCC
fur	**Forward**	CTGAAACAAGCGGGTCTGAA
fur	**Reverse**	CCACAATCCAGACACACCAG
hsp60	**Forward**	TCGTGGTTATCTGAGCCCAT
hsp60	**Reverse**	ACGACGATCACCAAAACCTG
zm	**Forward**	TTCAAGATCTGGGTCATCGTC
zm	**Reverse**	CAATTTCATGCGCCACCAC
vcpA	**Forward**	TACGCAGGGTCACATTTTCG
vcpA	**Reverse**	CACTTGCGCGGTTTGATTTT
toxR	**Forward**	AGCCCAGTGAATCATCCAGA
toxR	**Reverse**	TACGCAGGGTCACATTTTCG
ompU	**Forward**	TGACCGATAATAGCCGTGTG
ompU	**Reverse**	GCCGCAATTTTATTACCCGC
chi	**Forward**	TGCGTACCCCACAAATTAGC

chi	**Reverse**	GGTTTCCGCTTCCAGTTCAT
mshA	**Forward**	CCTGATTGAACTGGTGGTGG
mshA	**Reverse**	ATCGCCGCTTTACCATACAC
lip	**Forward**	GTGATGATGCGACCACCATT
lip	**Reverse**	TCGCCATATTCGCTTGTTCC
plp	**Forward**	TGGTGGATGTGAAATGGCTG
plp	**Reverse**	ACATGGGGTTTCACGATACG

Table S2. API 20 E and API 20 NE analysis.

API 20 E			
Isolate	**Profile**	**Api database match**	(Buller, 2004)**, Table 3.26**
V1	200600456	Not done	"200604 "*Pseudomonas aeuriginosa*
V2	324414556	Not done	"324414557" *Vibrio splendidus*
V3	200622454	Not done	No match
V4	304612556	Not done	Exact match, *Vibrio splendidus*
API 20 NE			
Isolate	**Profile**	**API database match**	(Buller, 2004)**, Table 3.26**
V1	5572344	Acceptable identification *Vibrio fluvialis*	No match
V2	7566345	Not done	No match
V3	3452344	Very good ID *Vibrio alginolyticus*	No match
V4	7576745	Very good ID to the genus *Vibrio fluvialis*	No match

Table S3. Survival analysis on spawning and sex effects on mussel mortality using Chi-square test analysis (p-value<0.001).

Strain	**Spawn versus Mortality**	**Sex versus Mortality**
V1	<0.001	0.195
V2	<0.001	0.311
V3	<0.001	0.227
V4	<0.001	0.416
Overall/stacked	<0.001	0.626

Table S4. The 16S rDNA consensus Sanger sequences in fasta format for isolates V1-V4.

Bacterial ID	Consensus sequence
V1	GGCCTTTCCGGGTTGGAAAAGTACTTTCAGCAGTGAGGAAGGTGGTAGTGTTAATAGCATTATCATTTG ACGTTAGCTGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAACCGGGGTAATAAGGAGGGTGCGAGC GTAAATCGGAATTACTGGGCGTAAAGCGCATGGATGGGGACTGTTAAGCAAGAAGGGAAAGCCCGGG GCTCTACCCCGGAAACGCATGTTGAAATGGCAGAAAAAAATCTTGGAAAGGGGGGTAAAAATTTTTGT GTAGCGGGCCAATGCTTTAACATAATAAGGAATAACGGTGGGTAGGGCGGGCCCCTGGAACAAGACTG GACGCT
V2	AAGAAGGCCTTCGGGTTGGTAAAGTACTTTCAGTTGTGAGGAAGGCGTTGTCGTTAATAGCGGCAGTGT TTGACGTTAGCAACAAAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCG AGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGCGGCTTGTTAAGCCTAGATGTGAAAGCCC GGGGCTC
V3	GAAGAAGGCCTTCGGGTTGAAAAGCACTTTCAGTCGTGAGGAAGGGTGTGCAGTTAATAGCTGTACATT TTGACGTTAGCGACARAAGAAGCACCGGCTAACTCCGGGCCAGCAACCCCGGTAATACGGAGGGTGCG AGCGTTAATCGGAATTACTGGGGGTAAAGCGCAAGCAGGCGGTCTGTTAAGCAAGATGTGAAAGCCCG GGGCTCTACCCCCGAACAGCATTTTGAACTGGCAAACTAAAATTTTGTAAAAGGGGGGAAAATATCAGG GGTAAAGGGGAAAAGCCGTAAAAATTTAAAGAAATAACGGCGGGTAAAGCCGCACCCTGGACAAAAA ATGACGCTCAGAATTGGAAACGTTGTGGACCAACA
V4	TGGAAAAGTAGCTTTCAGTTGTGAGGAAGGGGGTAGCGTGAATAGCGCTATCTCTTGACGTTAGCAACA AAAGAGGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAAT TACTGGGCGTAAAGCGCATGCAGGTGGTTCATTAAGTCAAATGTGAAAGCCCGGGGCTCAACCTCGGA ACTGCATTTGAAACTGGTGAACTAGATTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAAT GCGT

