Marine Environmental Research 196 (2024) 106392

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

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

Awanis Azizan [a], Leonie Venter [a], Jingjing Zhang [a,b], Tim Young [a,c], Jessica A. Ericson [d], Natalí J. Delorme [d], Norman L.C. Ragg [d], Andrea C. Alfaro [a,*]

[a] Aquaculture Biotechnology Research Group, Department of Environmental Sciences, School of Science, Auckland University of Technology, Private Bag 92006, Auckland, 1142, New Zealand
[b] The New Zealand Institute for Plant and Food Research Limited, Private Bag 92169, Auckland, 1142, New Zealand
[c] Centre for Biomedical & Chemical Sciences, School of Science, Auckland University of Technology, Auckland, New Zealand
[d] Cawthron Institute, Private Bag 2, Nelson, 7042, New Zealand

ARTICLE INFO

Keywords:
Bacteria
Biomarker
Greenshell™ mussel
Immune response
Multiple stresses
Perna canaliculus
Photobacterium
Temperature
Summer mortality

ABSTRACT

The New Zealand Greenshell™ mussel (*Perna canaliculus*) is an economically important aquaculture species. Prolonged increases in seawater temperature above mussel thermotolerance ranges 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 at 24, 48, 72, 96, 120 h 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 followed 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. In the gill tissue, 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), indicating the presence of oxidative stress due to temperature and pathogen stressors. Overall, this work confirms that *Photobacterium swingsii* is pathogenic to *P. canaliculus* and indicates that mussels may be more vulnerable to bacterial pathogens under conditions of elevated temperature, such as those predicted under future climate change scenarios.

1. Introduction

Endemic greenlipped mussels (*Perna canaliculus* Gmelin 1791; commercially branded as "Greenshell™") are the most valuable aquaculture species exported in New Zealand (NZ) (Castinel et al., 2019), with annual revenues of over $303M NZD in 2021 (Miller et al., 2023). Environmental changes in NZ coastal areas, such as marine heatwaves (MHWs), are increasingly common and result in mortalities and increased disease outbreaks on aquaculture farms (Heasman et al., 2020). Shellfish mortalities associated with increasing summer temperatures (termed 'summer mortalities') have been reported in different bivalve species, including *P. canaliculus* (Newton and Webb, 2019), *Mytillus galloproviancialis* (Anestis et al., 2010; Lattos et al., 2022), *Chamelea gallina* (Monari et al., 2007), *Crassostrea gigas* (Malham et al., 2009; Samain, 2011), and *C. virginica* (Encomio and Chu, 2005). The exact causes of summer mortalities often remain unclear, but thermal

* Corresponding author.
E-mail address: andrea.alfaro@aut.ac.nz (A.C. Alfaro).

https://doi.org/10.1016/j.marenvres.2024.106392
Received 20 October 2023; Received in revised form 1 February 2024; Accepted 2 February 2024
Available online 3 February 2024

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 have been 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 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 area located on the northern coast of the South Island of NZ), SSTs above 20 °C are currently recorded during summer months (Broekhuizen et al., 2021), and optimum mussel growth and survival start to decline once seawater temperatures exceed 22 °C (Ericson et al., 2023a). Notably, the projected increase in warming events in the coming decades will impact mussel aquaculture across most regions of NZ (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 (24h) laboratory experiment, *P. canaliculus* showed signs of physiological stress at 24 °C (Ericson et al., 2022). In a chronic (15-month) heat stress exposure experiment, the overall physiological performance of *P. canaliculus* was impacted by elevated temperature, with a survival tipping point noted between 21 and 24 °C resulting in net mortality of 6, 10, and 100% at 17, 21, and 24 °C, respectively (Ericson et al., 2023b). Additionally, Delorme et al. (2021c) 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 seawater temperatures above 24 °C exceed the optimum limits of fitness and survival of Greenshell™ mussels (Ericson et al., 2023b). As the temperature exceeds optimal ranges, mitochondrial shifts in mussels are increasingly induced as reported by Sokolova (2023), affecting ATP synthesis efficiency, maintenance costs, antioxidant defence and ultimately death of the shellfish organism (Eymann et al., 2020). Understanding these responses is crucial for deciphering the physiological effects of thermal stress on mussel performance. Few studies have investigated the combined impact of heat-stress and other drivers on Greenshell™ 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., 2020). 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 influences 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). The thermal window of *Photobacterium* and *Vibrio* bacteria varies among species and strains, with optimal growth temperatures typically ranging from 15 °C to 37 °C (Waters and Lloyd, 1985; Hauton et al., 2001; Thorvaldsen et al., 2007; Yung et al., 2015). Some strains exhibit psychrophilic behaviour, growing at lower temperatures, while others are more thermophilic and can thrive at higher temperatures. Understanding these thermal preferences is important for assessing bacterial infection risks in aquaculture. 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., 2019). 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: *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). Of particular interest for the current research are *P. swingsii* also implicated in infections of diverse aquatic organisms in aquaculture, including molluscs (Fichi et al., 2015), bivalves (Gomez-Gil et al., 2011; Eggermont et al., 2017), and crustaceans (Gomez-Gil et al., 2011), causing significant economic losses. Considering that mussel aquaculture areas are often cultivated near aquatic organism's, the potential exposure to pathogenic bacteria from these units is considerable. 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., 2022). 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 counts (THC) and haemocyte viability have been used to monitor changes due to temperature variations in *M. edulis* (Mackenzie et al., 2014), *M. coruscus* (Wu et al., 2016), *P. viridis* (Wang et al., 2011) and *P. canaliculus* (Delorme et al., 2021c). 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; Paillard et al., 2004; Allam et al., 2006; Nguyen et al., 2019a; 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-h, 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 removal 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; Delorme et al., 2021c). 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 frequently used in mussel studies to determine imbalances in the production of ROS (Azizan et al., 2023a; Delorme et al., 2021b). Antioxidants, such as superoxide dismutase, catalase and glutathione peroxidase help protect mussels against oxidative stress (Wenning and Di Giulio, 1988; Delorme et al., 2020, 2021a). 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 mussels 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., 2021c) and *Vibrio* sp. infection (Ericson et al., 2022).

Typically, ROS generated in infected cells are associated with the formation of lipid peroxides in cell membranes (Farmer and Mueller, 2013). When the free radical formation surpasses their rate of elimination by the antioxidant system, oxidative damage such as lipid peroxidation (LPO) occurs (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 the freshwater mussels, *Coelatura aegyptiaca*, *Mutela rostrata* and *Chambardia rubens* (Said and Nassar, 2022) and green marine mussels, *Perna viridis* (Wang et al., 2018) 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 of these stressors' effects mussel oxidative phosphorylation.

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. This experiment provides 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 a risk forecasting standpoint, which will assist the development of animal health strategies and policies for mitigating the impacts of future summer mortality events.

2. Methods and materials

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 'survival cohort'), and a separate group of mussels used for destructive sampling over a five-day period (referred to as the 'sampling cohort'). The two groups of animals were treated in the same manner, except where stated otherwise.

2.1.1. Animal husbandry

Adult *P. canaliculus* (n = 1200; 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 ~16 °C). The animals were transported to marine biocontainment facilities operated by the Cawthron Institute in Nelson, New Zealand. In the laboratory, the mussels were randomly assigned to three recirculating systems of 36 X 8 L tanks (108 tanks in total housing 11 mussels per tank) equipped with a UV filter, individual tank airlines and continuously recirculating seawater (34 ppt, 1 μm filtered). Water temperature was kept at an ambient 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 *Chaetoeceros muelleri* microalgae. Water ammonia (< 0.1 ppm), nitrite (< 0.1 ppm), and nitrate (< 5 ppm) 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 posterior adductor muscle remained unresponsive (mussels' shells stayed apart) after 10 gentle squeezes to stimulate valve closure, the animals was classified as dead and removed from the system (Dunphy et al., 2015).

2.1.2. Temperature exposure

Experimental temperature conditions were adjusted to achieve three stable temperature regimes (16, 20 and 24 °C), each incorporating 36 tanks containing 11 animals per tank. The 16 °C temperature represented the control (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 progressively increasing the seawater temperature of 1 °C per day over 4 days. Temperatures were regulated via a heat exchanger connected to a hot/cold loop, controlled by solenoid valves, and monitored daily (Fig. 1). Once the experimental temperatures of 20 °C and 24 °C were achieved (seawater temperatures reach the desired level on day 14 for monitoring cohort and day 15 for sampling cohort), the bacterial administration section of the experiment started.

2.1.3. Bacterial injection challenge

Prior to starting the injection challenge, a *P. swingsii* culture, obtained during austral summer of 2017–2018, during a mass mortality event (Azizan et al., 2022) 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 density (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) [as determined per previous publication (Azizan et al., 2022)]. 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 mantle water was drained out, but no injection was administered (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 careful 2 mm opening of the valves 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 for monitoring cohort and n = 66 total for the sampling cohort) (Fig. 1).

