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The effect of simulated marine heatwaves on green-lipped mussels, *Perna canaliculus*: A near-natural experimental approach

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ABSTRACT

Marine heatwaves (MHW) are projected for the foreseeable future, affecting aquaculture species, such as the New Zealand green-lipped mussel (*Perna canaliculus*). Thermal stress alters mussel physiology highlighting the adaptive capacity that allows survival in the face of heatwaves. Within this study, adult mussels were subjected to three different seawater temperature regimes: 1) low (sustained 18 °C), 2) medium MHW (18–24 °C, using a +1 °C per week ramp) and 3) high MHW (18–24 °C, using a +2 °C per week ramp). Sampling was performed over 11 weeks to establish the effects of temperature on *P. canaliculus* survival, condition, specific immune response parameters, and the haemolymph metabolome. A transient 25.5–26.5 °C exposure resulted in 61 % mortality, with surviving animals showing a metabolic adjustment within aerobic energy production, enabling the activation of molecular defence mechanisms. Utilisation of immune functions were seen within the cytology results where temperature stress affected the percentage of superoxide-positive haemocytes and haemocyte counts. From the metabolomics results an increase in antioxidant metabolites were seen in the high MHW survivors, possibly to counteract molecular damage. In the high MHW exposure group, mussels utilised anaerobic metabolism in conjunction with aerobic metabolism to produce energy, to uphold biological functions and survival. The effect of exposure time was mainly seen on very long-, and long chain fatty acids, with increases observed at weeks seven and eight. These changes were likely due to the membrane storage functions of fatty acids, with decreases at week eleven attributed to energy metabolism functions. This study supports the use of integrated analytical tools to investigate the response of marine organisms to heatwaves. Indeed, specific metabolic pathways and cellular markers are now highlighted for future investigations aimed at targeted measures. This research contributes to a larger program aimed to identify resilient mussel traits and support aquaculture management.

1. Introduction

The New Zealand (NZ) green-lipped mussel (*Perna canaliculus*, Gmelin 1791) is a culturally important endemic bivalve species, harvested commercially and recreationally (Webb et al., 2020). In NZ, the mussel farming industry is the largest aquaculture sector by means of production quantity and economic value, with frozen half shell mussels, whole animals and mussel oil mainly exported. As the increasing demand for farmed seafood surpasses production, mussel aquaculture will play an important part in the NZ government's aquaculture strategy of achieving a goal of $3 billion in annual sales by 2030 (Fisheries'New'Zealand, 2021). Climate extremes, such as marine heatwaves (MWH) affect the coastal regions of NZ which houses aquaculture facilities and commercial and recreational fishing grounds (Behrens et al., 2022). In brief, MHW are defined as periods of five days (or more) when temperature exceeds the 90th percentile of the local climatology (Smith et al., 2023). Recently MHW have been reported in NZ during the austral summer of 2021/22, 2018/19 and 2017/18 (Salinger et al., 2023). Marine organisms, such as mussels have limited mobility which in-effect makes them highly susceptible to MHW (Xu et al., 2023), with increased disease outbreaks and mortalities occurring in aquaculture species due to warm water anomalies (Heasman et al., 2020).

Green-lipped mussels typically thrive between temperatures of 16–19 °C, with deleterious effects often seen at higher temperatures. The

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species occurs naturally across the entire latitudinal range of New Zealand, with widely distributed sheltered areas, such as the Marlborough Sounds (41°S), Firth of Thames (37°S) and Stewart Island (46°S), supporting the majority of its aquaculture activity (Stevens et al., 2021). All these regions are being affected by increasing seawater temperatures. In the Firth of Thames, a 20% drop of mussels available for harvest from long-line culture was seen at temperatures of 25 °C (Peart, 2019), while sea surface temperatures upwards of 22 °C are regularly recorded in this region (Stevens et al., 2021). Climate change projections indicate that average seawater temperatures will continue to rise, and marine heatwaves will increase in frequency (Zhai et al., 2021). Mean ocean temperatures in the Cook Strait (41°S) are predicted to rise by +1–3 °C by the year 2100, and can possibly influence sea temperatures in the Marlborough Sounds, where current sea surface temperatures already exceed 20 °C during summer months (Broekhuizen et al., 2021). Prolonged exposure to artificially-maintained, stable temperatures of 21–24 °C caused *P. canaliculus* growth rates to decline and immune system stress, apparent as increased incidence of respiratory burst and apoptosis in haemocytes. The mussels also reduced investment in gonadogenesis and ultimately showed elevated mortality after a number of months of exposure (Ericson et al., 2023a). While this identifies chronic thermosensitivity that may influence the northern distribution limit for the species, the implications of transient heatwaves remain poorly understood.

The effect of such heatwave events on mussel physiology has become a popular recent research topic, with reports linking mortalities to temperature (and other stressors), in mussels, such as, *P. canaliculus* (Li et al., 2020; Nguyen and Alfaro, 2020), *Mytilus edulis* (Nielsen et al., 2021) and *M. galloprovincialis* (Lupo et al., 2021). Even though it is well known that thermal stress affects mussel physiological functions, general health and metabolic regulation (Marigomez et al., 2017), specific measurement of physiological response is crucial to better understand how a target organism interacts with specific environments at different seasonal and spatial scales. Data on biomarkers linked to the physiological response of mussels to a stressor can be utilised to gain resilience in a changing environment, to improve farming and processing efficiency and to add value in terms of market-desired traits (Fisheries'New'Zealand, 2021).

The routine monitoring of sublethal indicators, such as key haemolymph metabolites or immune parameters could serve as early warnings of declining conditions in farmed mussels (Waller and Cope, 2019). For example, changes in metabolic pathways due to abiotic stressors can compromise immune functions, resulting in organisms which are more susceptible to pathogen exposures. Such changes can be monitored utilising metabolomics approaches, targeting the phenotype of an organism, expanding on metabolic and physiological responses (Alfaro and Young, 2018). Immunological measures targeting haemocytes are also critical indicators of bivalve health and disease status (Waller and Cope, 2019), as demonstrated by flow cytometry assays performed on green-lipped mussels (Van Nguyen and Alfaro, 2019; Rolton and Ragg, 2020). The measurement of the total antioxidant capacity of a sample complements flow cytometry measures, enabling insights into the ability of organisms to scavenge free radicals in the haemolymph, and manage the generation of reactive oxygen species (ROS) (Delorme et al., 2021). Ultimately, environmental changes affect mussel energetics, resulting in different allocation patterns for investment in tissues, compromising mussel survival. By measuring the condition index, a ratio between the weight of soft tissue and the weight of the shell is achieved, which is a proxy for health and condition of the animal (Babarro et al., 2020). The use of integrated physiological assessments (biomarkers) to better understand cellular and biochemical responses of mussels in nature and experiments is an important step to develop and well-define normal and abnormal ranges. In the case of marine heatwaves, defined reference ranges can help to facilitate risk assessment and environmental management decisions, while advancing knowledge in this field and increasing the application of biomarkers as monitoring tools.

The aim of this study was to determine how near-natural experimental conditions, simulating marine heatwaves, affect the survival, condition index, selected immune parameters and the haemolymph metabolome of adult *P. canaliculus*. Particular attention was given to the *rate of onset* and the *duration* of the simulated heatwaves.

