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Behavioural and physiological responses of juvenile geoduck (*Panopea zelandica*) following acute thermal stress

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ABSTRACT

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

1. Introduction

New Zealand spans a large latitudinal distance across its two main Islands. The oceans around the main Islands are influenced by subtropical water in the upper north and by polar Antarctic currents in the south. The water temperature range coupled with a vast array of habitats provide the potential to consider of a variety of species for an emerging aquaculture industry. Indeed, aquaculture is the fastest growing primary sector in New Zealand with interests in expanding to new species in the near future (McGinnis and Collins, 2013; Stenton-Dozey et al., 2021). However, the aquaculture industry could face a major challenge due to the current global climate change crisis. Reports have confirmed that oceans around some regions in New Zealand are warming, and that climate extremes such as marine heatwaves are expected to become more intense and longer compared to the present day (Behrens et al., 2022 & Stevens et al., 2022). Marine heatwaves occur when water temperatures stay in the warmest 10% of historical observations for at least five days (Hobday et al., 2016). For example, a marine heatwave persisted around the South Island, NZ during February 2023 with an average sea surface temperature increase of 2.9 °C and a temperature spike of 6 °C (Noll, 2023) . Such events have significant implications for aquaculture and fisheries in New Zealand (de Burgh-Day et al., 2022). Increased temperatures and temperature anomalies affect the metabolic and physiological responses of commercially important fish and shellfish species (Rolton et al., 2022). However, the effects of sudden temperature fluctuations on the endemic geoduck clam *Panopea zelandica* Quoy and Gaimard 1835, are largely unknown.

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The New Zealand geoduck, *P. zelandica*, is a burrowing bivalve found in subtidal areas around the North and South Islands. Even though *P. zelandica* has a huge geographically spread (Gribben et al., 2004), there is limited information about its population dynamics and distribution (Gribben and Heasman, 2015). Currently, there are only a few known populations of *P. zelandica* which support wild harvests (Gribben and Heasman, 2015). Establishment of a geoduck aquaculture industry is still pending, with previous research efforts placed on investigating different aspects relevant for hatchery production of seed (Le et al., 2017a, 2017b; Sharma et al., 2020). Due to the current increase in global sea temperatures and the increased chances of heatwaves continuing in the future, organismal responses to temperature changes remain an important factor for consideration. Indeed, burrowing bivalves exposed to temperatures above their optimal ranges tend to exhibit lower burrowing capacity, decreased feeding and growth rates, and increased rates of mortality (Macho et al., 2016). A common behavioural response to thermal stress in bivalves includes valve closure and/or increased borrowing (Sakuari et al., 1996; Domínguez et al., 2021). Even though valve closure provides an escape from temperature extremes as seen in the mussel, *Limnoperna fortunei* (Andrade et al., 2018), valve closure is not an effective avoidance option for geoduck as these bivalves are unable to fully close their shells. As temperatures are likely to be cooler deeper within the sediment, compared to the surface layers, moving deeper within the sediment may provide animals a refuge from temperature stress (Macho et al., 2016). Consequently, Macho et al. (2016) documented the change in subsurface burrowing frequency and siphon activity as a useful measure of stress in clams.

As pointed out by Hernández-Méndez et al. (2020), haemocyte assessment allows one to detect the impact of environmental changes on animal health which can be used as a valuable measure of physiological response to stressors. Elevated temperatures have also been shown to affect clam haemocyte parameters, such as cell viability, adhesive capacity, and cell types (Monari et al., 2007; Pérez-Velasco et al., 2022). Haemocytes constitute the cellular component of haemolymph, which moves through the circulatory system, migrating to locations, such as connective tissue and epithelia (Hine, 1999). Apart from their main function in host defence mechanisms, bivalve haemocytes partake in physiological functions, such as nutrient digestion, transportation and distribution, wound healing, detoxification, shell mineralisation and excretion (De la Ballina et al., 2022). Bivalve haemocytes vary in size and abundance but are generally made up of three main cell types, which include small hyalinocytes, large hyalinocytes, and granulocytes, as demonstrated in *P. globosa* (Hernández-Méndez et al., 2020).

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

Assessing the effects of elevated temperatures on biological parameters, such as health, growth, and nutrition is important for understanding and predicting resilience (Ewere et al., 2021). Temperature has been the focus of several geoduck studies. For example, *P. globosa*, had increased metabolic rates to temperatures of 29 °C (Juárez et al., 2018). Whereas in *P. generosa*, 19 °C was deemed as a temperature resulting in significant growth rates (Arney et al., 2015). On the other hand, at 19 °C, both *P. zelandica* (Le et al., 2017a, 2017b) and *P. japonica* (Nam et al., 2015) had reduced aerobic scope and increased mortalities, respectively. However, acute temperature stress responses in geoduck is poorly described. The use of acute stress (hours to days) experiments enables one to compare the range over which a physiological stress response is observed with the actual body temperature animals experience under field conditions (Tomanek, 2008).

This study provides the first report on the behavioural and physiological responses of juvenile *P. zelandica* to acute thermal stress, aiding the development of strategies for aquaculture planning and fisheries management, of geoduck clams.

2. Materials and methods

To evaluate the thermal stress response mechanisms of *P. zelandica*, juveniles of this species were exposed to acute changes in temperature for a period of 24 h. The temperatures selected went from current summer ambient temperatures in their natural distribution (i.e., 17 °C) to two potential heatwave scenarios in which the temperature quickly rises by either 3 or 8 °C i.e., respectively representing mean temperature anomalies seen in recent marine heatwaves and extreme localised warming (Salinger et al., 2019; Noll, 2023). Overall responses were examined at three organisational levels: behavioural observations were conducted by taking hourly photographs of the experimental tanks to evaluate the borrowing status of the juvenile geoducks; changes in haemocyte subpopulations across different temperatures were investigated and expression of heat shock protein genes (*hsp70* & *hsp90*) were assessed in both haemolymph and gill samples.