After injection, each animal was returned to its tank. Mussels were treated in a block-wise manner to facilitate standardisation of subsequent sampling intervals; the no injection control group was followed by the marine broth injection group, the medium dose and, lastly, the high dose bacterial injection groups. The sequence was repeated for 16, 20 then 24 °C. As soon as infected mussels were placed back into their tanks, the recirculating water system was diverted to waste (i.e., flow-through configuration), to mitigate the compounding influence of reinfection by expelled bacteria.

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 120 h h post-challenge [hpc]). At the time of sampling, mussels (following the same sampling sequence) were blotted 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 valves were carefully prised 2 mm apart 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

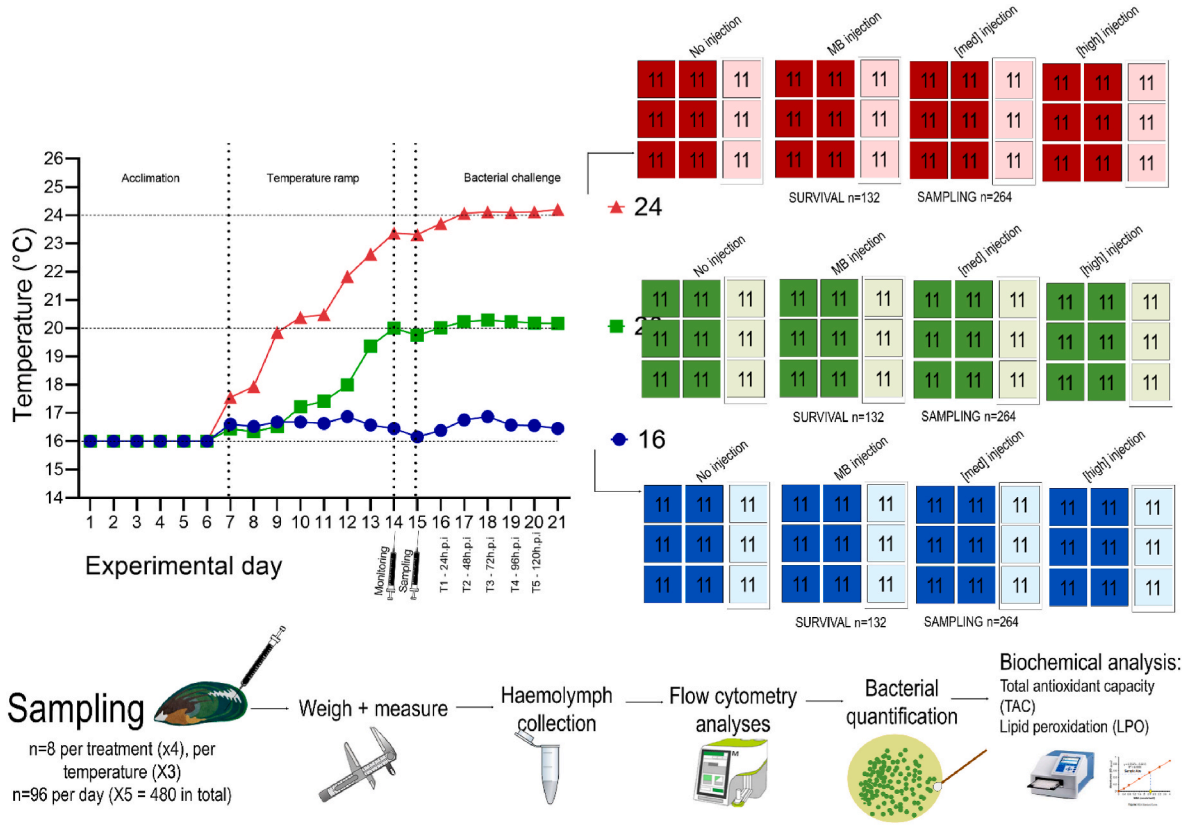

Fig. 1. Experimental design for the thermal stress and *Photobacterium swingsii* challenge trial. Mussels were exposed to three different temperature regimes and exposed to a medium and high dose of bacteria, along with comparative controls. Haemolymph and gill samples from three treatment groups (no 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 hours 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.

collected from each individual mussel 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). For bacteria culturing, haemolymph was chosen as the sample source. Given the size of many bacteria, including *P. swingsii*, which can be 1 μm or less, they may not be efficiently captured by the bivalve gill sieve when in free-living form (Rosa et al., 2018). Selecting haemolymph allows for the detection and quantification of pathogens circulating in the host's system, indicating systemic infections or pathogen dissemination throughout the organism (Parisi et al., 2019; Yang et al., 2023). For the biochemical analyses, gills were selected as the primary tissue of interest. Gills are directly exposed to aquatic environmental stressors, making them sensitive indicators of oxidative stress and overall mussel health (Saco et al., 2020; Yang et al., 2021; Xu et al., 2021). The gill tissues (3 × 3 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 (Nguyen et al., 2018).

For flow cytometry analyses, 50 μL haemolymph were diluted 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, followed by 1 μL of concentrated Muse® Count & Viability Kit (200X, MCH100104; Luminex). 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 and Wegner, 2019). All plates were incubated at 22 °C for 24–48h before being counted manually to obtain CFU/mL of Vibrionaceae (presumed to be predominantly introduced *P. swingsii*) in the haemolymph.

For biochemical analyses (TAC and MDA, Section 2.3), gill samples from two biological replicates were pooled to increase the overall biomass, resulting in four pooled samples per treatment.

2.3. Biochemical assays

2.3.1. 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 described previously by Delorme et al. (2021c). Frozen gill tissue samples from two biological replicates were homogenised using a liquid nitrogen-cooled mini mortar (Z756377, Merck). Approximately 100 mg 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 s at 1500 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).

2.3.2. Lipid peroxidation assay

Lipid peroxidation (LPO) in gill tissue was measured according to the 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 s at 1500 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 performed in triplicate in each plate to evaluate the intra-assay variability.

The LPO 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. A 1,1′, 3,3′-tetramethoxy propane (TMP) standard 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.

2.4. Statistical analyses

Statistical analyses for survival, bacterial load, total haemocyte count, haemocyte viability and biochemical analyses data were carried out RStudio version 4.2.3 and a significance level of 0.05.

2.4.1. 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.

2.4.2. 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, $p > 0.05$). 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) and 'dplyr' (Jockers et al., 2020). 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. The Pearson correlation coefficient was used in the correlation matrix. Differences were statistically significant when $p < 0.01$ and $p < 0.05$.

3. Results

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 ± 9 % survival rate (mean ± SE)) compared to 16 °C seawater (88 ± 6 % survival rate; $p = 0.004$) which, in turn, was not significantly different to net survival at 20 °C (73 ± 8 %; $p = 0.209$. Fig. 2a). 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 ± 9 %) were significantly different compared to the 20 °C (79 ± 7 %) and 16 °C groups (88 ± 6 % survival rate; $p = 0.016$ and $p < 0.001$, respectively. Fig. 2b). While significant differences were not detected between mussel survival at 16 °C and 20 °C (Fig. 2b; $p = 0.067$). The output from the coxme model showed that the variance attributed to tank as a random effect was ~0.

3.2. Effects of P. swingsii and temperature on P. canaliculus

3.2.1. Bacterial counts

Haemolymph from mussels in the no injection and marine-broth injected groups showed no bacterial colonies on TCBS agar at any of the sampling points (Fig. 3). Colony forming units (CFU) within the medium and high injection groups 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 S1, Fig. 3). Also, significant interactions were observed for CFUs in mussels within the high injection group collected at 48 and 120 hpc.

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). Significant interaction of THC was demonstrated between mussels held at temperature 20 °C and collected at 120 hpc (Table S2, Fig. 4).

3.2.3. Haemocyte viability

The proportion of viable haemocytes in the medium and high injection groups were significantly lower ($p < 0.05$) compared to the no injection group. No significant interaction was observed (Table S3, Fig. 5).