2. Materials and methods

2.1. Experimental design

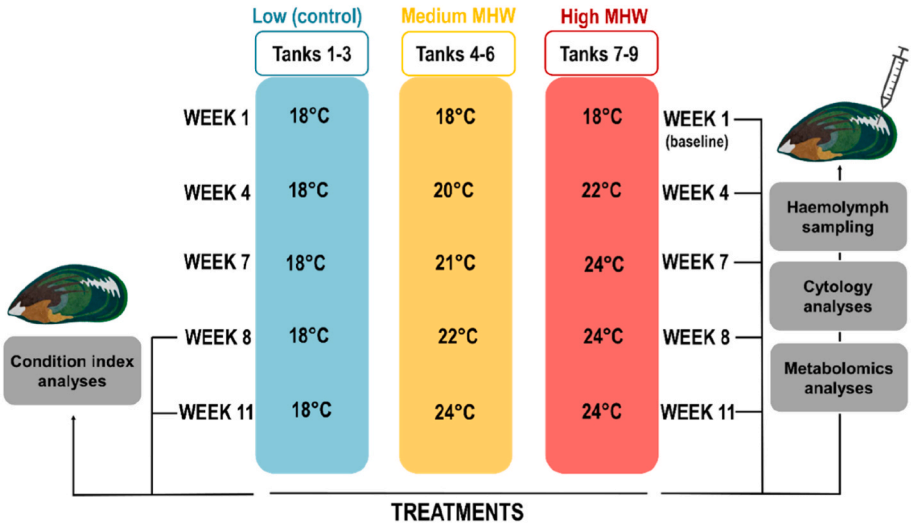
Adult *Perna canaliculus* (105 ± 9 mm shell length, 80 ± 18 g live mass [mean ± SD]) were obtained from a Sanford Limited mussel farm, located in the Marlborough Sounds (New Zealand). The mussels were transported to the Cawthron Aquaculture Park (Nelson, New Zealand), and placed in a flow-through seawater system at ambient temperatures (17.9 °C ± 0.3 [mean ± SD]) upon arrival. The tanks were aerated with air-stones and had a water flow rate of 2–3 L/min. During an eight-week acclimation period, mussels were fed a diet *ad libitum* (from eutrophic algal culture ponds), accompanied with *Tisochrysis lutea* (previously *Isochrysis galbana*) microalgae. Seawater temperatures were logged in 5 min intervals during the trial using HOBO water temp Pro v2 ONSET loggers.

For experimental purposes, mussels (n = 675) were divided into nine tanks (75 mussels per tank). All tanks were kept at 17.8 ± 0.4 °C for the first experimental week (Fig. 1). Three tanks remained at a stable temperature of 18.0 ± 0.5 °C for the duration of the 11-week trial (hereafter termed the 'low' temperature treatment), while another three tanks were increased to 24 °C at a moderate rate (approx. +1 °C per week; hereafter termed the 'medium' MHW temperature treatment) reaching 24 °C at week 10. The remaining three tanks were increased to 24 °C at a faster rate (approx. ± 2 °C per week; hereafter termed the 'high' MHW temperature treatment), reaching 24 °C at week 6. A controller malfunction during week 6 interrupted temperature ramping, causing water temperature to rise rapidly from 23.2 to 25.3 °C over 12 h and then to a maximum of 26.5 °C four days later, before decreasing steadily to ~24 °C over a further 2 d (see supplementary Fig. 1S for finer detail on the 6 d temperature event). The temperature was then held at 24 °C for the remainder of the experiment (11 weeks in total). The increase in water temperature of all tanks was achieved with the use of submersible titanium and glass aquarium heaters and was monitored daily. The increase in temperature was based on historic data collected from studies focusing on the thermotolerance of Greenshell™ mussels where subchronic thermotolerance is achieved when elevated seawater temperatures (22–27 °C) is sustained for days to weeks (Ericson et al., 2023b).

Nine animals were sampled in week 1 of the trial to obtain baseline measurements for all parameters. At weeks 4, 7, 8 and 11, animals were sacrificed and dissected for cytology and metabolomics analyses (n = 9 per sampling, except for weeks 8 and 11 where only 6 individuals were sampled from the high temperature treatment due to increasing mortalities, and less individuals were available for sampling). An additional nine animals were sampled per treatment for condition indices at weeks 1, 8 and 11 (with the exception of n = 6 and n = 4 mussels sampled from the high temperature treatment in weeks 8 and 11 respectively, due to higher mussel mortalities in those treatments). Mortalities were assessed and recorded daily (Dunphy et al., 2015) for the duration of the challenge.

2.2. Sample collection

Animals were sampled in a semi-random order from tanks, to remove any time bias from the sampling regime. Prior to sampling, mussels were weighed to the nearest 0.01 g and the shell lengths (along the longest axis) were measured to the nearest 0.10 mm. Mussel shells were gradually opened via the ventral posterior side to access the posterior adductor muscle. Using a pre-chilled needle and syringe, approximately 600 μL haemolymph were collected, with a subsection used for cytology



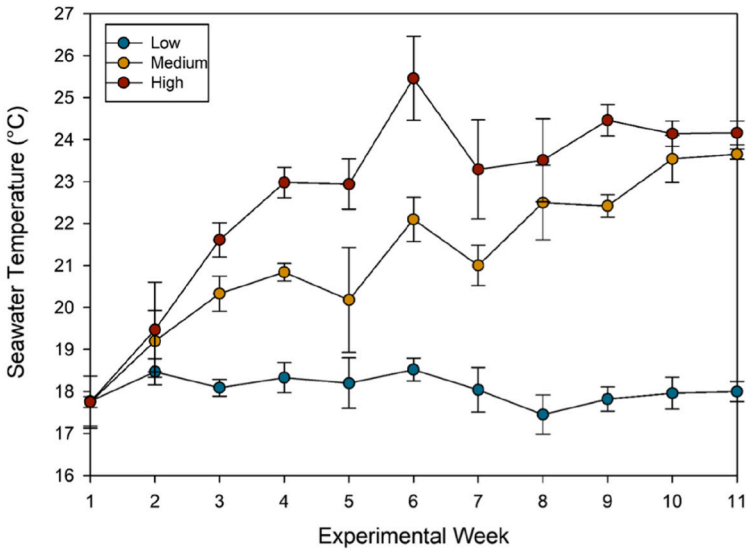


Fig. 1. Experimental approach highlighting the three experimental groups subjected to temperature exposures (low, medium, and high) during each experimental week of the heatwave simulation. Sampling was conducted at weeks 1 (baseline), 4, 7, 8 and 11. Seawater temperature is reported as mean ± SD temperature over the 11-week trial. A controller malfunction during week 6 interrupted temperature ramping in the high MHW treatment, causing water temperature to rise rapidly from 23.2 to 25.3 °C over 12 h and then to a maximum of 26.5 °C four days later, before decreasing steadily to ~24 °C over a further 2 d.

analyses (200 μL) kept on ice, and the second part used for metabolomics analysis (400 μL of haemolymph along with 20 μL internal standard - 10 mM L-alanine-2,3,3,3-d_4), which was snap-frozen using liquid nitrogen and placed in a −80 °C freezer until metabolomics analyses were performed. Additional mussels were utilised to determine the condition index with wet weights for both the flesh and shells recorded.