2.1. Animal acquisition and acclimation

Nine-month-old hatchery reared *P. zelandica* produced at the Cawthron Aquaculture Park (Nelson, New Zealand) were used for this study ($n = 27$, wet weight of 16.98 ± 0.41 g (mean ± S.E.), shell length of 32.96 ± 0.37 mm, shell width of 31.91 ± 0.23 mm). The juveniles were transported to the Auckland University of Technology (AUT) and were acclimated for two weeks prior to experimentation (Sharma et al., 2022). At AUT, the juveniles were kept in individual 760 mL containers (10 cm × 8.5 cm) with 9 cm depth of substrate (~500 μm grain sand). These holding containers were submerged in 2 × 180 L tanks in an 800 L recirculation system. Water temperature and salinity were maintained stable at 17 ± 0.5 °C and 35 ppt, respectively. Individuals were exposed to a light:dark cycle of 12 h:12 h throughout the two weeks using a 90 cm Blue Planet® LED track light set on a timer. No mortalities were recorded during the acclimation period.

2.2. Water parameters

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

2.3. Thermal stress exposure

At the end of the acclimation period, the 27 containers with animals were removed from the 180 L recirculation system and divided among three 40 L static tanks (nine replicates per temperature treatment) with aerated seawater (at 35 ppt) connected to an Aqua One® 200-watt heater. An Aqua One® Maxi Power head 101 submersible pump was placed in each 40 L static tank to keep the water circulating (Fig. 1A).

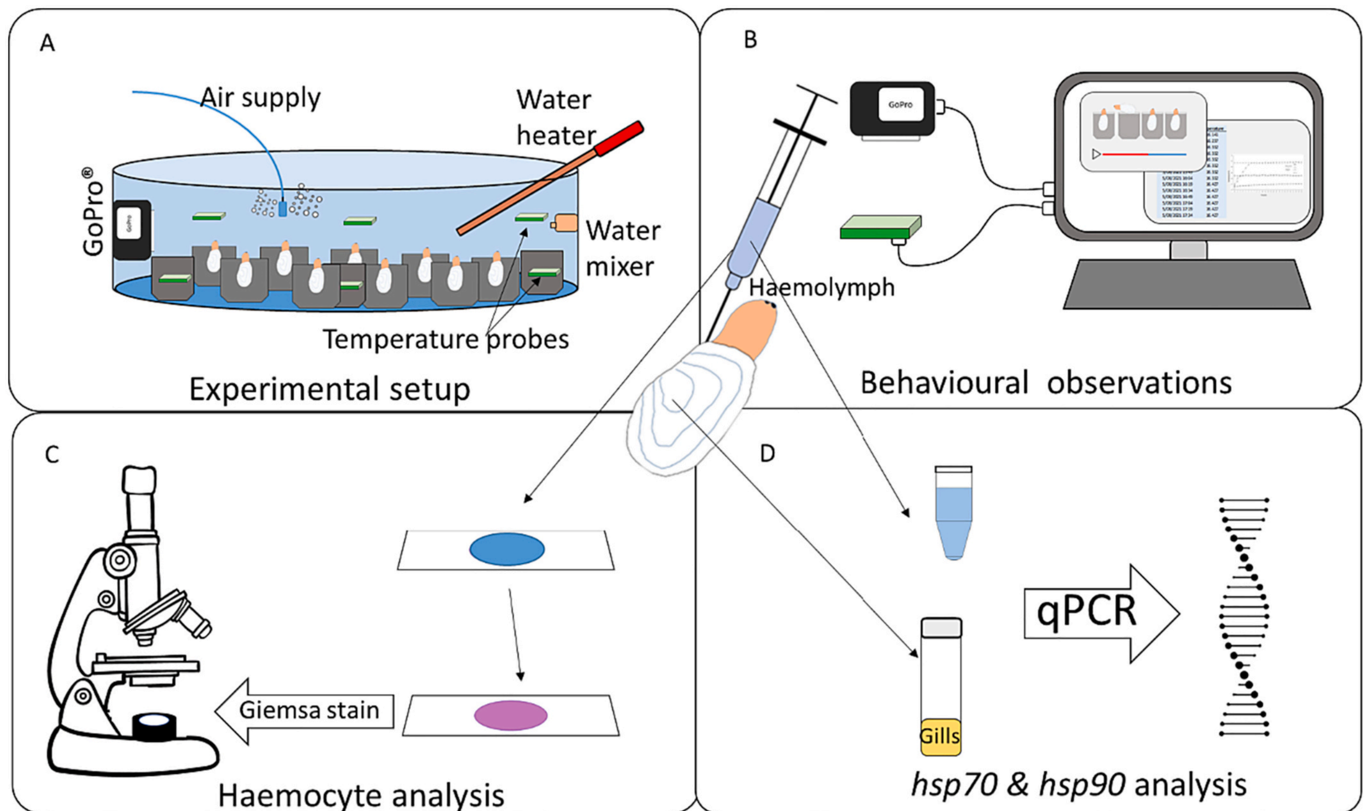

Fig. 1. Experimental outline starting with A) housing of *P. zelandica* juveniles (n = 9) at three different temperatures (17, 20, 25 °C) for 24 h, B) Video recording and temperature data were analysed, C) haemolymph collection and microscope slide preparation D) haemolymph and gill samples were processed for HSP gene expression analyses.

The static tanks were set up 12 h prior to the experiment to provide ample time for the tanks to reach the proposed temperatures of 17, 20, and 25 °C. Once the animals were placed in their temperature treatment the experimental time started (time 0). After 24 h of temperature exposure, all the animals were removed from the experimental tanks for sample collection giving 9 replicates (n = 9) for all the analysis conducted.