Table S5. Variation (standard deviation of the $2^{-\Delta\Delta CT}$ values) between the different strains in the expression levels of the 9 virulence-associated genes.

Genes	Variation
hsp60	0.142
zm	0.032
vcpA	0.082
toxR	0.129
ompU	0.112
mshA	0.190
chi	0.126
lip	0.115
plp	0.078

SUPPLEMENTARY MATERIAL FROM CHAPTER 7

Table S1: Metabolomics findings of *Perna canaliculus* haemolymph metabolites with an interaction effect between temperature and time. Compounds are listed in alphabetical order with a p-value, a Kyoto Encyclopaedia of Genes and Genomes (KEGG) identification number and metabolite class grouping visible in the table.

Metabolite	p-value	KEGG ID	Metabolite class
2-Deoxyadenosine 5-diphosphate	4.48E-05	C00206	Purine nucleotides
2-Deoxycytidine	2.86E-03	C00881	Pyrimidine nucleosides
2-Deoxycytidine 5-diphosphate	1.73E-08	C00705	Organic oxoanionic compounds
2-Deoxy-D-ribose	1.80E-03	Not Available	Organooxygen compounds
2-Deoxyguanosine 5-diphosphate	1.40E-05	C00361	Cinnamic acids and derivatives
2-Deoxyguanosine 5-monophosphate	3.23E-04	C00362	Purine nucleotides
2-Deoxyuridine	8.08E-07	C00526	Pyrimidine nucleosides
2-Methyl-1-butanol	4.48E-05	Not Available	Organooxygen compounds
3-Methylglutaric acid	6.17E-03	Not Available	Fatty Acyls
4-Methyl-2-oxovaleric acid	2.24E-08	Not Available	Keto acids and derivatives
5-Hydroxy-3-indoleacetic acid	6.17E-03	C05635	Indoles and derivatives
Adenosine 3-5-cyclic monophosphate	4.48E-05	C00575	Purine nucleotides
Adenosine 5-diphosphate	1.73E-04	C00008	Purine nucleotides
Adenosine 5-monophosphate	1.37E-04	C00020	Purine nucleotides
Adenylosuccinic acid	3.31E-03	Not Available	Purine nucleotides
AICAR	7.38E-04	C04677	Imidazole ribonucleosides and ribonucleotides
alpha-D-Glucose-1-phosphate	1.80E-06	C00103	Organooxygen compounds
alpha-Ketoglutaric acid	7.91E-11	C00026	Keto acids and derivatives
Arabinose-5-phosphate	3.31E-03	C01112	Organooxygen compounds
beta-Nicotinamide mononucleotide	7.78E-06	C00455	Pyridine nucleotides
Cellobiose	2.23E-06	C06422	Organooxygen compounds
cis-Aconitic acid	8.47E-05	C00417	Carboxylic acids and derivatives
Cytidine	8.80E-03	C00475	Pyrimidine nucleosides
Cytidine-5-monophosphate	2.00E-04	C00055	Pyrimidine nucleotides
Cytosine	4.27E-04	C00380	Diazines
D-+-Galactosamine	2.02E-04	Not Available	Organooxygen compounds
Deoxythymidine 5-triphosphate	3.31E-03	C00459	Pyrimidine nucleotides
D-Fructose 1-6-biphosphate	4.48E-05	C05378	Organooxygen compounds
D-pantothenic acid	3.31E-03	C00864	Organooxygen compounds
D-Xylulose-5-phosphate	1.73E-04	C00231	Organooxygen compounds
gamma-Aminobutyric acid	7.78E-06	C00334	Carboxylic acids and derivatives