2.3. Condition index

Flesh and shells that were separated from each mussel (as described in section 2.2) were dried at 100 °C for 24 h to ensure moisture was completely removed. A dry flesh weight and shell weight were then obtained for each individual after which the condition index was calculated using the following equation (Ibarrola et al., 2017):

$$\text{Condition index} = \frac{Dry\ flesh\ weight\ (g)}{Dry\ flesh\ weight\ (g) + Dry\ shell\ weight\ (g)}$$

2.4. Cytology analyses

Haemolymph samples on ice were analysed using a Muse® Cell Analyzer (Luminex Corporation), and Muse Oxidative Stress assay kit (MCH100111) to measure the haemocyte count and percentages of superoxide positive (SO+) haemocytes in each sample. All haemolymph samples were run on the flow cytometer within 1.5 h of haemolymph being removed from the animal (Rolton and Ragg, 2020). A 20 μL volume of haemolymph was pipetted into 180 μL of ROS assay kit reagent, vortexed and incubated at room temperature (17 °C) for 30 min. The Muse® Cell Analyzer was set to acquire results at 3000 events. The gate settings of the instrument were set using a positive control of haemolymph mixed with diluted hydrogen peroxide (H_2O_2) (Delorme et al., 2021).

A handheld device (e-BQC, Bioquochem S.L.) was used to measure the total antioxidant capacity (TAC). Levels of both fast-acting antioxidants (Q1) and slow-acting antioxidants (Q2) were obtained. Seventy μL of haemolymph were added onto a disposable e-BQC strip to obtain the antioxidant capacity values (Delorme et al., 2021).

2.5. GC-MS metabolomics analysis

Firstly, the frozen haemolymph (400 μL of haemolymph along with 20 μL internal standard - 10 mM L-alanine-2,3,3,3-d_4) were dried using a refrigerated vapor trap SpeedVac concentrator (Thermo Scientific, New Zealand). Metabolite extractions were performed by adding 500 μL of a cold 1:1 methanol:water solution to the dried samples, vortexing the mixture for 1 min and centrifuging the sample for 10 min at 20 800 *g* at -4 °C. The extraction step was repeated as above but using 500 μL of a cold 4:1 methanol:water solution. The supernatants of both extraction

steps were combined and dried using a SpeedVac (Venter et al., 2021).

Secondly derivatisation by methyl chloroformate (MCF) alkylation was performed (Smart et al., 2010). In brief 400 μL 1 M sodium hydroxide were added to the dried extracts, which was then transferred to salinized borosilicated glass tubes. Next 334 μL of methanol and 68 μL of pyridine was added to the glass tubes. The following reagents were added in a timely manner followed by vortexing before the addition of the next: 40 μL of MCF reagent - 30s, 40 μL of MCF - 30s, 400 μL of chloroform - 10s, and 800 μL of 50 mM sodium bicarbonate – 10s. Next the tubes were centrifuged at 1174 *g* for 5 min at 6 °C. Following removal of the upper aqueous layer and the addition of anhydrous sodium sulphate, the chloroform phase (with the MCF derivatives) was transferred to inserts in a GC glass vial and subjected to GC-MS analyses. The MCF derivatives were analysed with an Agilent GC7890B and autosampler coupled to a MSD5977A (Agilent Technologies) as described by Venter et al. (2021).

Quality controls (QC) samples (pooled from volumes of the biological samples) (Broadhurst et al., 2018), were included amongst the biological samples, to assess instrument repeatability and assist with potential batch effects in the data. Furthermore, a derivatised sample blank containing the internal standard, an in-house prepared derivatised standard amino acid mix, a non-derivatised standard alkane mix (Supelco 49451-U, Merck), and a sample of pure chloroform solvent were also injected and analysed for QC purposes (Young et al., 2019).

2.6. Data processing and statistical analyses

2.6.1. Survival data

Survival data were analysed in RStudio (v April 1, 1717) using the "survival" package. A Kaplan Meier analysis and a log-rank test were conducted on survival data, with temperature treatment as a factor (low, medium- and high MHW ramp).

2.6.2. Condition index data

Baseline measurements of condition index (n = 9 animals) are included in the results and figures but were not included in the statistical analysis because they represented the starting population before animals had been allocated to experimental treatments. Condition index data for weeks 8 and 11 were analysed in RStudio (v April 1, 1717). Data were analysed using a Two-Way ANOVA, with week (2 levels: weeks 8 and 11) and treatment (low, medium- and high MHW ramp) as factors. A Shapiro-Wilk and Levene's test were run to ensure that data met ANOVA assumptions of normality and homogeneity of variances. Tukey pairwise comparisons were conducted to compare differences between factor levels. Alpha was set at 0.05 and all tests were two-tailed.

2.6.3. Cytology data

Baseline measurements of haemocyte count, (n = 9 animals), oxidative stress (n = 9 animals) and antioxidant capacity (n = 9 animals) are included in the results and figures but were not included in the statistical analysis because they represented the starting population before animals had been allocated to experimental treatments. Cytology data for weeks 4, 7, 8 and 11 were analysed in Sigmaplot (v 14.0). Data were analysed using Two-Way ANOVA, with week (4 levels: weeks 4, 7, 8 and 11) and treatment (low, medium- and high MHW ramp) as factors. Shapiro-Wilk and Brown-Forsythe tests were run to ensure that data met ANOVA assumptions of normality and homogeneity of variances. Haemocyte count data were log10 transformed and superoxide positive (SO+) data were arcsin-square root transformed to ensure they met these assumptions. Bonferroni tests were used to compare differences between factor levels. Alpha was set at 0.05 and all tests were two-tailed.

2.6.4. Metabolomics data

Metabolite spectra were processed with Automated Mass Spectral Deconvolution and Identification System (AMDIS v2.66) software, while peak integration and metabolite identifications (≥70% match to MS spectra and retention time) were done with Chemstation Software (Agilent Technologies) and customised R-XCMS based scripts (Aggio et al., 2011). Compounds, with metabolites names were identified to the highest confidence level, (and level 2 in the case of unknowns) (Schymanski et al., 2014). Data were blank-corrected, peak intensities were quality checked to meet the distributional requirements prior to online statistical analyses with MetaboAnalyst 5.0 (Pang et al., 2021). Data were normalised against the internal standard, and in combination with a systematic error removal using random forest normalisation approach (Fan et al., 2019).

Within MetaboAnalyst, generalised log (glog) transformed was performed to alleviate the dependency of the variance on the compound concentrations (van den Berg et al., 2006). Two-way ANOVA was used to determine the influence of time (week 4, 7, 8, 11) and treatment (low, medium-, high MHW ramp) on the metabolite response of mussels (between subject, $p < 0.05$; false discovery rate [FDR] ≤ 0.05). Clustering as a heatmap and principal component analysis (provided as supplementary data) were used to visualise metabolic difference influence by time and treatment, based on the average abundance of the metabolites ($p < 0.05$) detected (Chong et al., 2019). Features with an interaction effect were plotted as mean values. Metabolites affected by temperature treatments were manually incorporated into a metabolic map.

3. Results

3.1. Survival

Seawater temperature had a significant effect on adult mussel survival (Kaplan-Meier Log-rank $X^2 = 344$, $p < 0.0001$). No mussel mortalities occurred in the low and medium temperature treatments, resulting in 100% survival after 11 weeks (Fig. 2). In the high MHW treatment, a transient 6 d increase in temperature to 25–26.5 °C during week 6 (Fig. 1; summarised in Supplementary Fig. 1S) resulted in significant mortalities, whereafter only 39% of the mussels survived by week 8. Unsurprisingly, the proportion of mussels surviving in the high temperature treatment was significantly lower than the other treatments ($p < 0.001$).