2.4. Behavioural observations

To record the borrowing status of individual juvenile geoduck during the experiment, each tank had a GoPro® Hero 5 camera placed in the water column set to take hourly snapshots. The wide-angle setting was used to ensure all geoducks were in the recording frame. Photographs and time stamps were used to obtain the burrowing status of individual juvenile geoduck over the 24 h period (Fig. 1B). Position of the geoduck (buried or disinterred) were assessed and counted at hourly intervals over 24 h. This was assessed as there have been previous accounts of geoduck actively exiting the sediment when stressed in the hatchery.

2.5. Sample collections

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

2.6. Haemocyte preparations and assessment

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

Haemocyte subpopulations were differentiated based on descriptions by Hernández-Méndez et al. (2020). In brief, haemocytes were identified and classified as granulocytes, and small and large hyalinocytes. A differential haemocyte count was conducted in triplicate by tallying up the first 200 cells observed while moving from left to right on the microscope. Haemocyte cellular aggregations were separated into three categories based on number of haemocytes and the size of aggregations. Aggregations were classified as 'small' if 4–10 cells with a net diameter of 10–80 μm were present, as 'medium' if 11–30 cells of 80–150 μm were seen and as 'large' if >30 cells of >150 μm were detected (Fig. 1C).

Individual haemocyte and aggregation diameters were determined using CellSens® software (Version 1.16). Targeted images were taken with a camera (OMAX 14.0mp attached to the microscope).

2.7. Heat shock protein (HSP) analyses

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

Specific gene primers for *hsp70* and *hsp90*, and the housekeeper β-Actin, were designed using the Geneious Prime (version 2022.0). A single housekeeper β-Actin was used in the analysis as it had an adequate efficiency of 91% and demonstrated stable amplification and efficiency across temperatures and treatments. Target sequences were designed based on the alignment of conserved domains aligned against the assembled genome for the closely related *Panopea generosa*. Resulting target and housekeeper amplicons were Sanger sequenced, with sequences confirmed through the National Center for Biotechnology Information (NCBI) database, nucleotide BLAST (https://blast.ncbi.nlm.nih.gov, accessed on 30 Nov 2022). Quantitative Polymerase Chain Reaction (qPCR) analyses were performed in duplicate using 2 μL of 10 ng/μL template cDNA in a total reaction volume of 20 μL, using SYBR® Green PCR Master Mix (Applied Biosystems™) according to manufacturer's instructions (publication Part Number 4310251 Rev. G). Three non-template controls (Delorme et al., 2020), *hsp70*, *hsp90* and *β-Actin* (Table 1) were tested in triplicate along with controls in the Mastercycler Realplex 2, programmed with the Sybr Green I detection system as follows: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 50 °C or 60 °C for 1 min. The relative mRNA expression fold change for all target genes was determined by the delta-delta Ct (2- ddCT) method (Livak and Schmittgen, 2001)(Fig. 1D).

2.8. Statistical analyses

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

All data were checked for normality and homoscedasticity using the Shapiro-Wilk and Levene's test, respectively (Quinn and Keough, 2002).

Table 1
qPCR Primers for *hsp70*, *hsp90* and β-Actin.

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

Significant differences among treatments for all the analysis were determined using Tukey pairwise comparisons with significance taken at 0.05. All statistical analysis were conducted in R Studio® (version 1.4.1103).

3. Results

3.1. Experimental parameters

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

3.2. Behavioural observations

Behavioural observations of geoduck across treatments indicated that within the 17 °C experimental tank, all nine individuals remained within the sediment for the duration of the experiment. In the 20 °C experimental tank, the first geoduck completely disinterred itself from the sediment and ended up in a horizontal position on top of the sediment after 13 h. By 24 h in the 20 °C experimental tank, 4 animals (44.4%) had completely emerged from the sediment and were lying on the sediment surface. In the 25 °C experimental tank the first juvenile geoduck was observed to emerge from the sediment at 8 h, with all 9 animals (100%) leaving the sediment by 24 h (Fig. 3). There were no mortalities recorded in any of the experimental tanks

3.3. Haemocyte characterisation

Three subpopulations of haemocytes were found and characterised as either granulocytes, large hyalinocytes & small hyalinocytes (as described per Pila et al., 2016; Hernández-Méndez et al., 2020; Nuria et al., 2022). Granulocyte ranged in size between 10 and 30 μm, and exhibited a round, eccentric basophilic (blue stained) nucleus with abundant granules (Fig. 4A). The hyalinocytes were primarily acidophilic (red stained) and were differentiated size and morphological differences. Large hyalinocytes (15–55 μm) had a crescent shape with a central or eccentric nucleus with cytoplasm spread out in a crescent shape (Fig. 4B). The small hyalinocytes (5–18 μm) were characterised by small round cells with a central or eccentric nucleus (Fig. 4C). Haemocyte aggregations were mainly comprised of a central core of hyalinocytes surrounded by granulocytes (Fig. 4 D, E, & F).

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

A significant increase in the number of haemocyte aggregations (ANOVA, $F_{(2,21)} = 57.9$, $p < 0.001$) was recorded with increasing temperatures. At 17 °C, 3 ± 10.5 (mean ± S.E.) haemocyte aggregations