Glyceric acid	6.17E-03	C00258	Organooxygen compounds
Guanosine	4.48E-05	C00387	Purine nucleosides
Inosine	6.17E-03	C00294	Purine nucleosides
Inosine 5-diphosphate	8.47E-05	C00104	Purine nucleotides
Isopentyl acetate	2.23E-06	C12296	Carboxylic acids and derivatives
Itaconic acid	1.98E-08	C00490	Fatty Acyls
Lactic acid	1.54E-07	C00186	Hydroxy acids and derivatives
L-Arabinose	7.78E-06	C02479	Organooxygen compounds
L-Citrulline	6.79E-04	C00327	Carboxylic acids and derivatives
L-Dihydroorotic acid	6.20E-06	C00337	Carboxylic acids and derivatives
L-Gluthathione (oxidized)	3.80E-06	C00051	Carboxylic acids and derivatives
L-Malic acid	2.67E-03	C00149	Hydroxy acids and derivatives
L-Methionine	8.08E-07	C00073	Carboxylic acids and derivatives
L-Phenylalanine	3.31E-03	C00079	Carboxylic acids and derivatives
L-Tryptophan	3.31E-03	C00078	Indoles and derivatives
Maleic acid	1.78E-04	C01384	Carboxylic acids and derivatives
Mevalonic acid 5-phosphate	1.76E-03	C01107	Organic phosphoric acids and derivatives
m-Hydroxybenzoic acid	3.31E-03	C00587	Benzene and substituted derivatives
N-Acetylglutamic acid	4.41E-05	C00624	Carboxylic acids and derivatives
Nicotinic acid	1.18E-04	C00253	Pyridines and derivatives
Nicotinic acid mononucleotide	3.31E-03	C01185	Pyridine nucleotides
o-Hydroxy hippuric acid	5.47E-04	Not Available	Benzene and substituted derivatives
Riboflavin	1.30E-03	C00255	Pteridines and derivatives
Shikimic acid	1.73E-04	C00493	Organooxygen compounds
Succinic acid	6.08E-04	C00042	Carboxylic acids and derivatives
Succinic semialdehyde	6.92E-10	C00232	Fatty Acyls
Taurine	8.80E-03	C00245	Organic sulfonic acids and derivatives
trans-Aconitic acid	3.31E-03	C02341	Carboxylic acids and derivatives
Uridine 5-diphosphate	4.48E-05	C00015	Pyrimidine nucleotides
Xylitol	8.80E-03	C00379	Organooxygen compounds