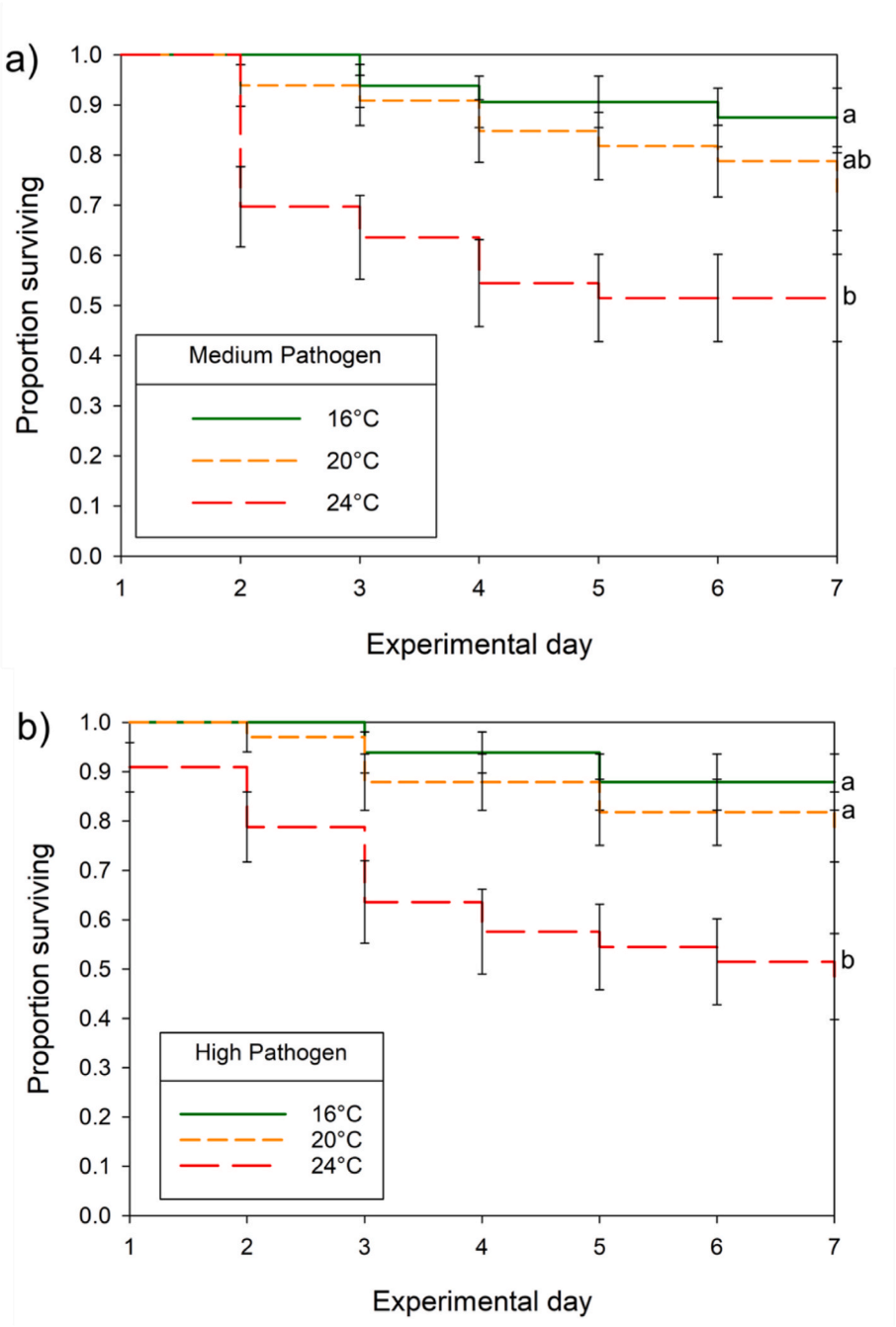

Fig. 2. Kaplan-Meier survival trajectories (±SE) for *P. canaliculus* after injection with a) medium and b) high doses of *Photobacterium swingsii* at three different seawater temperatures.

3.2.4. Correlation among haemocyte parameters

The correlation analysis was illustrated in Fig. S1. There was no significant correlation between THC and bacterial counts ($r = -0.01$, $p = 0.90$). A moderate negative correlation observed between haemocyte viability and bacterial counts ($r = -0.31$, $p < 0.05$). The correlation between THC and haemocyte viability was not statistically significant ($r = -0.05$, $p = 0.27$).

3.3. Effects of sex on selected measurements

The sex of individual mussels was assessed, documenting 206 females, 228 males and 46 not identified mussels (mantle shows glycogen accumulation but no gonad tissue that can be differentiated by colour. Fig. 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. 6b). No significant difference in the THC (Fig. 6c) and haemocyte viability (Fig. 6d) were found between sexes ($p < 0.05$).

3.4. Biochemical analyses

3.4.1. Total antioxidant capacity (TAC)

The mean total antioxidant capacity (calculated as Trolox-equivalent antioxidant capacity [TAC]) of gill tissues was 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. 7). TACs within the marine broth injection group were significantly lower ($p < 0.05$) than the no injection control treatment.

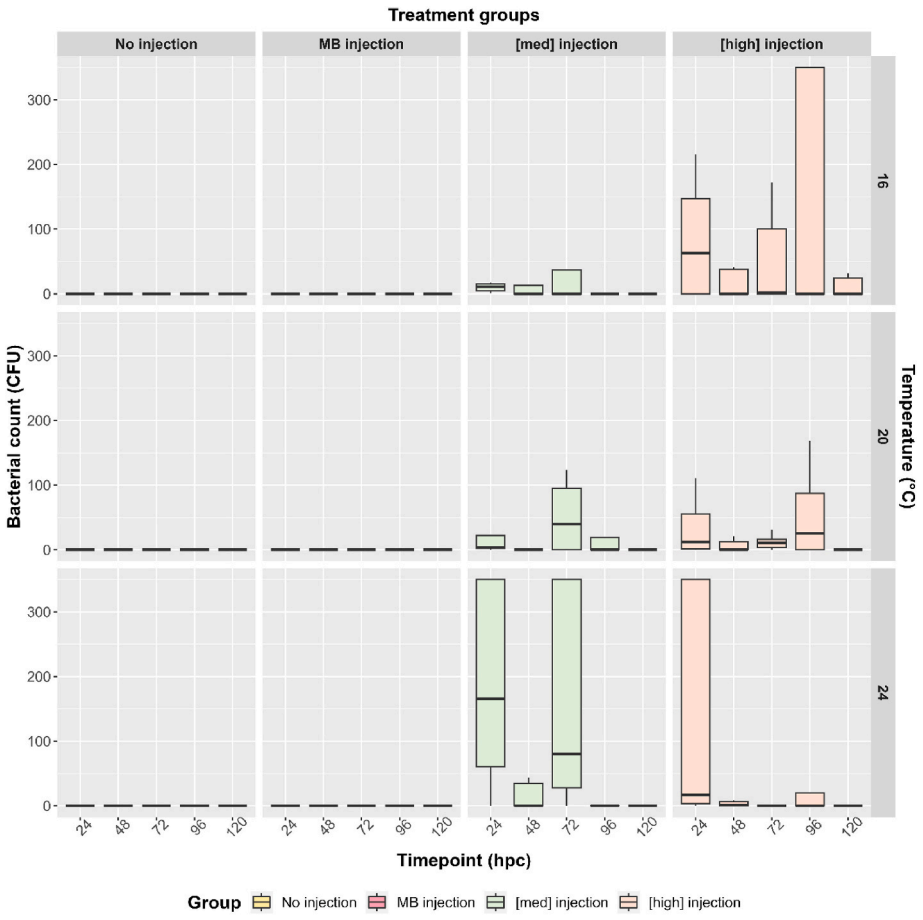

Fig. 3. The combined effects of temperature, treatment and time (hours post-challenge) on bacteria count. Each panel compares the mean TCBS agar (Vibrionaceae) bacterial counts (CFU per 10 μL of haemolymph sample) from *P. canaliculus* haemolymph from the no injection control, the marine browth (MB) injection control, and bacterial injections of medium and high doses of *P swingsii,* at low and high seawater temperatures (16, 20 and 24 °C), across five timepoints (24, 48, 72, 96, 120 hpc). All values represent average of 8 replicates per treatment at each specified timepoint. Refer to Supplementary Excel File S1 for detailed pairwise comparisons based on the emmeans post-hoc test results.

TAC levels in mussels exposed to 24 °C were significant higher ($p < 0.05$) than at 16 °C. A significant interaction ($p < 0.05$) was demonstrated for TACs in mussels within the high injection group collected at 120hpc (Table S4, Fig. 7).

3.4.2. Lipid peroxidation (LPO)

Malondialdehyde concentrations (representative of TBARS concentration) were highest in gill tissues obtained from mussels injected with a high dose of *P. swingsii* at both temperatures (16 and 24 °C) and timepoints (24 and 120 hpc) (Fig. 8). MDA levels within the high injection group were significantly increased ($p < 0.05$) when compared to the no injection control treatment. A significant interaction ($p < 0.05$) was observed in the MDA levels of mussels held at 24 °C and subjected to a high infection dose in the gill tissues sampled at 120 hpc, as compared to mussels held at 16 °C (Table S5, Fig. 8).