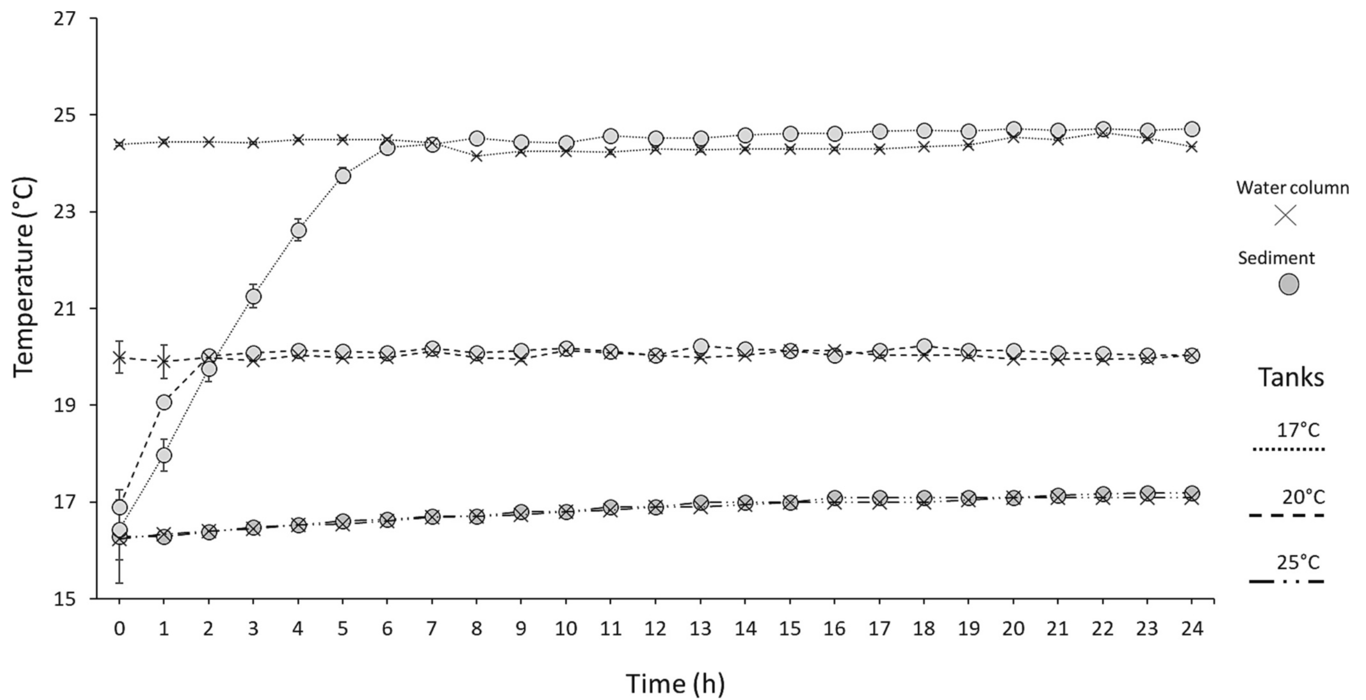

Fig. 2. Temperature monitoring during acute thermal stress experiment. In all instances, water temperature (water column X) was set at the target temperature of 17, 20 and 25 °C prior to the start of the experiment. The temperature of the sediment (sediment ●) housing pots is also shown.

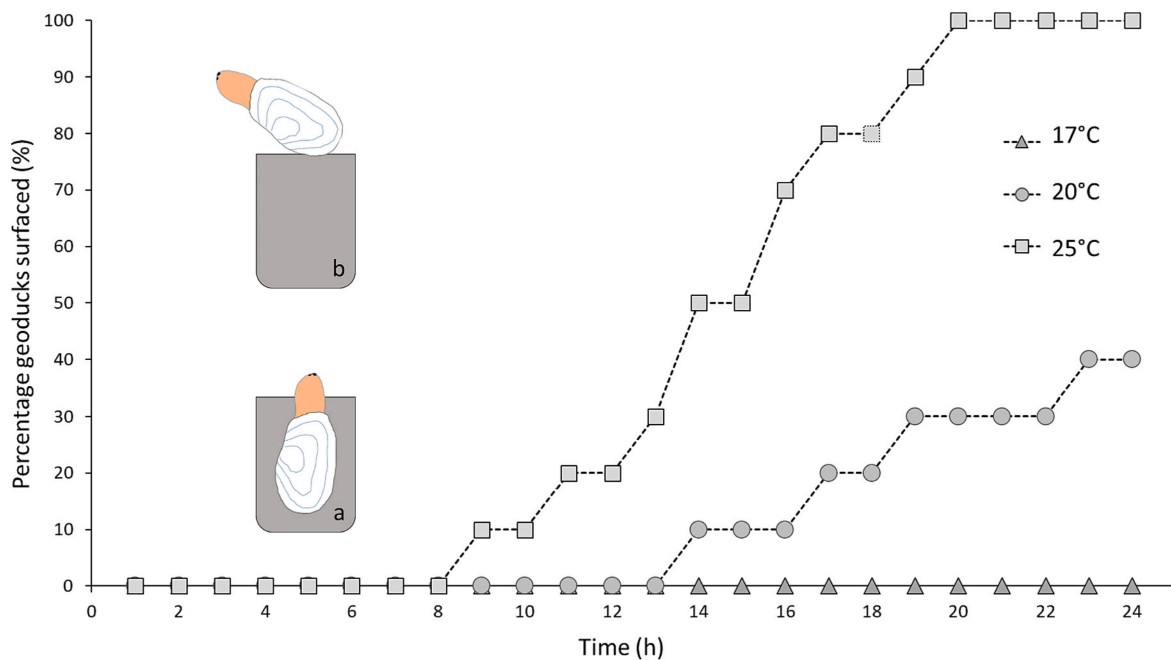

Fig. 3. Behaviour of *Panopea zelandica* juveniles with regards to their position within the sediment (a –geoduck buried in the sediment; b – emerged from the sediment) was monitored for 24 h among all experimental temperatures (17 °C ▲, 20 °C ●, 25 °C ■).

were detected, while at 20 °C, 15 ± 3 and at 25 °C, 35 ± 2 cell aggregations per 200 haemocytes were recorded (Fig. 6A). Small and medium haemocyte aggregations were observed mainly comprising of large hyalinocytes, while large haemocyte aggregations were observed as a mass of granulocytes. There was a significant increase (ANOVA, $F_{(2,78)} = 10.68$, $p < 0.001$) in the respective size of these aggregations with 58.31 ± 3.85 μm (mean ± S.E) seen at 17 °C; 106.61 ± 8.10 μm at 20 °C and 172.82 ± 20.55 μm at 25 °C (Fig. 6B). Pairwise comparisons showed differences in number of aggregations at all tested temperatures, whereas the size of the aggregations were only significantly different between the 17 and 25 °C treatments.