SUPPLEMENTARY MATERIA FROM CHAPTER 9

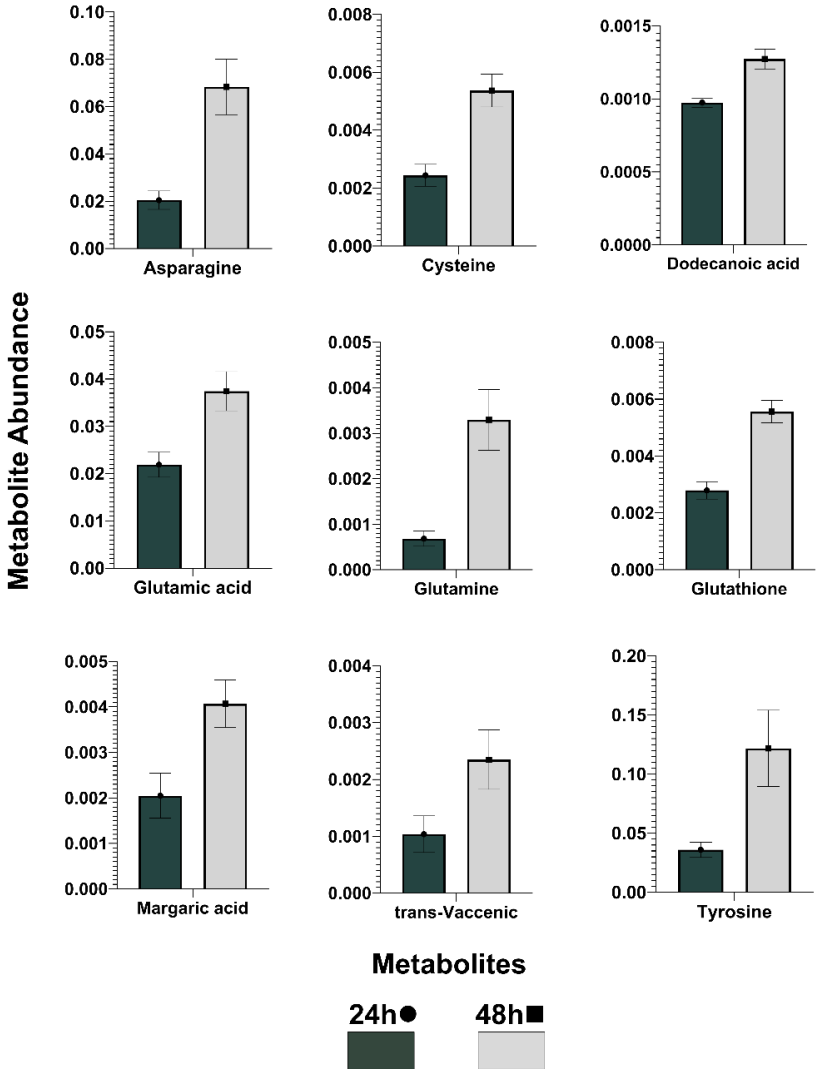

Fig S1: Metabolite differences of *Perna canaliculus* haemolymph relating to experimental time following injection with marine broth.

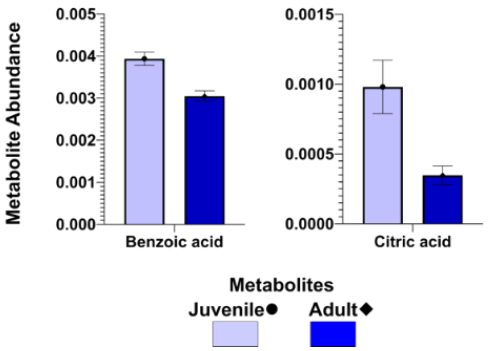

Fig S2: Metabolite differences of *Perna canaliculus* haemolymph life stage following injection with *Vibrio mediterranei.*