4. Discussion

4.1. 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). *Photobacterium* tends to thrive in warmer temperatures, with optimal growth occurring between 15 °C (Waters and Lloyd, 1985) to 37 °C (Matanza and Osorio, 2020). Lower temperatures can slow down bacterial growth, while temperatures above the optimal range can hinder bacterial growth, virulence and viability. In the current study, significant mussel mortalities occurred at 20 and 24 °C when *P. swingsii* was present, suggesting that the presence of bacteria drives mortality once thermal stress is already in place. It is know that temperatures in this range cause mussel mortality over long time periods (> four weeks) (Ericson et al., 2023b; Venter et al., 2023), opposed to short term exposures, which result in few or no mortalities (Delorme et al., 2021c; Ericson et al., 2022). This is confirmed in the current experiment where mussels subjected to temperature stress alone survived the five-day exposure period. The use of *P swingsii* as an 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 for the mortalities observed. In this study, injection of *P. swingsii* bacteria directly into the mussels adductor muscle was chosen as infection method, rather than relying on natural exposure through seawater, as a means to control the dosage and exposure duration, enhancing the reproducibility of the bacterial administration in laboratory trials. While recognising the ecological importance of natural exposure, our approach prioritises targeted examination of host responses. 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*

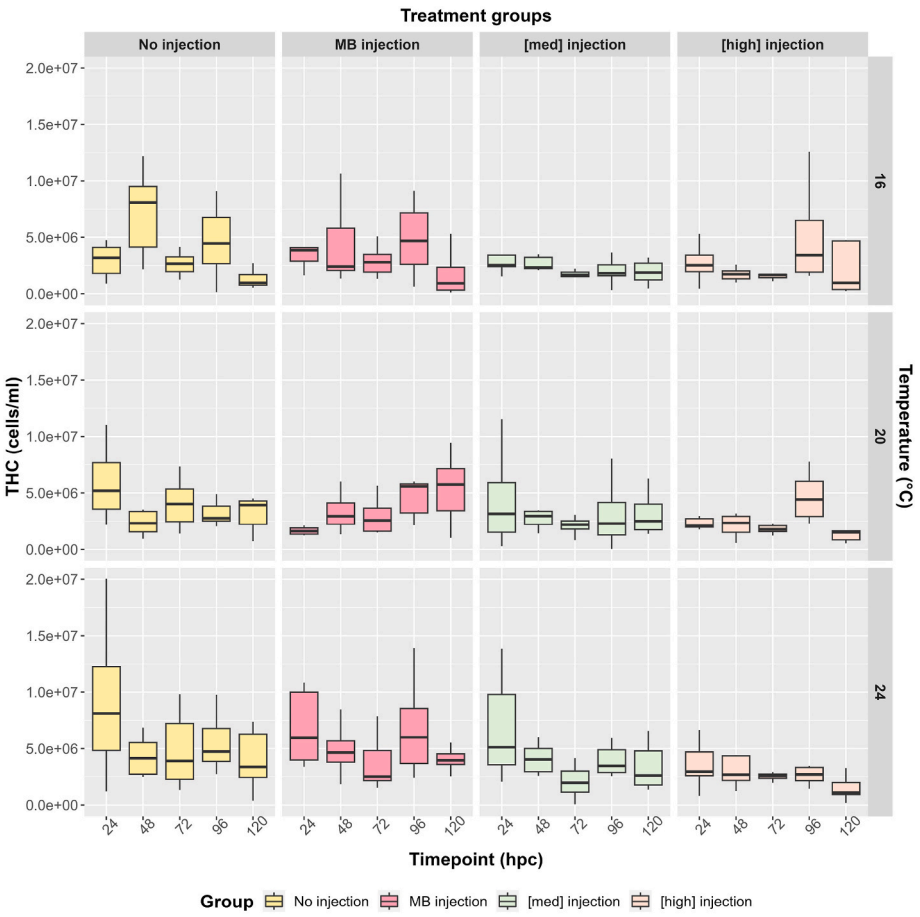

Fig. 4. The combined effects of temperature, treatment and time (hours post-challenge) on total haemocyte count. Each panel compares the mean haemocyte counts from *P. canaliculus* haemolymph from the no injection control, the marine browth (MB) injection control, and bacterial injections of medium and high doses of *P swingsii*, at 16, 20 or 24 °C, across five timepoints (24, 48, 72, 96, 120 hpc). Refer to Supplementary Excel File S1 for detailed pairwise comparisons based on the emmeans post-hoc test results.

are presented together. This current study parallels findings on Pacific oysters (*Crassostrea gigas*) where oysters infected with *Vibrio alginolyticus* at high temperatures 33 °C), had a higher mortality rate than oysters at an optimal temperature (23 °C) (Li et al., 2023).

4.2. Bacterial quantification from host haemolymph

From literature is it hypothesised that mussels that survive infection have likely cleared the infecting pathogen from their system (Duneau et al., 2017). From the current experiment, there was variability or differences in the results obtained from each of the eight replicates for some groups, particularly in cases like high injection, 16 °C, 96 hpc; medium injection, 24 °C, 24 hpc; medium injection, 24 °C, 72 hpc and high injection, 24 °C, 24 hpc. This variation is a common occurrence and can be attributed to natural variability (biological variability, dynamics of bacterial infections, variations in individual host responses, and stages of infection) (Ben Cheikh et al., 2017).

Additionally, 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* can 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, 2024) 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. In line with previous research indicating *Photobacterium*'s preference for temperatures within the range of 15–37 °C, this study observed a decline in bacterial counts within the first three sampling timepoints at 24 °C. This finding suggests that *P. swingsii* clearance occurs at this temperature when subjected to a high bacterial dose, highlighting its unique response within this thermal window (15–37 °C) (Waters and Lloyd, 1985; Thorvaldsen et al., 2007; Yung et al., 2015; Matanza and Osorio, 2020). 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 initiated by the mussels' immune response in the early hours of the experiment (Canesi et al., 2002; Parisi et al., 2008). Infection of *M. galloprovincialis* with *V. splendidus*, (bacterial suspension 10^7 CFU/ml) at 20 °C showed an increase in bacterial CFU at 1- and 6-h post infection, with very few colonies subsequently detected at 24, 48 and 72 hpc (Parisi et al., 2019). The bacterial count showed an increase in mussels exposed to 16 °C after 96 hpc in the current study and decrease at 24 °C at all sampling times when compared to 24 hpc may be due to the slower metabolic rate of mussels at lower temperatures. This slower metabolic rate might result in delayed cellular responses and slower clearance of bacteria from the haemolymph (Eisenreich et al., 2013). Moreover, the higher bacterial counts in the medium injection group at 24 and 72 hpc compared to the high injection group may be attributed to several factors. These include a potential dose-response relationship (Xia et al., 2015), where the medium injection group received an effective initial bacterial dose, while the high injection group may have faced an overwhelming dose initially, slowing bacterial growth. Additionally, variations in the host's

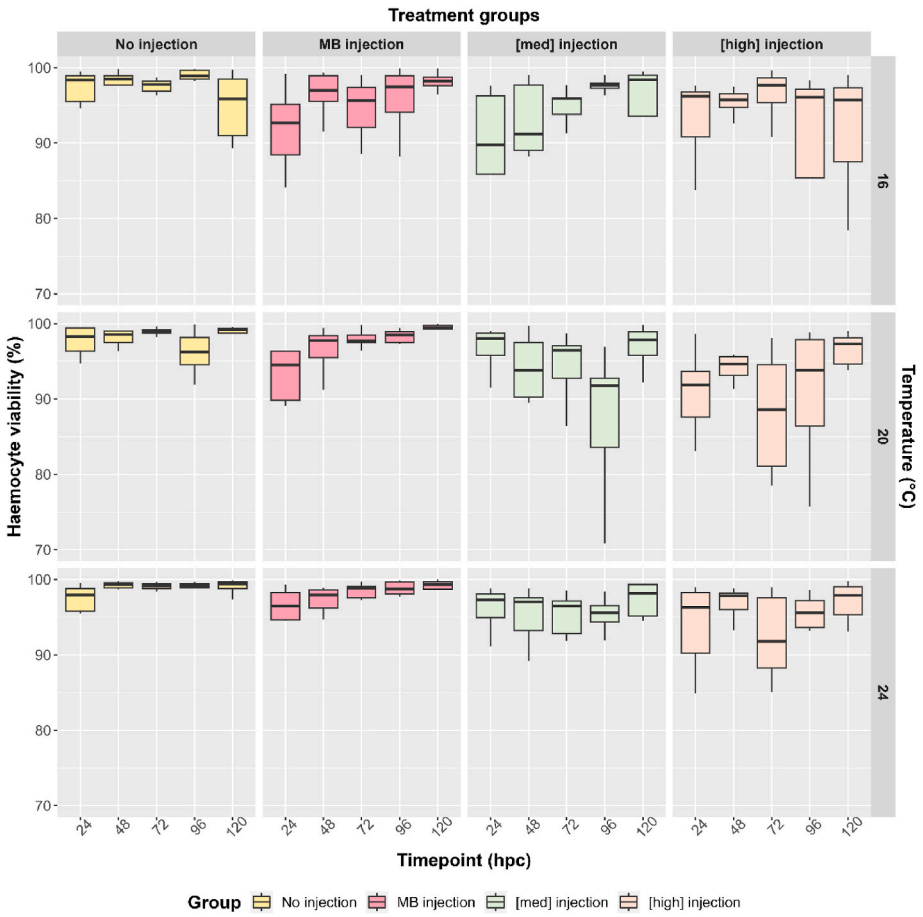

Fig. 5. The combined effects of temperature, treatment and time (hours post-challenge) on haemocyte viability (viable cells as % of total haemocyte population). Each panel compares the mean values from *P. canaliculus* haemolymph from the no injection control, the marine browth (MB) injection control, and bacterial injections of the medium and high doses of *P swingsii*, at 16, 20 or 24 °C, across five timepoints (24, 48, 72, 96, 120 hpc). Refer to Supplementary Excel File S1 for detailed pairwise comparisons based on the emmeans post-hoc test results.

immune response could play a role, with the high injection group initially limiting bacterial growth but potentially succumbing to adaptation by the bacteria over time (Boulo et al., 1991). Mussels in the current study were not sampled within the first 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., 2002).