3.4. *hsp70* and 90 expression

There were no significant differences in the expression of *hsp70* in the gill tissue at the different experimental temperatures (Fig. 7A), however the expression of *hsp70* was highest (albeit insignificant) in the gills at 25 °C, with a 16-fold increase from 17 °C to 25 °C. In haemolymph

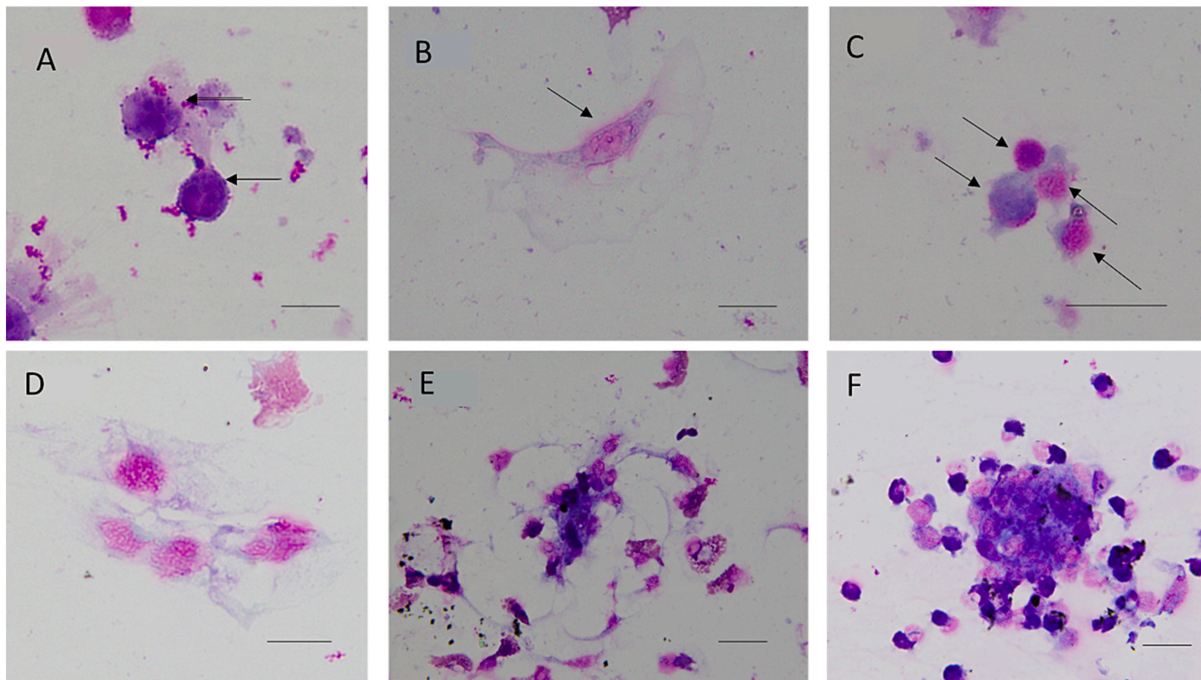

Fig. 4. Haemocyte cell types and aggregations observed in the haemolymph of *Panopea zelandica* juveniles prepared with Giemsa stain. A: granulocytes, B: large hyalinocyte, C: small hyalinocyte, (arrows indicate individual cell types) D: small haemocyte aggregation, E: medium haemocyte aggregations, and F: large haemocyte aggregations (Scale bar: 20 μm).