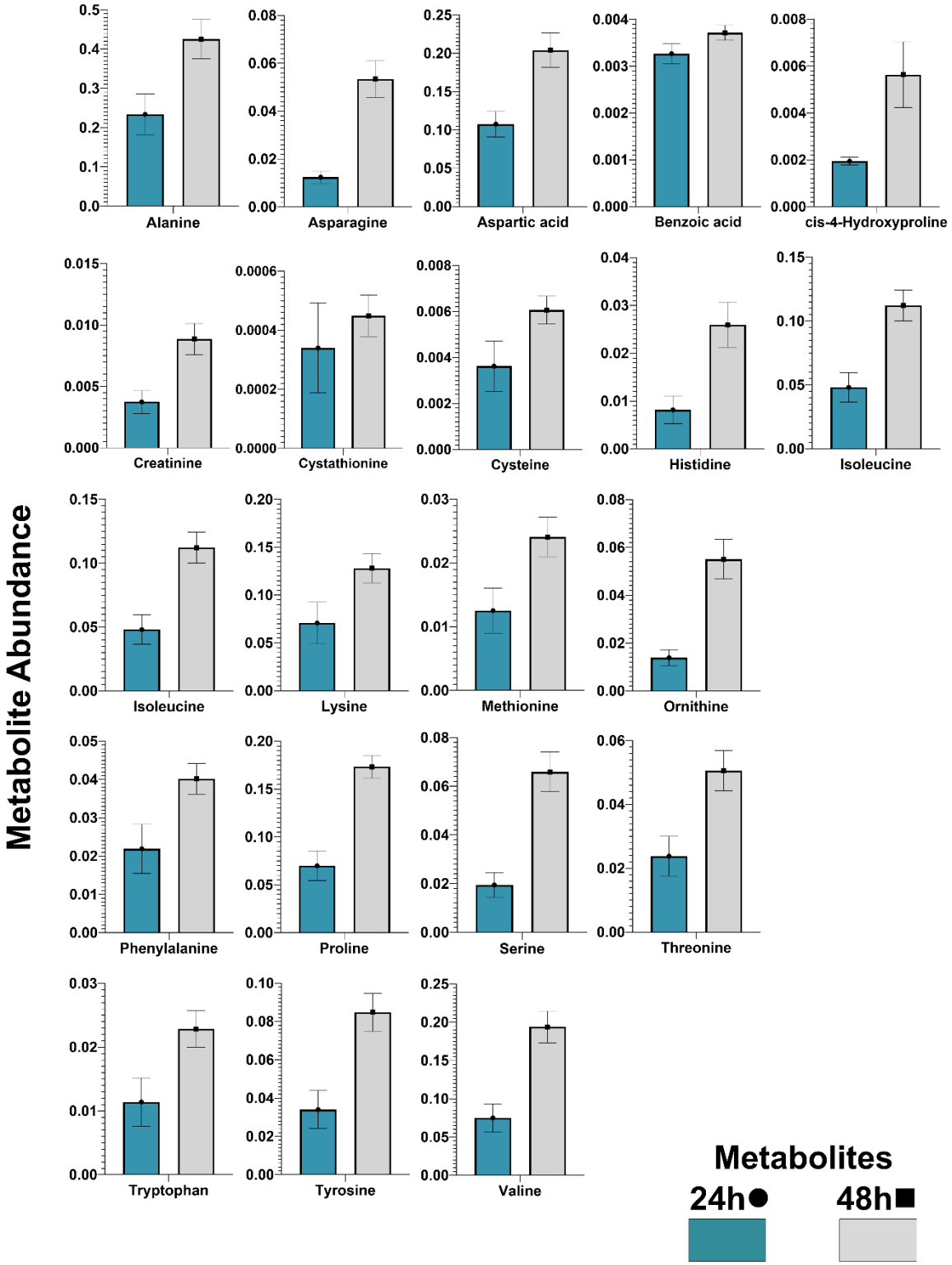

Fig S3: Metabolite differences of *Perna canaliculus* haemolymph relating to experimental time following injection with *Vibrio mediterranei.*