It is worth noting from the results of bacterial quantification, that the choice of infection route whether through injection or bath infection, can influence the specific sites of invasion of pathogens (Chiang et al., 1999), mortality rate (Romero et al., 2014) and bacterial clearance measurement. For mussels, the gills are suggested as possible routes for infection when exposed to bacteria in the surrounding seawater, as these organs come into direct contact with the microbial environment (Saco et al., 2020). Additionally, intramuscular injections revealed bacterial pathogens can potentially be penetrated when injected directly (Romero et al., 2014). Bacterial injection may lead to more extensive and rapid mortality, making it an efficient method for ensuring pathogen infection in mussels (Xia et al., 2015). However, it can also have drawbacks, such as potential stress on the mussels, increased labour and time requirements, and safety concerns for researchers (Xia et al., 2015). In contrast, immersion mimics natural infection routes and can be effective for pathogen colonization and infection in mussels, particularly when studying specific target organs and tissues (Saulnier et al., 2000). Therefore, the selection of the infection route should align with the research objectives and practical considerations of the study.

4.3. Mussel cellular response

The number of circulating haemocytes often reflects the magnitude of a systemic response to a stressor 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) occurred over time 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), possibly due 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 where *P. canaliculus* was infected with *P. swingsii,* an increase in THC was seen from 12 to 48 hpc (housed at 16 °C), possibly as a sign of stress (Azizan et al., 2024"). The current study also revealed an increase in THC levels within the evaluated timeframe, which is consistent with the findings of the previous study suggesting the crucial role of circulating haemocytes in the encapsulation and phagocytosis of pathogens. The control treatments fluctuated 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 *M.edulis desolationis* can exit the haemolymph after a few hours post infection, and move to the mantle fluid before being

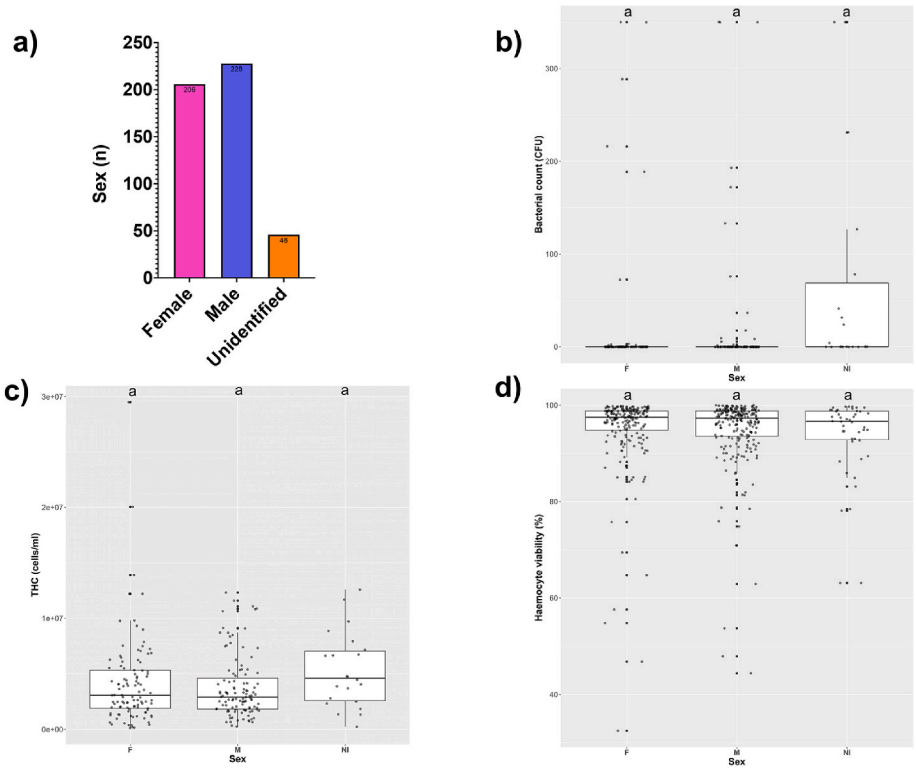

Fig. 6. a) Proportion of female (F), male (M) and not identified (NI) *P. canaliculus* in different treatment groups across timepoints. Individual mussel sex data (±SE) collected from b) bacterial counts, c) total haemocyte counts and d) haemocyte viability. Within each graph, different letters indicate means that are significantly different from each other ($p < 0.05$).

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) typically had the lowest haemocyte viability (at all temperatures), potentially due to handling stress. In a transport stress study on *P. canaliculus,* 24 h 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, indicating a diverse response pattern between individuals. 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., 2019b) and *V. tapetis* (Allam et al., 2006) infections. The decreasing numbers of circulating haemocytes found in the current study could be attributed to cell death induced by stressors, or signs of damage to the cell membrane (Nogueira et al., 2017; 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 the 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) of 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 cellular 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.

In the present study the correlation between THC and bacterial counts was very weak and almost negligible correlation. This suggests that the abundance of immune cells does not significantly change with variations in bacterial counts. However, the correlation between haemocyte viability and bacterial counts is significant, suggesting that there is a meaningful relationship between haemocyte viability (the health and functionality of immune cells) and bacterial counts. Hence, it could be hypothesised a higher bacterial infection load is associated with reduced health and functionality of the mussels' immune cells (Ellis et al., 2011). Moreover, these correlations revealed the disorder of physiological response of mussels under combined stress and explained the association of high mussel mortality in bacterial injection groups (Xu et al., 2021).

4.4. 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 provided 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 maintained at different seeding densities (Reyden et al., 2023). The present study showed increased TAC and MDA

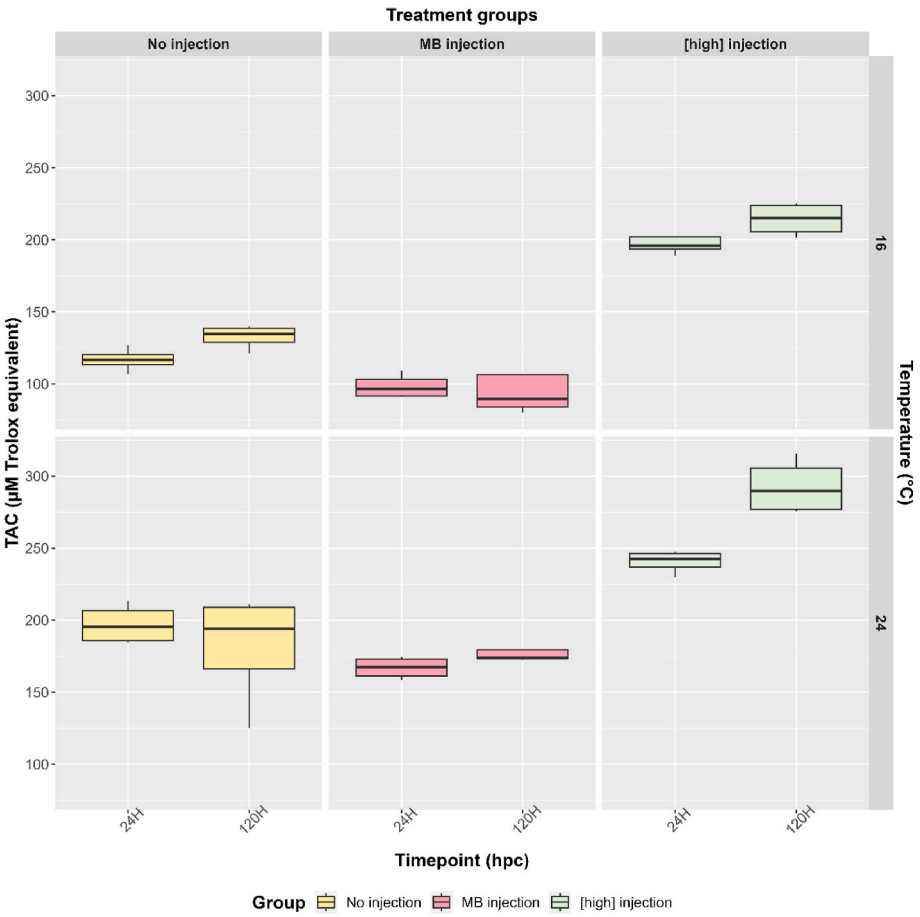

Fig. 7. The combined effects of temperature, treatment and time (hours post-challenge) on Trolox-equivalent antioxidant capacity (TAC) of gill tissue. Each panel compares the mean TAC from *P. canaliculus* haemolymph of the no injection control, the marine broth (MB) injection control and injection with a high dose of *P swingsii*, at low and high seawater temperatures (16 and 24 °C), across two timepoints (24 and 120 hpc). Refer to Supplementary Excel File S1 for detailed pairwise comparisons based on the emmeans post-hoc test results.