3.2. Condition index

The baseline condition index of *P. canaliculus* at week 1 was 0.16 ± 0.01 (mean ± SE). From week 8 onwards, the condition index of mussels

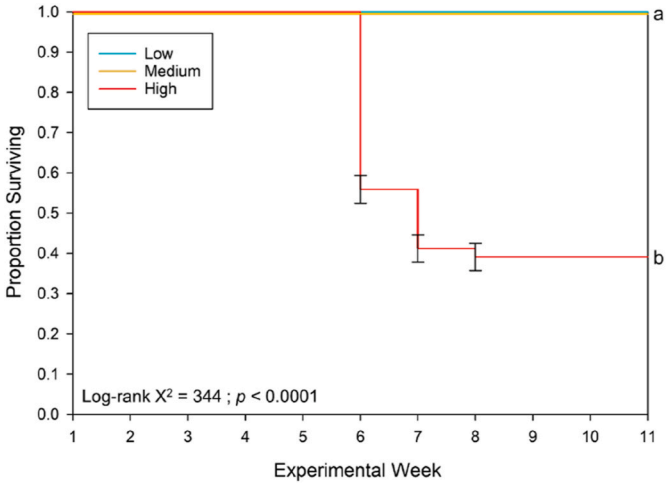


Fig. 2. Kaplan-Meier survival trajectories of *P. canaliculus* in each of the MHW scenarios (low, medium, and high) over the 11-week experiment. Error bars are standard error, demonstrating between-tank variation. Differences between treatments are denoted with lower case letters.

differed between temperature treatments, but not weeks (Fig. 3) (Two-Way ANOVA; treatment $p < 0.001$, week $p = 0.701$). The effect of treatment was not different between weeks (Two-Way ANOVA; treatment*week $p = 0.212$). Mussels in the high MHW treatment had a lower condition index than mussels in the low and medium MHW treatments during both sampling weeks (Tukey post-hoc; low vs medium $p = 0.187$, low vs high $p = 0.0005$, medium vs high $p = 0.033$).

3.3. Cytology

Baseline haemocyte counts were $7.9 \times 10^5 \pm 1.6 \times 10^5$ cells/mL (mean ± SE) during week 1 of the experiment (Fig. 4). Haemocyte counts did not differ between weeks or treatments, and there was no interaction between week and treatment (Two-Way ANOVA; sample week $p = 0.372$, temperature $p = 0.706$, temperature*week $p = 0.095$).

Baseline percentages of superoxide positive (SO+) haemocytes were 8.9% ± 1.3 (mean ± SE) at week 1 (Fig. 5). Percentages of SO+ haemocytes in *P. canaliculus* differed between the low, medium- and high MHW treatments during some sampling weeks (Two-Way ANOVA; temperature*week $p < 0.001$). SO+ percentages were significantly higher in mussels in the high temperature treatment (17.5 ± 2.1) compared with the low temperature treatment (10.0% ± 1.5) during week 7 (Bonferroni post-hoc; $p = 0.018$). During week 8, SO+ percentages were lower in mussels in the medium treatment (14.2% ± 1.0) compared to the low treatment (24.5% ± 3.4) (Bonferroni post-hoc; $p = 0.005$), and in week 11, SO+ percentage levels were higher in mussels at the low temperature (8.1% ± 1.4) compared to those in the high MHW treatment (3.1% ± 0.5) (Bonferroni post-hoc; $p = 0.047$) (Fig. 5A).

During week 1, baseline levels of fast-acting antioxidants (Q1) were 1.8 µC ± 0.1 (mean ± SE) and slow-acting antioxidants (Q2) were 11.5 µC ± 0.3 (mean ± SE) (Fig. 5B and C). Levels of Q1 differed between weeks, but not between temperature treatments (Two-way ANOVA; week $p = 0.010$, temperature $p = 0.835$). Levels of Q2 also differed between weeks, but not between temperature treatments (Two-way ANOVA; week $p < 0.001$, temperature $p = 0.511$). Levels of Q1 were slightly lower in week 7 (1.30 µC ± 0.1) compared to week 4 (1.64 µC ± 0.1) and week 11 (1.65 µC ± 0.1) (Bonferroni post-hoc; $p < 0.028$). Levels of Q2 were lower in week 7 (10.0 µC ± 0.4) compared to week 4 (11.6 µC ± 0.2), week 8 (11.8 µC ± 0.5) and week 11 (12.0 µC ± 0.3) (Bonferroni post-hoc; $p < 0.013$). For both Q1 and Q2, there was no interaction between temperature and week (Two-Way ANOVA; temperature*week $p = 0.920$ and $p = 0.656$, respectively).

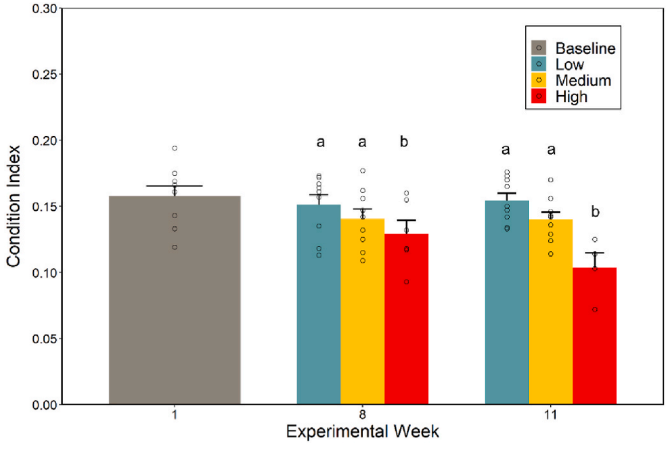


Fig. 3. Mean condition indices (±SE) of adult *P. canaliculus* during week 1 (baseline measurement), week 8 and week 11 of the experiment. Differences between temperature treatments (low, medium- and high MHW ramp) within each experimental week are denoted with different lower-case letters. Open circles on the bars represent the raw data. Baseline measurements during week 1 were not included in the statistical analyses but are shown for reference.

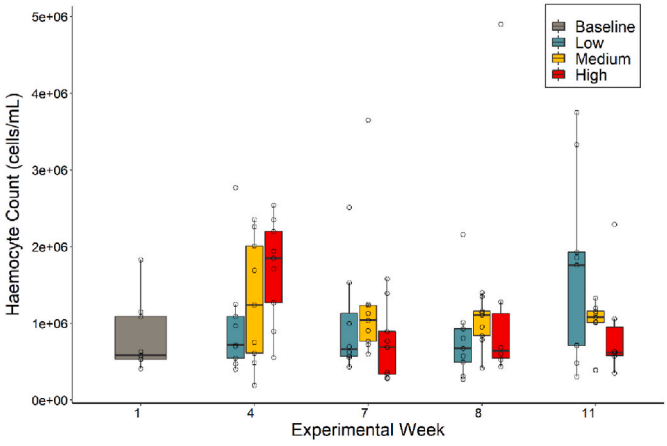


Fig. 4. Haemocyte counts (cells/mL) of adult *P. canaliculus* exposed to three different thermal treatments (low, medium- and high MHW ramp) for 11-weeks. Baseline measurements before treatments were applied are shown in week 1. Individual boxes represent the median, upper, and lower quartiles, and minimum and maximum values.