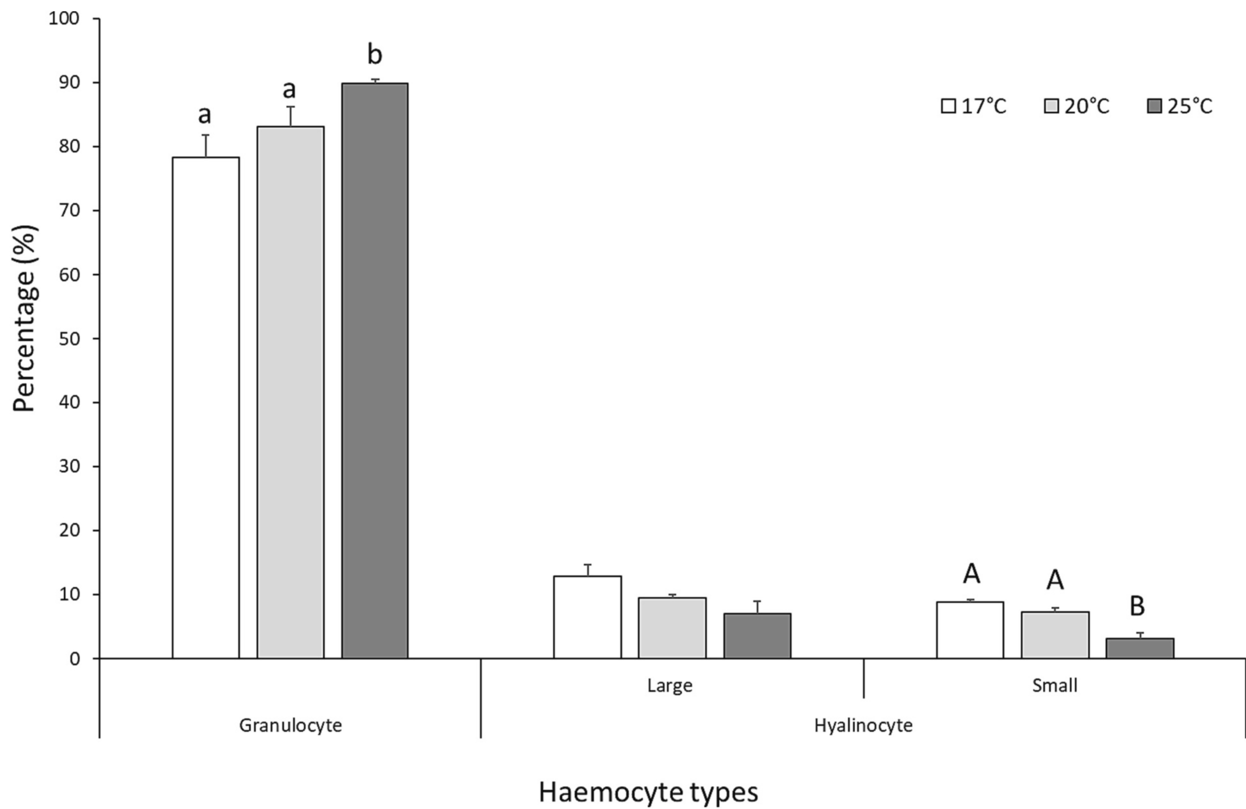

Fig. 5. Percentage of individual haemocyte subpopulation types: granulocytes (left) large hyalinocytes (middle) small hyalinocytes (right), recorded in *Panopea zelandica* juveniles (n = 9) at different temperatures. Significant differences in the proportion of haemocyte cell type are denoted by upper- and lower-case letters above each bar within each subpopulation (significance taken at $p < 0.05$).

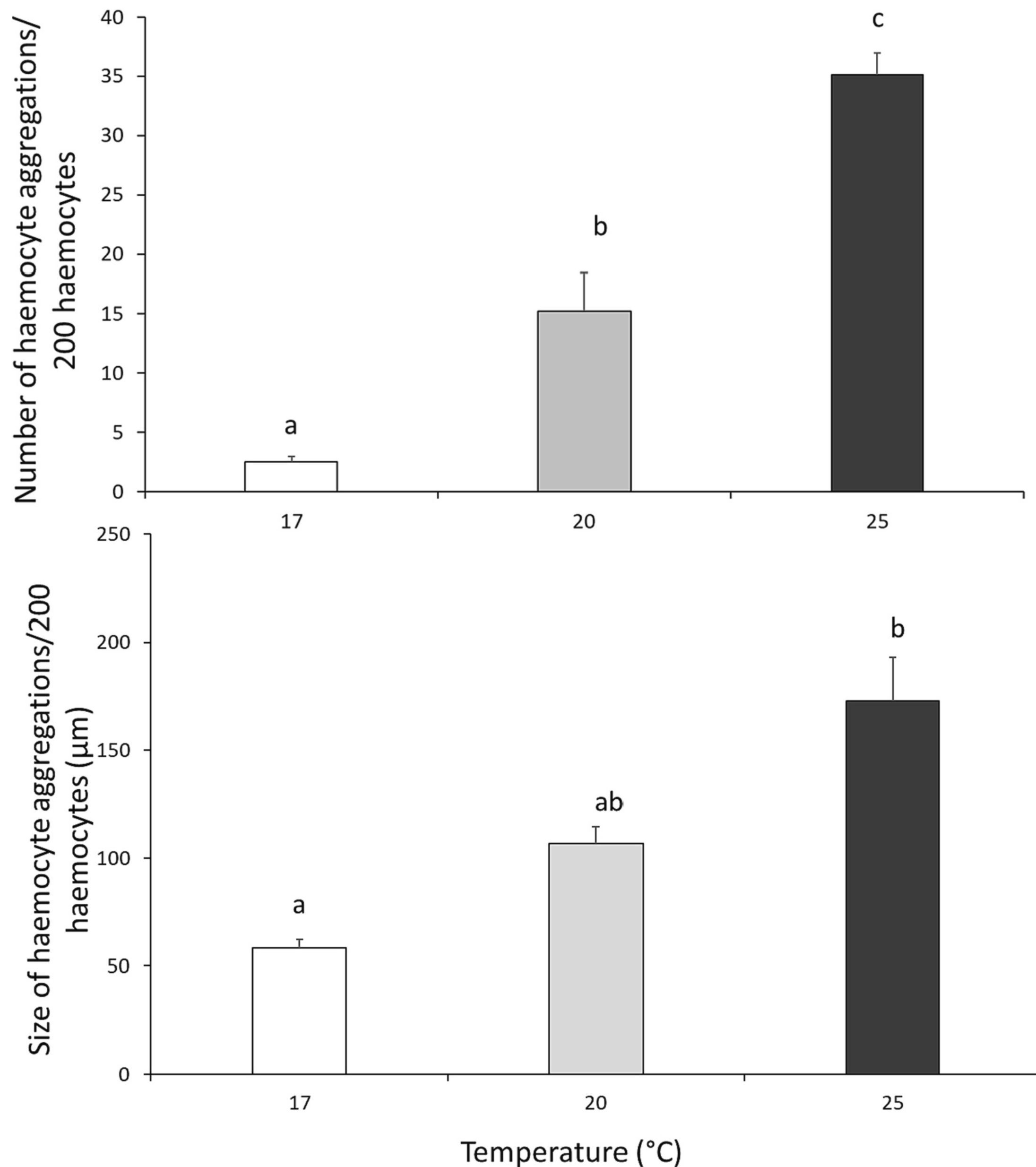

Fig. 6. Number (top) and size of haemocyte aggregations (bottom) recorded per 200 haemocytes in *Panopea zelandica* juveniles ($n = 9$) at different experimental temperatures. Significant differences ($p < 0.05$) are denoted by lowercase letters above each bar.

samples, the expression of *hsp70* was recorded to significantly increase with increasing temperatures (ANOVA, $F_{(2,20)} = 15.17$, $p < 0.001$). Relative comparisons between 17 °C and 25 °C showed a 543-fold increase in *hsp70* expression within the haemolymph (Fig. 7B). Results from *hsp90* expression showed no significant differences in either gill (Fig. 7C) or haemolymph samples (Fig. 7D). During the experimental treatments, the levels of *hsp90* remained similar at different experimental temperatures. There was an increase in *hsp90* expression in haemolymph samples at 25 °C, but this increase was not statistically significant.