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 combined with reduced energy production hindered the mussels' ability to mount an effective antioxidant response against the pathogen challenges in the above-mentioned studies (Genard et al., 2011; Box et al., 2020). A similar outcome may apply 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 ($O_2^{\bullet-}$), 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). Increase in TAC in response to stressors has not been widely reported. Exceptions include *P. canaliculus* juveniles, where increases in antioxidant activities have been observed when mussels were exposed to air at different humidity levels (Delorme et al., 2021a). In clams (*Laternula elliptica*), an increase in transcription of antioxidant genes were seen in response to heat stress (suggesting antioxidant defence against oxidative stress), which results in increased antioxidant activity in the clams (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 dose 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).

4.5. Dual stressors as trigger for summer mortality

For summer mortality events various biotic and abiotic 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 can 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

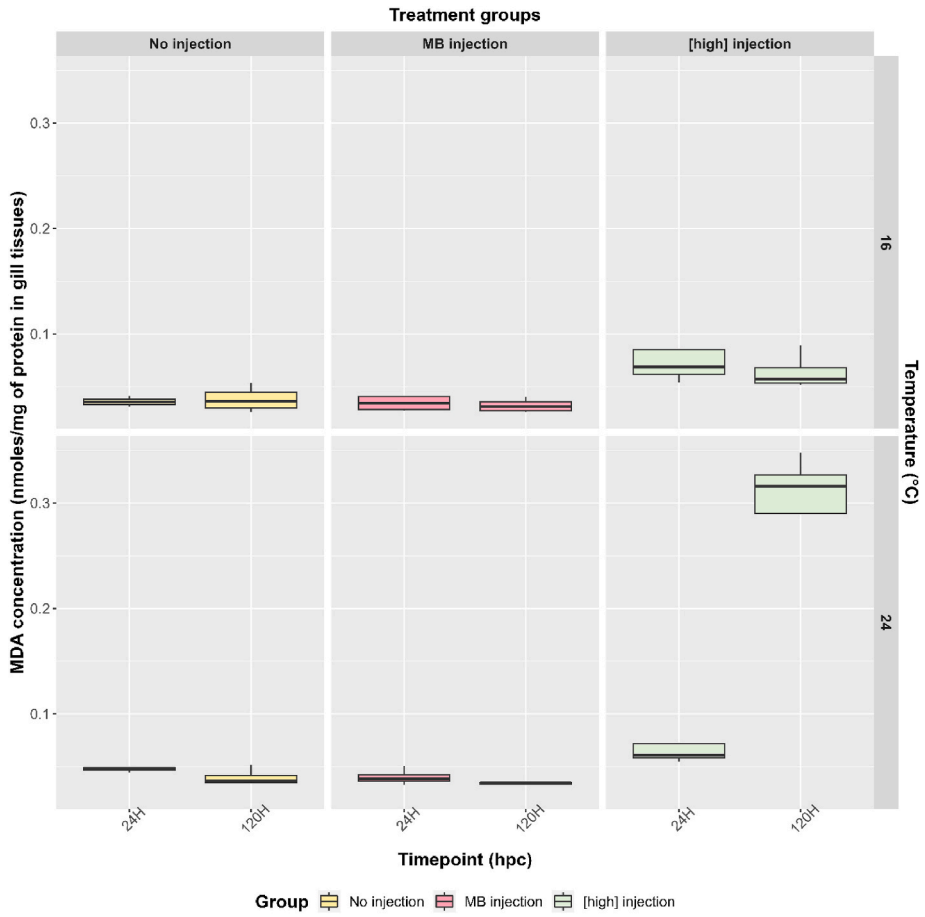

Fig. 8. The combined effects of temperature, treatment and time (hours post-challenge) on lipid peroxidation (LPO) measured as MDA equivalents normalised by total protein (TBARS) determined from mussel gill samples. Each panel compares the mean TBARS from *P. canaliculus* haemolymph of the no injection control, the marine broth (MB) injection control and injection with a high dose of *P swingsii*, at low and high seawater temperatures (16 and 24 °C), across two timepoints (24 and 120 hpc). Refer to Supplementary Excel File S1 for detailed pairwise comparisons based on the emmeans post-hoc test results.

proliferation in water and bacterial accumulation in mussel tissues, leading to stress and mortality (Rahman et al., 2019). The lack of mortality in the non-injected individuals at 24 °C, despite being suboptimal for the examined species, could be due to factors such as the relatively short 24-h observation period, during which immediate mussel mortality may not occur. Additionally, mussels may exhibit thermal tolerance and acclimate to gradual temperature changes, potentially explaining their survival under these conditions (Azizan et al., 2023b). 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 higher 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 confirms that *P. swingsii* is an aquatic pathogen that affects *P. canaliculus*.

To the best of our knowledge, previous studies on *P. canaliculus* have primarily focused on its antioxidant defence mechanisms in response to environmental abiotic factors (Dunphy et al., 2015; Delorme et al., 2021c), anthropogenic activities (Chandurvelan et al., 2012) or handling procedures (Nguyen et al., 2020; Venter et al., 2021). However, the influence of pathogens, either alone or synergistically with abiotic factors, has been relatively understudied. The current research aimed to investigate the activity of antioxidant and oxidative response in *P. canaliculus* in the context of summer mussel mortality, particularly considering the potential association with elevated temperatures. While previous research has extensively examined *P. canaliculus* populations facing mortality, it has mainly concentrated on the impacts of environmental stressors related to climate change. Surprisingly, the role of potential pathogenic microorganisms contributing to mussel mortality in New Zealand's aquaculture has received less attention. Our findings reveal that lipid peroxidation levels in gill tissue samples increased in mussels exposed to *P. swingsii* alone, with a further elevation observed at 24 °C in the presence of *P. swingsii*. These results are consistent with studies on various marine bivalves, suggesting a heightened oxidative stress response due to rising temperatures and pathogen presence (Rahman et al., 2019; Lattos et al., 2023a, 2023b). Therefore, it can be inferred that *P. swingsii* may induce an oxidative stress response in *P. canaliculus*, particularly under temperature conditions favourable for this pathogen's infestation. This highlights the potential role of pathogenic microorganism in the mass mortalities observed in this species.

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., 2023). Additionally, the rapid interpretation 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.

Funding

We are grateful to the New Zealand Ministry for Business, Innovation, and Employment, through the Cawthron Institute's Aquaculture Health Strategies to Maximise Productivity and Security programme and the Shellfish Aquaculture Research Platform (Contract Nos. CAWX1707 & CAWX, 1801) for providing funding for this research.

Ethical statement

Mussels do not fall under the Animal Welfare Act, 1999. Therefore, ethical approval for work using molluscs is not needed (except for cephalopods).

CRediT authorship contribution statement

Awanis Azizan: Writing – original draft, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Leonie Venter:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Formal analysis. **Jingjing Zhang:** Writing – review & editing, Validation, Software, Formal analysis, Data curation. **Tim Young:** Writing – review & editing, Validation. **Jessica A. Ericson:** Writing – original draft, Visualization, Software, Resources. **Natalí J. Delorme:** Writing – review & editing, Validation, Methodology. **Norman L.C. Ragg:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Andrea C. Alfaro:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

Thank you to SPATnz for supplying the mussels used in this experiment. A massive thank you to the scientists and technicians (Shenae Muirhead, Joanna Copedo, Jolene Berry, Hayley Chapman, Chaya Bandaranayake, Daniel Cross, Christopher Chamberlain, Jordan Elvy, Michael Scott, and Karthiga Kumanan) at the Cawthron Institute, Nelson, for assisting with logistics, experimental setup, and sampling. We are thankful to the Aquaculture Biotechnology Research Group at Auckland University of Technology (AUT) for their intellectual, technical, and financial support. A special thanks is given to the technicians at the School of Science at AUT for support during the preparation phases of this experiment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2024.106392.

References

Abele, D., Puntarulo, S., 2004. Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. Comp. Biochem. Physiol. Mol. Integr. Physiol. 138, 405–415.

Alfaro, A.C., Nguyen, T.V., Merien, F., 2019. The complex interactions of Ostreid herpesvirus 1, *Vibrio* bacteria, environment and host factors in mass mortality outbreaks of *Crassostrea gigas*. Rev. Aquacult. 11, 1148–1168.

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 Shellfish Immunol. 20, 384–397.

Allam, B., Paillard, C., Ford, S.E., 2002. Pathogenicity of *Vibrio tapetis*, the etiological agent of brown ring disease in clams. Dis. Aquat. Org. 48, 221–231.