3.4. Metabolomics

From the Two-way ANOVA results, the concentration of 54 features (42 identified metabolites) were affected by experimental exposure time. For the most part, an increase of metabolite concentration was seen at weeks 7 and 8, followed by a decrease at week 11 (Fig. 6A). A total of 50 features (34 identified metabolites) were significantly different amongst the temperature treatment groups, showing an increase in metabolite concentration with an increase in temperature exposure (Fig. 6B). These differences are reflected in the 2D colour heatmap, providing an overview of the data values. In both instances the metabolites detected as statistically significant between experimental groups are seen in rows with colour changes to portray differences in peak intensity values. The interaction between time and treatment resulted in 4 significant features (1 identified metabolite), with varying metabolite responses (Fig. 6C). The interaction between time and treatment is future visualised via principal component analysis (PCA), focusing on the grouping of metabolite findings per sampling timepoint (Fig. 2S). Additionally, heatmaps of significantly different features are highlighted per timepoint (Fig. 3S). A metabolic map (Fig. 7) was manually constructed to visualise the significant metabolite markers (Supplementary Table 1), in the medium and high temperature groups (unknown compounds not included). Significantly altered metabolites are depicted by an increase (▲) or decrease (▼) in metabolite abundance.

4. Discussion

Elevated ocean temperatures can influence bivalve immune systems, metabolic rate, growth and survival (Ewere et al., 2021), requiring an integrated view of the physiological response of marine organisms to the thermal stress induced by marine heatwaves (Xu et al., 2021). In the current study, simulated heatwaves to 24 °C, using a medium (approx. +1 °C per week) and high (approx. ± 2 °C per week) ramp approach, altered the condition, haemocyte response and metabolic functioning of *P. canaliculus* over an 11-week sampling period.

The haemocytes with the ability to respond to environmental stress, showed and interesting response, reflecting a tendency to increase in total haemocyte counts during week 4 of this experiment, especially in the high MHW ramp treatment (albeit statistically insignificant due to high variability between individuals). This result corresponds to findings in *M. galloprovincialis*, where high temperature exposure (25 °C) increased the total haemocyte counts after 14 days (Rahman et al., 2019). Haemocyte counts in the current study in the heat-ramp

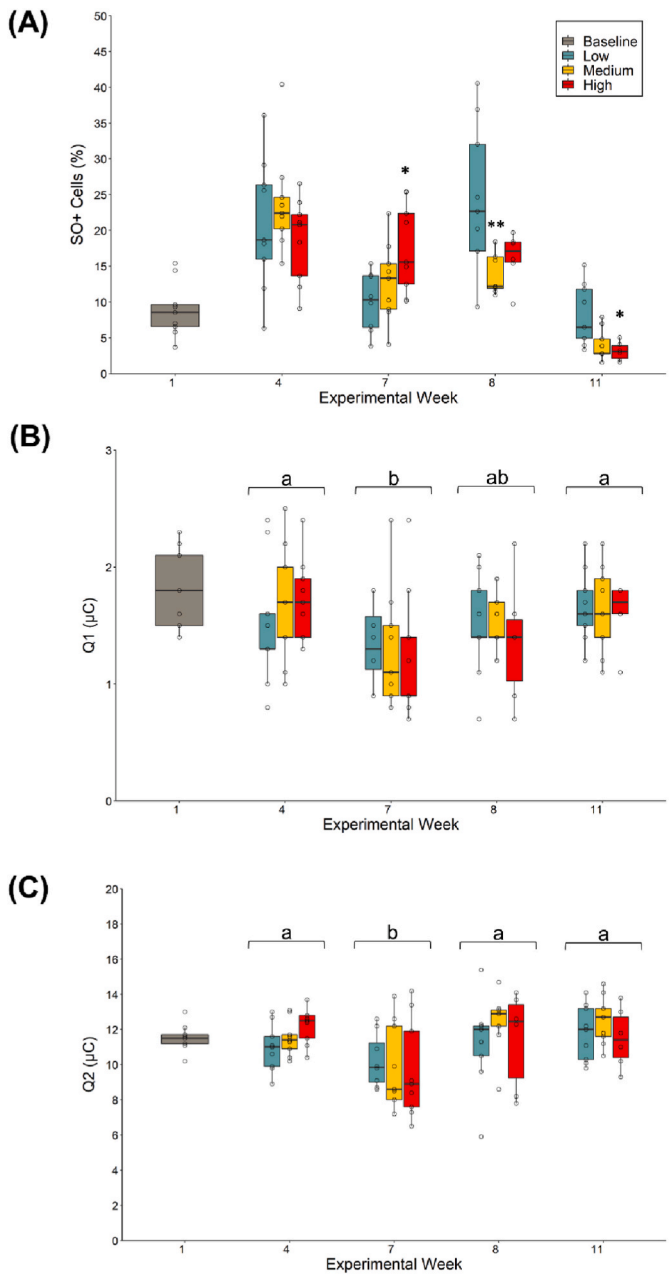


Fig. 5. Percentages of superoxide positive (SO+) haemocytes (A), levels (µC) of Q1 fast-acting (B) and Q2 slow-acting (C) antioxidants in haemolymph of adult *P. canaliculus* exposed to three different thermal treatments (low, medium- and high MHW ramp) for 11 experimental weeks. Baseline measurements before treatments were applied are shown in week 1. In panel A, differences to control temperature (low MHW) treatments are denoted by asterisks (* = $p < 0.05$, ** = $p < 0.001$). In panels B and C, differences between weeks are denoted by different lower-case letters. Individual boxes represent the median, upper, and lower quartiles, and minimum and maximum values.

treatments subsequently returned to levels resembling those in the stable 18 °C control treatment during weeks 7 and 8. Interestingly, some individuals in the stable low temperature treatment showed elevated haemocyte counts at the end of the trial (week 11, Fig. 4), reflecting a possible response to infection and husbandry stress.

Mussels in the high MHW ramp treatment experienced unintended further heating during week 6, as target temperatures approached 24 °C, resulting in a 6 d exposure to a ramp from 25 to 26.5 °C, before returning to the target 24 °C. A mortality pulse was subsequently observed over the following 10 days, with 61% of the treatment's mussels dying; the remaining 39% of mussels survived until the end of the experiment. Mortalities are a common occurrence in populations where organisms are unable to adjust to heatwaves (Stillman, 2019), as previously seen in a wild population of green-lipped mussels (Nguyen and Alfaro, 2020). The mortalities observed in the current experiment in the high ramp group can likely be attributed to the rapid temperature increase experienced, forcing mussels to rely on stored fuels to replenish adenosine triphosphate (ATP) used, which in effect depletes metabolic stores, contributing to cell death, as previously reported in *M. galloprovincialis* (Anestis et al., 2007). Interestingly cellular apoptotic events and cell death were reported in *M. galloprovincialis* after 10 days of exposure to 26 °C suggesting a negative energetic balance with subsequent mussel mortality at this temperature (Feidantsis et al., 2020). Although cell apoptosis was not measured in *P. canaliculus* under investigation it is a common response to thermal stress and potentially a contributing factor to the mortalities reported in this study.