4. Discussion

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

Behavioural responses are often difficult to quantify, especially for animals with limited visibility, such as infaunal clams (Woodin et al., 2020). In the majority of thermal studies bivalves are subjected to continuous thermal exposure, as opposed to considering the buffering effects of the sediment, thus limiting information on behavioural adaptations, (such as burial speed or depth or sediment temperature) (Rubio-Portillo et al., 2016; Whiteley and Mackenzie, 2016 ; Pansch et al., 2018; Shanks et al., 2020). With limited studies reporting on combined conditions, the common consensus is that burrowing bivalves tend to dig further into the sediment in response to thermal stress, as the sediment tends to partially buffer changes in temperature (Archambault et al., 2013; Zhang et al., 2020; Domínguez et al., 2021). In a natural setting, the ocean water and sediment pore water are usually separated,

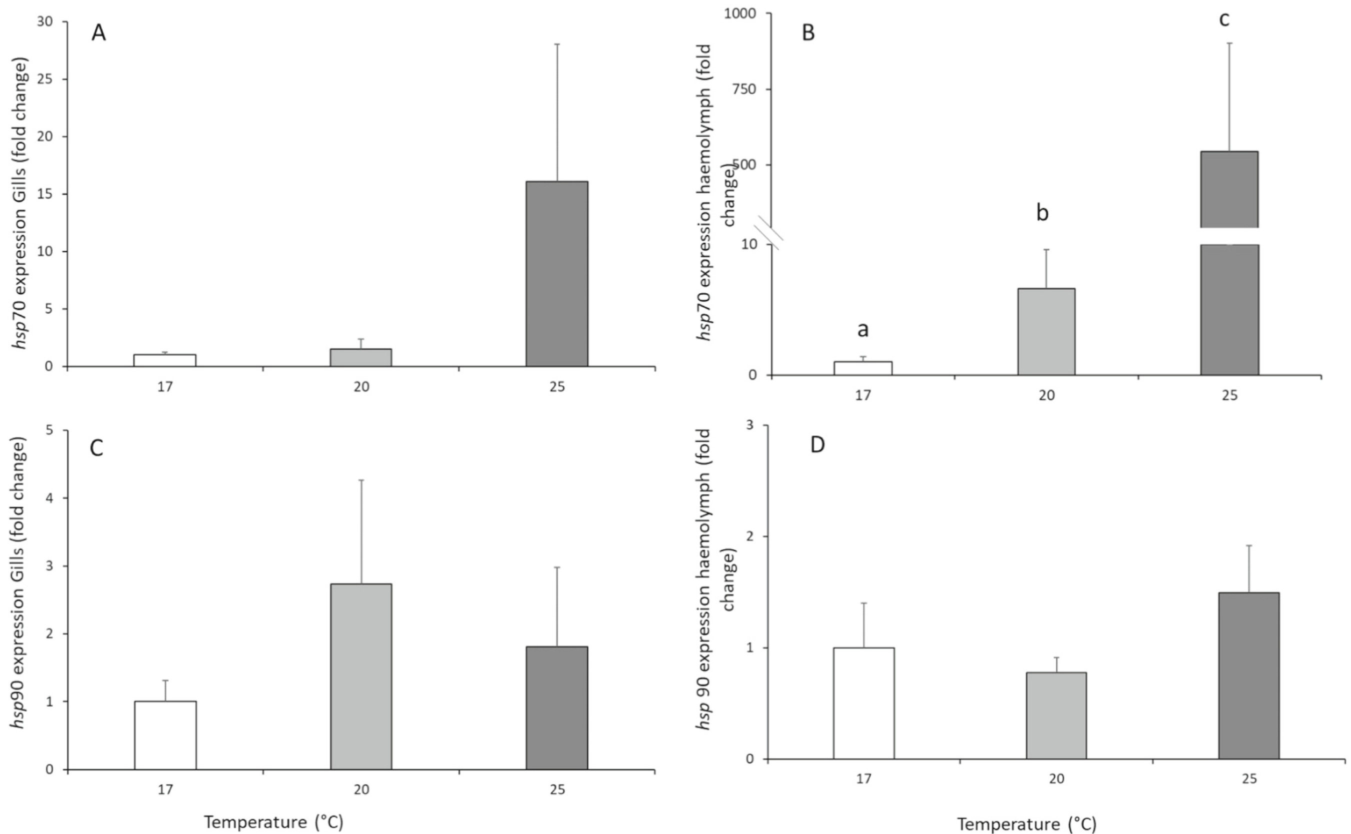

Fig. 7. The gene expression of heat shock proteins in *P. zelandica* juveniles (n = 9) exposed to 17, 20 and 25 °C. Fold change values of *hsp70* gene expression detected in A) gill and B) haemolymph samples, along with gene expression of *hsp90* in C) gill and D) haemolymph samples. Significant differences ($p < 0.05$) are denoted by lower case letters on top of each bar.