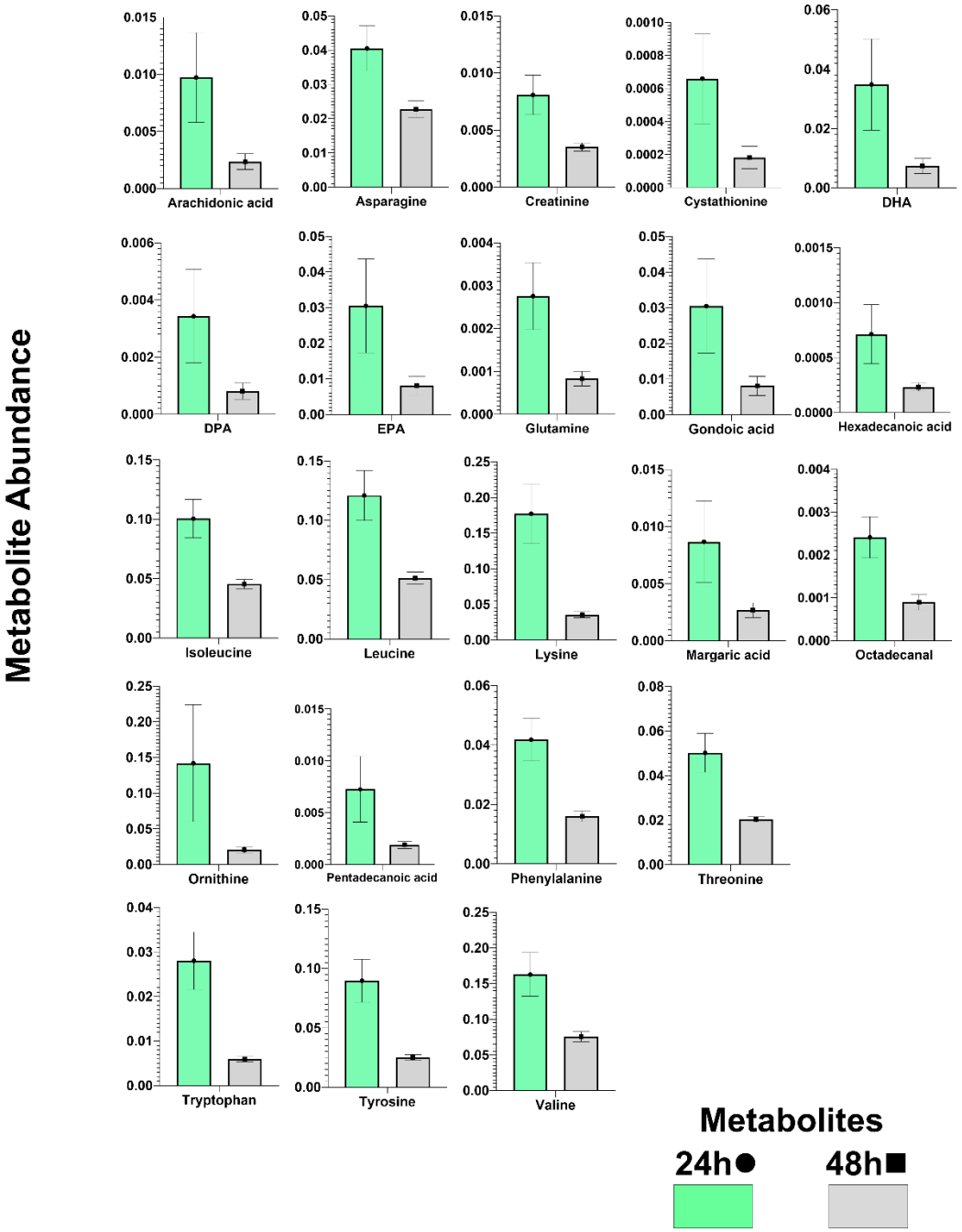

Fig S4: Metabolite differences of *Perna canaliculus* haemolymph relating to experimental time following injection with *Vibrio mediterranei* and *Photobacterium swingsii.*

Table S1: Two-way ANOVA metabolomic findings of *Perna canaliculus* haemolymph following marine broth injection.