4.1. Aerobic ATP production, sustaining energy for defence mechanisms

A metabolic shift to manage ATP supplies was evident in *P. canaliculus* exposed to higher temperatures. Involvement of the main energy producing systems, i.e., glycolysis, the tricarboxylic acid cycle (TCA) and electron transport chain (ETC), along with phosphagen breakdown, allowed sufficient energy production in the mussels in this study. An upregulation of aerobic metabolism during heatwaves is typically required to supply metabolic energy to active molecular defence mechanisms, such as heat shock proteins and antioxidant enzymes (Leung et al., 2019). Firstly, added energy requirements were seen in this study in the glycolysis pathway, where the amino acids serine, glycine and threonine were increased in *P. canaliculus* exposed to higher temperatures. These amino acids feed into the glyceraldehyde-3-phosphate (G-3-P) reaction of the glycolysis pathway, allowing the regeneration of cytoplasmic nicotinamide adenine dinucleotide (NAD^+) enabling the synthesis of ATP directly from the phosphorylation of adenosine diphosphate (ADP) (Venter et al., 2018a). This is supported by the increase of phosphoenolpyruvate, suggesting enhanced usage of the glycolysis pathway for energy production in heat-stressed mussels. Secondly, an accumulation of metabolites as energy supply, to potentially protect cells from damage was seen when considering increased anaplerotic reactions of the TCA cycle. Anaplerotic reactions in terms of branch-chained amino acids (BCAAs) isoleucine and valine, glutamate, alanine, tyrosine and phenylalanine, all increased in this study, can replenish the TCA cycle to ensure production of essential energy (Inigo et al., 2021). For example carbon produced by BCAA catabolism enters the TCA cycle where it can be oxidised to provide electron carriers for oxidative phosphorylation and energy production (Neinast et al., 2019). Glutamate plays a key part in amino acid transamination, but what may be of more importance for this study is the requirement of glutamate during glutathione production (Brosnan, 2000). Elevated alanine assists with the buffering of H^+ ions, regulation of intracellular osmotic pressure, and serves as a substrate for alanopine production during anaerobic conditions (Venter et al., 2018c). Tyrosine and phenylalanine are important precursors of hormones and neurotransmitters involved in stress responses (Salamanca et al., 2021), and are known to play a part in the antioxidant defence pathway (Zhang et al., 2021). All together, these amino acids support TCA cycle functioning in heat-stressed mussels, while also fulfilling important survival functions (Chen et al., 2021). A third mechanism utilised by heat-stressed mussels to ensure rapid metabolic energy production, is the substrate-level phosphorylation in terms of phosphagen breakdown. Phosphagens like phosphocreatine, can be broken down rapidly in conjunction with ADP, resulting in ATP and creatine as end products. The current study detected an increase in creatinine, the nitrogenous waste product of creatine and phosphocreatine, suggesting an increase of the aforementioned metabolites (even though not detected), contributing to energy metabolism (Brosnan and Brosnan, 2010). In

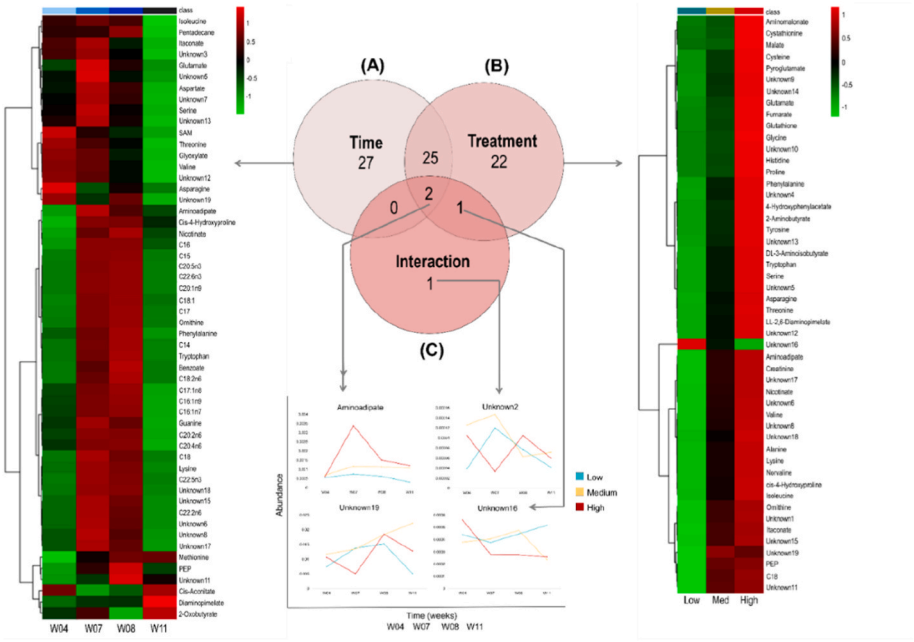


Fig. 6. Venn diagram of the number of metabolomic features in *P. canaliculus* haemolymph significantly influenced by experimental exposure time (weeks [W] 04, 07, 08, and 11) and temperature treatment (low, medium- and high MHW ramp) and the interaction of both, with associated data overviews in terms of heatmaps (A and B) and plotted features (C).

effect, increased functioning of the urea cycle is also implicated, where excess urea from ammonia production is excreted (Mohamed et al., 2005). Metabolites like proline and ornithine, both increased in the heat-stressed mussels in this study, facilitates numerous metabolic functions. For example, proline and its associated pathway transfers reducing potential which can contribute to ATP production, enhances metabolites with anaplerotic roles, and serves as an antioxidant and bioenergetic substrate (Wyse and Netto, 2011). Then again, ornithine plays a pivotal role in several metabolic pathways as a precursor to other urea cycle metabolites, while also partaking in ammonia detoxification (Wu, 2009).

4.2. Anaerobic ATP production, supplying energy for survival

Temperature stress generally affects aerobic metabolism to meet increased energy demands (Leung et al., 2019). However, when aerobic metabolism fails to increase with temperature, a shift towards anaerobic metabolism takes place, with detailed processes well described for mussels (Livingstone, 1978; Müller et al., 2012). Rather than a shift towards anaerobic metabolism, it is suggested that temperature stressed *P. canaliculus* utilised anaerobic pathways (by means of the succinate pathway) to supplement aerobic metabolism, as previously reported in the clam, *Mya arenaria* (Ouillon et al., 2023). Despite the absence of well-known markers such as lactate and opines, which indicate anaerobic metabolism, increased amounts of alanine, malate, glutamate (detected in the current study), aspartate (alternative form asparagine detected) and succinate (not detected in the current study) are markers of the succinate pathway, commonly liked to anaerobic activity, as reported in temperature stressed *M. edulis* (Livingstone, 1978; Torossian et al., 2020) and *M. galloprovincialis* (Georgoulis et al., 2022). In essence, the production of oxaloacetate (via phosphoenolpyruvate) can generate propionate and succinate using the reversal of the second half of the TCA cycle, which supports the synthesis of NAD^+ and FAD (Venter et al., 2018b). Although this process results in a smaller number of ATP molecules, it allows survival at a lower energy state with lower free radical production (Stefano et al., 2015). Mussels experiencing the effects of a heatwave in the current study, likely utilised anaerobic sources like the reversal of the TCA cycle to ensure energy production for survival. The TCA cycle was further affected in the current study due to temperature stress, when considering increased itaconate (derived from the decarboxylation of aconitate), an anti-bacterial agent with the ability to inhibit mitochondrial ROS (Noe and Mitchell, 2019). Increased itaconate has previously been reported in *P. canaliculus* in response to acute thermal stress (Ericson et al., 2022).