Allam, B., Raftos, D., 2015. Immune responses to infectious diseases in bivalves. J. Invertebr. Pathol. 131, 121–136.

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.

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 (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 98, 1–14.

Azizan, A., Alfaro, A.C., Venter, L., Jaramillo, D., Bestbier, M., Bennet, P., , et al. Young, T., 2024. Quantification of Photobacterium swingsii and characterisation of disease progression in the New Zealand Greenshell™ mussel, Perna canaliculus. Journal of Invertebrate Pathology 108065 submitted for publication.

Azizan, A., Venter, L., Alfaro, A.C., 2023a. Physiological Biomarkers of Mussel Vibrionaceae: A Review on the Constraints and Potentials. Aquaculture international, Submitted.

Azizan, A., Venter, L., Van Resburgn, P.J.J., Ericson, J.A., Alfaro, A.C., Ragg, N.L.C., 2023b. Metabolite changes of *Perna canaliculus* following a laboratory marine heatwave exposure: insight from metabolomics analysis. Metabolites 13, 815.

Baker-Austin, C., Trinanes, J., Gonzalez-Escalona, N., Martinez-Urtaza, J., 2017. Non-cholera vibrios: the microbial barometer of climate change. Trends Microbiol. 25.

Barrick, A., Marion, J.M., Perrein-Ettajani, H., Châtel, A., Mouneyrac, C., 2018. Baseline levels of biochemical biomarkers in the endobenthic ragworm *Hediste diversicolor* as useful tools in biological monitoring of estuaries under anthropogenic pressure. Mar. Pollut. Bull. 129, 81–85.

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 Immunol. 70, 515–523.

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*. Comp. Biochem. Physiol. Mol. Integr. Physiol. 152, 278–289.

Bi, Y., Xue, B., Briscoe, D., Vennell, R., Zhang, M., 2023. A new artificial intelligent approach to buoy detection for mussel farming. J. Roy. Soc. N. Z. 53, 27–51.

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. Ecotoxicol. Environ. Saf. 138, 105–112.

Boulo, V., Hervio, D., Morvan, A., Bachere, E., Mialhe, E., 1991. In vitro culture of mollusc hemocytes. Functional study of burst respiratory activity and analysis of interactions with protozoan and procaryotic pathogens. 8th International conference on Invertebrate and Fish Culture. 56–64.

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.

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. N. Z. J. Mar. Freshw. Res. 55, 46–64.

Canesi, L., Gallo, G., Gavioli, M., Pruzzo, C., 2002. Bacteria-hemocyte interactions and phagocytosis in marine bivalves. Microsc. Res. Tech. 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.

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. Sci. Rep. 10.

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, p. 1.

Chandurvelan, R., Marsden, I.D., Gaw, S., Glover, C.N., 2012. Impairment of green-lipped mussel (*Perna canaliculus*) physiology by waterborne cadmium: relationship to tissue bioaccumulation and effect of exposure duration. Aquat. Toxicol. 124–125, 114–124.

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 Immunol. 70, 515–523.
Chiang, S.L., Mekalanos, J.J., Holden, D.W., 1999. In vivo genetic analysis of bacterial virulence. Annu. Rev. Microbiol. 53, 129–154.
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.
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 Immunol. 80, 71–79.
Delorme, N., Biessy, L., South, P., Zamora, L., Ragg, N., Burritt, D., 2020. Stress-on-stress responses of a marine mussel, *Perna canaliculus*: food limitation reduces the ability to cope with heat stress in juveniles. Mar. Ecol. Prog. Ser. 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., Schmidt, A.J., Zamora, L.N., Burritt, D.J., Ragg, N.L., 2021b. A new method to localise and quantify oxidative stress in live juvenile mussels. Biology Open 10, bio059030.
Delorme, N.J., Venter, L., Rolton, A., Ericson, J.A., 2021c. Integrating animal health and stress assessment tools using the green-lipped mussel *Perna canaliculus* as a case study. J. Shellfish Res. 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. J. Anim. Ecol. 88, 427–438.
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. melanogaster*. Elife 6, e28298.
Dunphy, B.J., Watts, E., Ragg, N.L.C., 2015. Identifying thermally-stressed adult green-lipped mussels (*Perna canaliculus* Gmelin, 1791) via metabolomic profiling. Am. Malacol. Bull. 33, 127–135.
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 (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 93, 1–11.
Eisenreich, W., Heesemann, J., Rudel, T., Goebel, W., 2013. Metabolic host responses to infection by intracellular bacterial pathogens. Front. Cell. Infect. Microbiol. 3, 24.
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 Immunol. 30, 1209–1222.
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). J. Shellfish Res. 24.
Ericson, J.A., Delorme, N.J., Ragg, N.L.C., 2023a. Heat Tolerance of Greenshell™ Mussels (*Perna canaliculus*) : Collated Research Findings and Implications for Mussel Farming. Report no: 3914, 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 Immunol. 119, 452–455.
Ericson, J.A., Venter, L., Copedo, J.S., Nguyen, V.T., Alfaro, A.C., Ragg, N.L.C., 2023b. 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 Shellfish Immunol. 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. Mar. Biol. 167, 1–15.
Farmer, E.E., Mueller, M.J., 2013. ROS-mediated lipid peroxidation and RES-activated signaling. Annu. Rev. Plant Biol. 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. Dis. Aquat. Org. 115, 147–156.
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. J. Invertebr. Pathol. 61, 49–57.
Fraga, C.G., Oteiza, P.I., Galleano, M., 2014. In vitro measurements and interpretation of total antioxidant capacity. Biochim. Biophys. Acta Gen. Subj. 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. Mar. Pollut. Bull. 102, 30–35.
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*. Front. Mol. Biosci. 8.
Froelich, B., Oliver, J.D., 2013. The iteractions of *Vibrio vulnificus* and the oster *Crassostrea virginica*. Microb. Ecol. 65, 807–816.
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*). Aquat. Living Resour. 24, 247–260.
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. Int. J. Syst. Evol. Microbiol. 61, 315–319.
Gutteridge, J.M.C., Halliwell, B., 2006. Free radicals and antioxidants in the year 2000: a historical look to the future. Ann. N. Y. Acad. Sci. 899, 136–147.
Hauton, C., Hawkins, L., Hutchinson, S., 2001. Response of haemocyte lysosomes to bacterial inoculation in the oysters *Ostrea edulis* L. and *Crassostrea gigas* (Thunberg) and the scallop *Pecten maximus* (L.). Fish Shellfish Immunol. 11, 143–153.
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. Front. Mar. Sci. 7, 565686.
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. J. Exp. Mar. Biol. Ecol. 293, 249–265.
Ivanina, A.V., Sokolova, I.M., 2016. Effects of intermittent hypoxia on oxidative stress and protein degradation in molluscan mitochondria. J. Exp. Biol. 219, 3794–3802.
Jimenez, A.G., Alves, S., Dallmer, J., Njoo, E., Roa, S., Dowd, W.W., 2016. Acclimation to elevated emersion temperature has no effect on susceptibility to acute, heat-induced lipid peroxidation in an intertidal mussel (*Mytilus californianus*). Mar. Biol. 163, 1–10.
Jockers, M.L., Thalken, R., L Jockers, M., Thalken, R., 2020. Introduction to Dplyr. *Text Analysis With R: for Students Of Literature*, pp. 121–132.
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. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 149, 631–639.
Kassambara, A., 2021. Rstatix: Pipe-Friendly Framework for Basic Statistical Tests. R package version 0.7. 0. Computer software]. CRAN.R-proje ct. org/packa ge= rstatix.
Kesarcodi-Watson, A., Kaspar, H., Lategan, M.J., Gibson, L.F., 2009. Challenge of New Zealand GreenshellTM mussel Perna canaliculus larvae using two *Vibrio* pathogens: a hatchery study. Dis. Aquat. Org. 86, 15–20.
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. Microb. Ecol. 77, 502–512.
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.
Lattos, A., Papadopoulos, D.K., Feidantsis, K., Giantsis, I.A., Georgoulis, I., Karagiannis, D., Michaelidis, B., 2023a. Antioxidant defense of *Mytilus galloprovincialis* mussels induced by marine heatwaves in correlation with *marteilia pathogen* presence. Fishes 8, 408.
Lattos, A., Papadopoulos, D.K., Feidantsis, K., Karagiannis, D., Giantsis, I.A., Michaelidis, B., 2022. Are marine heatwaves responsible for mortalities of farmed *Mytilus galloprovincialis*? A pathophysiologicalanalysis of *Marteilia* infected mussels from Thermaikos Gulf, Greece. Animals 12, 2805.
Lattos, A., Papadopoulos, D.K., Giantsis, I.A., Feidantsis, K., Georgoulis, I., Karagiannis, D., Carella, F., Michaelidis, B., 2023b. Investigation of the highly endangered *Pinna nobilis*' mass mortalities: seasonal and temperature patterns of health status, antioxidant and heat stress responses. Mar. Environ. Res. 188, 105977.
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. Int. J. Parasitol. 50, 75–83.
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). Front. Microbiol. 6.
Lesser, M.P., 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. Annu. Rev. Physiol. 68, 253–278.
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, 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.
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. Rev. Aquacult. 13, 504–536.
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.
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.
Matanza, X.M., Osorio, C.R., 2020. Exposure of the opportunistic marine pathogen *Photobacterium damselae* subsp. damselae to human body temperature is a stressful condition that shapes the transcriptome, viability, cell morphology, and virulence. Front. Microbiol. 11, 1771.