Metabolite	Size	Time	Interaction
Asparagine	9.94E-01	1.77E-02	9.31E-01
Cysteine	9.94E-01	1.77E-02	9.69E-01
Dodecanoic acid	9.94E-01	1.77E-02	9.31E-01
Glutamic acid	9.94E-01	1.77E-02	9.41E-01
Glutamine	9.94E-01	1.77E-02	9.57E-01
Glutathione	9.94E-01	5.78E-03	9.41E-01
Margaric acid	9.94E-01	2.65E-02	9.69E-01
trans-Vaccenic	9.94E-01	4.71E-02	9.69E-01
Tyrosine	9.94E-01	2.65E-02	9.31E-01

Table S2: Two-way ANOVA metabolomic findings of *Perna canaliculus* haemolymph following *Vibrio mediterranei* injection.

Metabolite	Size	Time	Interaction
Alanine	1.27E-01	1.14E-02	6.79E-01
Asparagine	4.52E-01	1.22E-03	5.41E-01
Aspartic acid	4.59E-01	4.82E-03	4.24E-01
Benzoic acid	5.30E-03	3.45E-02	4.98E-01
cis-4-Hydroxyproline	2.53E-01	1.67E-02	5.41E-01
Citric acid	2.65E-02	3.83E-01	9.53E-01
Creatinine	1.67E-01	8.15E-03	4.65E-01
Cystathionine	1.06E-01	4.04E-02	4.24E-01
Cysteine	1.34E-01	1.25E-02	4.24E-01
Histidine	1.92E-01	4.82E-03	4.24E-01
Isoleucine	1.38E-01	4.82E-03	4.65E-01
Leucine	1.34E-01	4.82E-03	4.65E-01
Lysine	1.27E-01	1.25E-02	4.24E-01
Methionine	2.29E-01	1.67E-02	6.85E-01
Ornithine	1.34E-01	1.22E-03	4.65E-01
Phenylalanine	1.34E-01	1.25E-02	4.53E-01
Proline	1.38E-01	2.54E-03	4.65E-01
Serine	9.73E-01	4.82E-03	4.98E-01
Threonine	1.34E-01	4.82E-03	4.24E-01
Tryptophan	1.27E-01	8.15E-03	4.24E-01
Tyrosine	1.67E-01	4.82E-03	4.53E-01
Valine	1.67E-01	4.82E-03	4.77E-01

Table S3: Two-way ANOVA metabolomic findings of *Perna canaliculus* haemolymph following *Vibrio mediterrane*i and *Photobacterium swingsii* injection.

Metabolite	Size	Time	Interaction
Arachidonic acid	7.18E-01	7.57E-03	2.41E-01
Asparagine	6.27E-01	3.12E-02	9.64E-01
Creatinine	6.27E-01	2.32E-02	9.90E-01
Cystathionine	1.86E-01	1.59E-02	2.41E-01
DHA	8.25E-01	2.32E-02	2.41E-01
DPA	7.18E-01	3.79E-02	2.65E-01
EPA	8.25E-01	3.45E-02	2.41E-01
Glutamine	7.18E-01	2.32E-02	6.54E-01
Gondoic acid	8.25E-01	3.45E-02	2.41E-01
Hexadecanoic acid	9.36E-01	3.24E-02	2.65E-01
Isoleucine	7.18E-01	7.57E-03	9.64E-01
Leucine	7.18E-01	1.18E-02	9.60E-01
Lysine	7.18E-01	6.84E-04	4.56E-01
Margaric acid	8.04E-01	4.58E-02	2.41E-01
Octadecanal	7.18E-01	1.18E-02	3.83E-01
Ornithine	9.36E-01	1.93E-02	9.64E-01
Pentadecanoic acid	9.36E-01	2.32E-02	2.41E-01
Phenylalanine	7.18E-01	5.45E-03	4.27E-01
Threonine	7.18E-01	5.45E-03	9.64E-01
Tryptophan	7.18E-01	6.84E-04	4.86E-01
Tyrosine	8.04E-01	1.73E-03	4.56E-01
Valine	7.18E-01	2.32E-02	9.64E-01

A good thesis
is a finished thesis.

A great thesis
is a published thesis.

A perfect thesis
is neither.