4.3. Management of oxidative stress in heat-stressed mussels

The occurrence of oxidative stress, additional ROS production through aerobic respiration in mitochondria, damage to cellular components and up-regulation of antioxidant defence mechanisms are well known consequences of thermal stress (Torossian et al., 2020). Under normal physiological conditions, superoxide anion, one of the major species of ROS, is produced from complexes I and III of the ETC, but are kept within redox balance thanks to antioxidant enzymes (Burgos-Morón et al., 2019). Yet, an increase in ROS production has been reported in *M. galloprovincialis* and *P. viridis*, under heat-stress, likely due to the disruption of oxygen-metabolic homeostasis due to the temperate exposure (Wang et al., 2018a, 2018b). A similar result was seen in the current study, where *P. canaliculus* showed an increase in SO+ haemocytes, after 7 weeks in the high ramp and 8 weeks in the medium ramp MHW treatments. At this point high ramp temperatures were ~23.5 °C and mussels had experienced a transient exposure to 25–26.5 °C water, whereas the medium ramp had steadily risen to 22.5 °C, suggesting the integrated thermal history, rather than absolute temperature, was influencing the redox response. Although the differences were significant, superoxide is only moderately reactive and does not cause extensive damage by itself, and is more likely to transform to more reactive and toxic ions, which do cause damage (Das and Roychoudhury, 2014). Interestingly, once mortalities occurred, a decrease of SO+ haemocytes were seen in surviving animals in the high temperature ramp group. This may reflect the capacity of the surviving animals to neutralise ROS in response to a stressor or be associated with the levels of increased itaconate in the temperature stressed mussels, which can act as a ROS inhibitor. What is more is that a switch from NADH to NADPH producing pathways are common in mussels during thermal stress, where NADPH can be used to support antioxidant

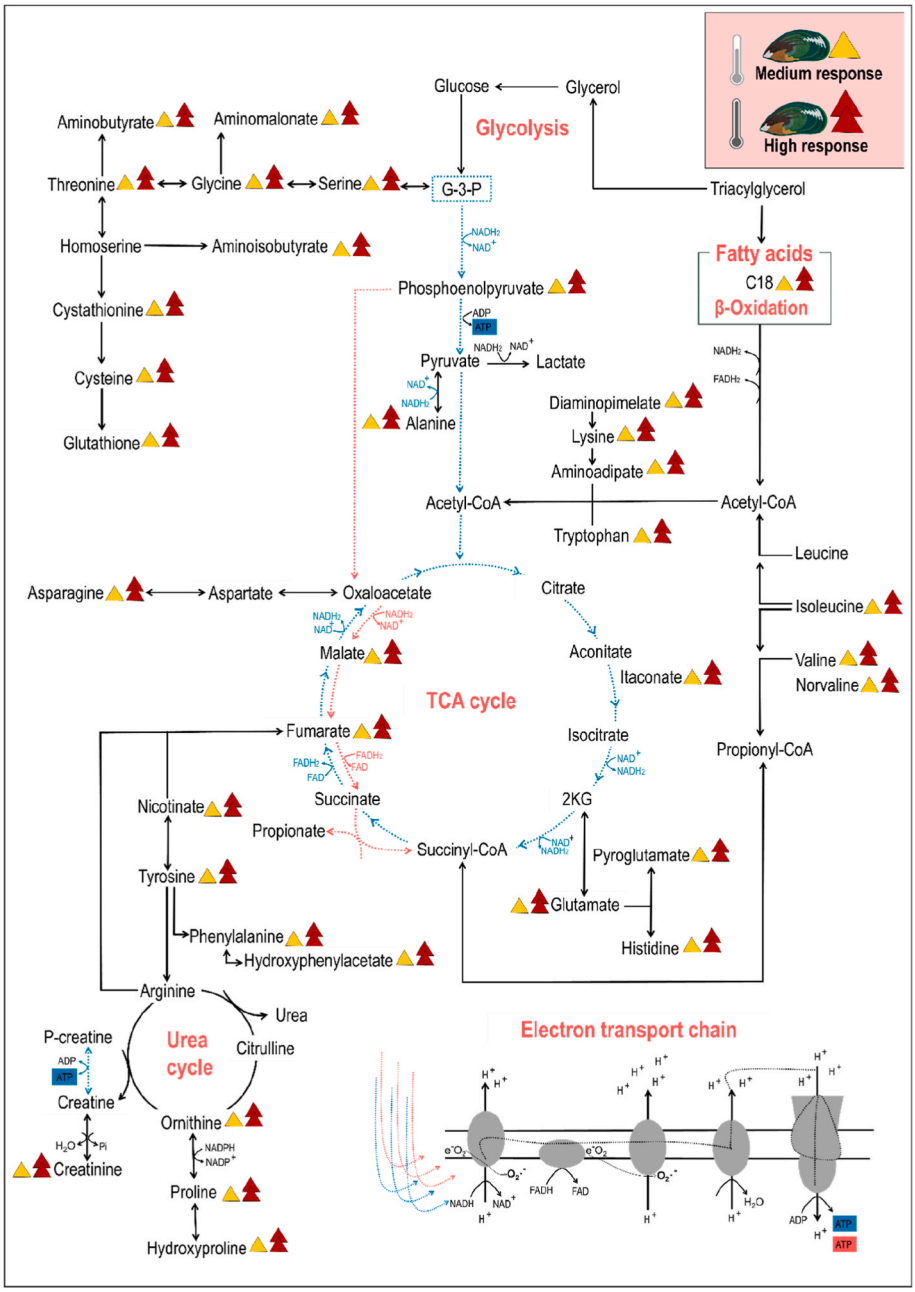


Fig. 7. Overview of the metabolic response of *P. canaliculus* following medium (yellow) and high (red) marine heatwave scenario exposures. Increased metabolic activity shown by the medium group (compared to the low group) are represented by ▲, while ▲▲ suggests increased metabolic activity within the high group (compared to medium and low groups). The blue lines are representative of aerobic energy producing pathways, while the pink lines represent anaerobic pathways. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

defences. Typically such a shift enhances cellular protection thereby also increasing mussel survival (Georgoulis et al., 2021), a likely explanation for the response seen by the surviving animals. This is an area of the metabolism that will benefit from future target analyses encompassing pathways such as the pentose phosphate pathway and nucleotide metabolism.

In the event of heatwaves, molluscs can also increase their antioxidant capacity as defence mechanisms in the presence of oxidative stress (Leung et al., 2019), a likely scenario for *P. canaliculus* when considering the metabolite profile of increased antioxidant metabolites. From the current study, metabolites within the glutathione metabolic pathway (cysteine, glutamate, glutathione, glycine, and ornithine) had a higher concentration, suggesting the function of glutathione as an antioxidant molecule, as seen previously in *P. canaliculus* (Nguyen et al., 2018). Glutathione is a strong fast acting antioxidant, which in this study was also quantified as part of Q1. Additionally, amino acids are considered as precursors of antioxidants, with numerous amino acids increased following temperature stress in this study, corresponding to a previous study where *P. canaliculus* was subjected to heat-stress (Delorme et al., 2021). The levels of fast (Q1) and slow (Q2) acting antioxidants were not significantly different among treatments in this study due to high inter-individual variability. However, there was a significant effect of time on the antioxidant response, with lower antioxidant capacity at 7 weeks. At this time, there was a trend for both fast and slow antioxidants to decrease in mussels experiencing the high MHW treatment. It is possible that antioxidants at this time were being used to scavenge the high ROS production, resulting in a decrease in ROS together with a stabilisation of the antioxidant capacity by the end of the experiment at 11 weeks. An increase in total antioxidant capacity has been reported in *P. canaliculus* following a short term (i.e., acute) heat-shock (Delorme et al., 2021), yet data corresponding to long-term thermal exposures are scarce for *P. canaliculus*. An antioxidant defence response comes at an energetic cost for organisms, further reducing available ATP, resulting in impaired mitochondrial functioning (Stillman, 2019), linking back to

the metabolomics outcomes from this study, where anaerobic respiration assists with energy production.