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*). Comp. Biochem. Physiol. Mol. Integr. Physiol. 164, 545–553.
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.
Monari, M., Matozzo, V., Foschi, J., Cattani, O., Serrazanetti, G.P., Marin, M.G., 2007. Effects of high temperatures on functional responses of haemocytes in the clam *Chamelea gallina*. Fish Shellfish Immunol. 22, 98–114.
Muznebin, F., Alfaro, A.C., Venter, L., Young, T., 2022. Acute thermal stress and endotoxin exposure modulate metabolism and immunity in marine mussels (*Perna canaliculus*). J. Therm. Biol. 110, 103327.
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.
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.
Nguyen, T.V., Alfaro, A.C., Merien, F., Young, T., Grandiosa, R., 2018. Metabolic and immunological responses of male and female New Zealand Greenshell™ mussels (*Perna canaliculus*) infected with *Vibrio* sp. J. Invertebr. Pathol. 157, 80–89.
Nguyen, T.V., Alfaro, A.C., Young, T., Merien, F., 2019a. 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., Merien, F., 2019b. Tissue-specific immune responses to *Vibrio* sp. infection in mussels (*Perna canaliculus*): a metabolomics approach. Aquaculture 500, 118–125.
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.
Nogueira, L., Mello, D.F., Trevisan, R., Garcia, D., Da Silva Acosta, D., Dafre, A.L., De Almeida, E.A., 2017. Hypoxia effects on oxidative stress and immunocompetence biomarkers in the mussel *Perna perna* (Mytilidae, Bivalvia). Mar. Environ. Res. 126, 109–115.
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. Nat. Commun. 8, 16101.
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). Aquat. Living Resour. 9, 137–143.
Paillard, C., Allam, B., Oubella, R., 2004. Effect of temperature on defense parameters in Manila clam *Ruditapes philippinarum* challenged with *Vibrio tapetis*. Dis. Aquat. Org. 59, 249–262.
Parisi, M.G., Li, H., Jouvet, L.B.P., Dyrynda, E.A., Parrinello, N., Cammarata, M., Roch, P., 2008. Differential involvement of mussel hemocyte sub-populations in the clearance of bacteria. Fish Shellfish Immunol. 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*. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 221, 1–9.
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.
Pruzzo, C., Gallo, G., Canesi, L., 2005. Persistence of vibrios in marine bivalves: the role of interactions with haemolymph components. Environ. Microbiol. 7, 761–772.
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 Immunol. 86, 868–874.
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. Comp. Biochem. Physiol. Mol. Integr. Physiol. 164, 629–637.
Reverchon, S., Nasser, W., 2013. Dickeya ecology, environment sensing and regulation of virulence programme. Environmental microbiology reports 5, 622–636.
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*. Aquacult. Int. 1–17.
Ripley, B., Venables, B., Bates, D.M., Hornik, K., Gebhardt, A., Firth, D., Ripley, M.B., 2013. Package 'mass'. Cran r 538, 113–120.
Rolton, A., Ragg, N.L., 2020. Green-lipped mussel (*Perna canaliculus*) hemocytes: a flow cytometric study of sampling effects, sub-populations and immune-related functions. Fish Shellfish Immunol. 103, 181–189.
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. Org. 108, 149–163.
Rosa, M., Ward, J.E., Shumway, S.E., 2018. Selective capture and ingestion of particles by suspension-feeding bivalve molluscs: a review. J. Shellfish Res. 37, 727–746.
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. Front. Immunol. 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. J. Therm. Biol. 104, 103154.
Samain, J.F., 2011. Review and perspectives of physiological mechanisms underlying genetically-based resistance of the Pacific oyster *Crassostrea gigas* to summer mortality. Aquat. Living Resour. 24, 227–236.
Saulnier, D., Haffner, P., Goarant, C., Levy, P., Ansquer, D., 2000. Experimental infection models for shrimp vibriosis studies: a review. Aquaculture 191, 133–144.
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 Res. 153.
Sokolova, I., Evans, S., Hughes, F., 2004. Cadmium-induced apoptosis in oyster hemocytes involves disturbance of cellular energy balance but no mitochondrial permeability transition. J. Exp. Biol. 207, 3369–3380.
Sokolova, I.M., 2023. Ectotherm mitochondrial economy and responses to global warming. Acta Physiol. 237, e13950.
Soon, T.K., Zheng, H., 2020. Climate change and bivalve mass mortality in temperate regions. Rev. Environ. Contam. Toxicol. 251, 109–129.
Srinivasan, R., Carey-Smith, T., Macara, G., 2021. Trend analysis on frequency of New Zealand climate extremes. Weather Clim. 41, 18–29.
Stevens, C.L., O'Callaghan, J.M., Chiswell, S.M., Hadfield, M.G., 2021. Physical oceanography of New Zealand/Aotearoa shelf seas–a review. N. Z. J. Mar. Freshw. Res. 55, 6–45.
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. J. Exp. Mar. Biol. Ecol. 497, 172–179.
Thorvaldsen, S., Hjerde, E., Fenton, C., Willassen, N.P., 2007. Molecular characterization of cold adaptation based on ortholog protein sequences from Vibrionaceae species. Extremophiles 11, 719–732.
Tresnakova, N., Famulari, S., Zicarelli, G., Impellitteri, F., Pagano, M., Presti, G., Filice, M., Caferro, A., Gulotta, E., Salvatore, G., 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.
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*. J. Exp. Mar. Biol. Ecol. 391, 65–72.
Venter, L., Alfaro, A.C., Ragg, N.L., Delorme, N.J., Ericson, J.A., 2023. The effect of simulated marine heatwaves on green-lipped mussels, *Perna canaliculus*: a near-natural experimental approach. J. Therm. Biol., 103702
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.
Wang, J., Dong, B., Yu, Z.-X., Yao, C.-L., 2018. The impact of acute thermal stress on green mussel *Perna viridis*: oxidative damage and responses. Comp. Biochem. Physiol. Mol. Integr. Physiol. 222, 7–15.
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*. Mar. Pollut. Bull. 63, 201–208.
Waters, P., Lloyd, D., 1985. Salt, pH and temperature dependencies of growth and bioluminescence of three species of luminous bacteria analysed on gradient plates. Microbiology 131, 2865–2869.
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*). Mar. Environ. Res. 24, 301–305.
Wickham, H., Chang, W., Wickham, M.H., 2016. Package 'ggplot2'. *Create Elegant Data Visualisations Using the Grammar of Graphics*, 2. Version, pp. 1–189.
Wickham, H., Wickham, M.H., 2017. Package Tidyverse. *Easily Install And Load The 'Tidyverse*.
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 Shellfish Immunol. 56, 554–562.
Xia, Q., Wang, B., Liu, M., Jiang, K., Wang, L., 2015. A new method to evaluate the effects of bacterial dosage, infection route and *Vibrio* strain in experimental challenges of *Litopenaeus vannamei*, based on the Cox proportional hazard model. Fish Shellfish Immunol. 46, 686–692.
Xu, D., Wu, J., Sun, L., Qin, X., Fan, X., Zheng, X., 2021. Combined stress of acute cold exposure and waterless duration at low temperature induces mortality of shrimp *Litopenaeus vannamei* through injuring antioxidative and immunological response in hepatopancreas tissue. J. Therm. Biol. 100, 103080.
Yang, Q., Guo, K., Zhou, X., Tang, X., Yu, X., Yao, W., Wu, Z., 2021. Histopathology, antioxidant responses, transcriptome and gene expression analysis in triangle sail mussel *Hyriopsis cumingii* after bacterial infection. Dev. Comp. Immunol. 124.
Yang, Q., Xiao, G., Chen, R., Huang, X., Teng, S., 2023. Immune responses of hemocytes in the blood clam *Tegillarca granosa* in response to in vivo *Vibrio harveyi* infection. Fish Shellfish Immunol. 132, 108447.
Yung, C.M., Vereen, M.K., Herbert, A., Davis, K.M., Yang, J., Kantorowska, A., Ward, C. S., Wernegreen, J.J., Johnson, Z.I., Hunt, D.E., 2015. Thermally adaptive tradeoffs in closely related marine bacterial strains. Environ. Microbiol. 17, 2421–2429.
Zhang, Y.-Y., Yu, S.-E., Wang, W.-L., Yang, L.-E., Lu, Q.-Q., Xie, C.-T., Liu, T., Dong, Y.-W., 2023. Temperature Sensitivity of Marine Macroalgae for Aquaculture in China. Aquaculture, 739262.