4.4. Influence of time on mussel fatty acid metabolism

Reactive oxygen species not only contribute to oxidative stress, but also oxidise macromolecules such as lipids (lipid peroxidation), causing the removal of electrons from lipids (containing carbon-carbon double bonds, especially polyunsaturated fatty acids) in cell membranes, largely by the working of hydroxyl radicals (Ito et al., 2019). Even though lipid peroxidation assays, as a measure of the oxidative status of mussels were not utilised in this study, changes in polyunsaturated fatty acids did occur. Temperature changes are often managed by changes in levels of polyunsaturated fatty acids within membrane phospholipids (Fokina et al., 2015), likely due to the strong effect of temperature on physical properties of membrane lipids, as reported in *M. edulis* (Pernet et al., 2007; Matoo et al., 2021). However, the changes of long chain fatty acids (C14; C15; C16:0; C16:1n9; C16:1n7; C17; C17:1n8; C18; C18:1; C18:2n6; C20:1n9; C20:4n6; C20:2n6; C20:5n3) and very long chain fatty acids (C22:2n6; C22:5n3; C22:6n3) seen in the current study, can be attributed to experimental time, and not temperature, with increases of fatty acids seen at weeks 7 and 8 of experimental holding, followed by a decrease at week 11. This is an interesting observation where either more lipids were broken down resulting in higher fatty acids or more fatty acids were produced, also resulting in the increases seen. From the literature, it is clear that marine invertebrates are not able to effectively synthesise n-3 and n-6 polyunsaturated fatty acids, and that these are largely obtained from dietary nutrients (Zhukova, 2019). Yet, increases of polyunsaturated fatty acids detected in *M. edulis*, has been linked to the regulation of membrane-bound proteins, supporting survival under environmental fluctuations (Nemova et al., 2013), a likely response for *P. canaliculus* kept at experimental conditions in the current study. Additionally, the study by Nemova et al. (2013) reported increased fatty acid concentrations of mussels in laboratory conditions. Also, the known functions of fatty acids (and lipids) as a source of metabolic energy and essential compounds for cell and membrane tissues (Venter et al., 2021) can been seen as reasons for increased fatty acid production in the current study. The involvement of fatty acids in processes important for survival, has been reported in *M. galloprovincialis* (Andrade et al., 2018), linking to the current findings, where increased fatty acids were seen at weeks 7 and 8, notably following the largest mortalities in the high MHW ramp treatment (at week 6). Arguably, fatty acid sources were depleted by week 6, attributing to mortalities, whereafter lipogenesis takes place to convert dietary carbohydrates into fatty acids, starting with palmitate (C16, highest at week 8), followed by desaturation and elongation of various fatty acids which are mostly stored as an energy source (Lee et al., 2018), resulting in the high fatty acid content at weeks 7 and 8. The decrease in long and very-long chain fatty acids at week 11 in the mussels under investigation, can be attributed to the usage hereof to produce medium- and short-chain fatty acids assisting with cell signalling and energy metabolism (Schönfeld and Wojtczak, 2016).

4.5. Mussel condition not affected by time but by temperature

Over the time of 11 weeks, the condition index did not change significantly for mussels kept at lower temperatures of 18 °C or mussels subjected to the medium MHW ramp. Yet, a decrease in mussel condition index was observed in mussels experiencing the high MHW. A similar outcome was reported in *M. edulis*, where elevated temperature was associated with reduced condition index, likely as a consequence of increased metabolism (Clements et al., 2018), as seen in the current study.

5. Conclusions

Shellfish aquaculture production is threatened by marine heatwaves and associated mortalities, with both the North and South Islands of New Zealand reaching new maximum seawater temperatures yearly. By assessing the survival, condition, selected immune parameters and the metabolome of *P. canaliculus* subjected to ramp-and-hold temperature regimes, valuable insights into the sub-chronic (days/weeks) responses of mussels to simple MHW scenarios were obtained. Mussels experiencing the highest MHW treatment utilised aerobic ATP to fuel molecular defence mechanisms, resulting in increased phosphoenolpyruvate, amino acids and metabolites linked to anaplerotic reactions. Complementation of aerobic metabolism by means of anaerobic metabolism implementing the succinate pathway and consequently the reversal of the TCA cycle, was also seen in this study, mainly as a method to ensure energy supply for biological functions and survival. Additional results support the use of antioxidant metabolites to counteract the production of oxidative stress due to the increasing temperature, coinciding with superoxide positive haemocytes and total antioxidant capacity results. The effect of experimental time was largely seen on fatty acids, likely due to the membrane storage functions of fatty acids and energy metabolism attributes. Time did not affect mussel condition index, but temperature did, with the highest temperature resulting in the lowest mussel condition.

The unintended transient 6 day warming event in the 'high MHW' treatment (peaking at 26.5 °C) serendipitously highlighted a critical threshold in *P. canaliculus*, resulting in acute mortality of 61% of the animals exposed to a thermal profile that is becoming increasingly likely during the northern New Zealand summer (Salinger et al., 2020). Importantly, many individuals survived this exposure, presenting the metabolic responses described above, highlighting important plasticity mechanisms that may support the persistence of the species in warming regions and may inform selective breeding strategies in aquaculture. Mussels that did not experience temperatures exceeding 24 °C showed relatively subtle signs of metabolic disruption. Sustained exposure to this temperature for periods exceeding 4–5 months has been shown to cause severe stress and may be lethal (Ericson et al., 2023a), but the present study suggests the effects of transient exposure may be reversible if benign temperatures are restored. It would be valuable to confirm this suggestion experimentally in future work, and further studies would also benefit from the inclusion of measuring enzymes that scavenge excessive ROS, and heat shock proteins. Equally important for future studies is to consider what happens on a metabolic level during a recovery phase and the achievement of a homeostatic setpoint or new metabolic status when a recovery period is implemented.

These results provide critical insight into the physiological response of *P. canaliculus* to heatwaves, highlighting metabolic and cellular markers worth investigating using more targeted methods going forward. Further studies will enable a comprehensive view of mechanisms activated by *P. canaliculus* during heatwave events, contributing to the building of a resilient aquaculture industry in the face of changing environments.

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Credit authorship statement

Leonie Venter & Jessica A. Ericson: Conceptualisation; Data curation; Formal analysis; Investigation; Methodology; Project administration; Software; Visualisation; Roles/Writing - original draft. Natalí J.

Delorme: Formal analysis; Investigation; Writing - review & editing. Andrea C. Alfaro & Norman L. C. Ragg: Conceptualisation; Funding acquisition; Resources; Supervision; Writing - review & editing.

Declaration of competing interest

All authors declare that they have no competing interests.

Data availability

The data that support the findings of this study are openly available in Zenodo at https://zenodo.org/record/8267565.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2023.103702.

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