and temperature changes are gradual due to pore water circulation (Rato et al., 2022). In the present study, geoduck were left in situ, and with increasing temperatures the proportion of geoduck emerging from the sediment significantly increased. In a study on the Manila clam, *Ruditapes philippinarum*, it was found that at higher sediment temperatures the clam actively reduced its burial depth, approaching the sediment-water interface (Liu et al., 2022). The response of *R. philippinarum* was attributed to the rapid consumption of dissolved oxygen due to increased metabolic rate. In other studies, temperatures above the sublethal limit have also been documented to affect the burrowing ability of several bivalves, such as *Cerastoderma edule*, *Venerupis corrugata*, *Lampsilis siliquoidea*, *L. cariosa*, *L. fasciola,* and *L. abrupta* (Archambault et al., 2013; Domínguez et al., 2021). Therefore, the emergence of geoduck juveniles in this study could have been a sign of morbidity, if the temperature experienced was ultimately lethal, or could reflect active avoidance as the animals exit the sediment in search of a more suitable environment. However, the ability of geoduck to re-bury themselves decreases as the animal grows to a point where the animal is unable to rebury once out of the sediment; this is mainly due to their poorly developed foot muscles (Reidy and Cox, 2013). The behaviour of geoduck actively emerging from the sediment has not been observed in the natural environment, thus making this an interesting topic for future research.

High water temperature has been reported to influence haemocyte parameters, such as haemocyte motility and viability in bivalves (Fısıer, 1988; Monari et al., 2007). The higher percentage of granulocytes detected in geoduck with increasing temperatures, has also been documented in other bivalve species, such as the oyster, *Crassostrea virginica* (Chu and La Peyre, 1993), the clam, *Ruditapes philippinarum* (Liu and Zhao, 2018), and the mussel, *Mytilisepta virgata* (Hong et al., 2021). Monari et al. (2007) hypothesised that the increase in granulocytes due to increased temperature exposure is an immune response as bacteria tend to proliferate at higher temperatures. Indeed, Monari et al. (2007) observed a significantly higher number of bacteria surrounding granulocytes of the clam *Chamelea gallina* exposed to a water temperature of 30 °C. Therefore, the increase in percentage of granulocytes recorded in *P. zelandica,* in the present study, could be a prophylactic immune response to prepare for possible bacterial infection. On the other hand, in the present study, there was a decrease in the percentage of hyalinocytes observed with increasing temperatures. One likely cause for the decrease of hyalinocytes is the increase in the size of haemocyte aggregations with increasing temperatures, which made it harder to get an accurate count of hyalinocytes. Bivalve hyalinocytes have been reported to play an important role in haemocyte aggregations processes following wounding (Ruddell, 1971; Suzuki et al., 1991; Aladaileh et al., 2007; Gosling, 2015). Nakayama et al. (1997) found that when the haemolymph of the giant clams (*Tridacna crocea*) is exposed to seawater, the haemocytes tended to coagulate, with hyalinocytes forming the core of these clots. As the formation of haemocyte aggregations in uninfected individuals would seem to be maladaptive, it would be valuable to further investigate the pathways mobilised by both heating and wounding. Having observed an increase in the size of haemocyte aggregations with increasing temperatures in Manila clam (*R. philippinarum*), the authors offered the simple suggestion that haemocyte activity and cell-cell interaction increase with temperature, thus explaining the correlation between aggregate size and temperature (Flye-Sainte-Marie et al., 2009). Unlike other infaunal clams, *Panopea* genus has poorly developed adductor muscles and thus actively requires sediment to keep its valves fully closed. Once out of the sediment (in the case of animals in the 25 °C experimental group), the adductor muscles

are extended beyond the normal limits, potentially causing muscle tears; a hypothesis that will benefit from histological investigations in future studies.

In the present study, the expression of heat shock proteins (HSP70 and HSP90) in the gills and haemolymph were analysed. Heat shock protein expression has been reported to change in response to external stressors, such as temperature, potentially as a response to stress-induced tissue and protein damage (Fabbri et al., 2008; Liu et al., 2014; Velez et al., 2017; Jahromi et al., 2020; Timmins-Schiffman et al., 2020). From the current investigation, higher temperatures influenced the upregulation of HSP70 in the haemolymph. This is particularly important, considering the role haemolymph plays in eliciting mounting thermoprotection against severe cellular stress during times of heat shock (Silver and Noble, 2012). Although there was a trend of increasing *hsp70* gene expression in the gill tissue with increasing temperatures in this study , this was not significant. This result could be attributed to the difference in peak HSP gene expression at different times in different tissues. Li et al. (2016) found that in the pearl oyster *Pinctada martensii* the upregulation of HSP was faster in in the haemolymph compared to the gills. Moreover, it has been found that different tissues upregulate *hsp70* at different rates, with peak *hsp70* expression achieved in the gills of the scallop *Chlamys nobilis* by 6 h, declining thereafter (Cheng et al., 2020).

There was no significant difference in the expression of *hsp90* in either the gills or the haemolymph of *P. zelandica* in the current study. A number of investigations have shown that the expression of *hsp90* behaves differently compared to *hsp70*, with expression either not changing, or only being upregulated immediately following the initiation of heat-shock, declining thereafter (Lyons et al., 2003; Masanja et al., 2022). In *Pinctada maxima*, *hsp90* expression in the gills and haemolymph were upregulated within the first 6 h after exposure to heat-shock, peaking at 6 h and declining to the end of the experiment at 72 h (Masanja et al., 2022). Conversely, in a study on the Venus clam, *Paphia undulata*, *hsp90* was substantially elevated in the gonad and gills, and poorly expressed in the haemocytes following 2 h of heat stress. It has been suggested that this could be as a result of modifying biochemical reactions and changes in the resource energy allocation, with energy being reallocated towards more beneficial proteins, such as HSP70 (Masanja et al., 2022). It is possible that in the current investigation, HSP90 may be involved in the acute stages of heat stress with HSP70 being upregulated when *P. zelandica* experiences chronic stages of heat-shock. However, as sampling did not incorporate the initial stages of heat shock, this cannot be confirmed. Considering that samples for *hsp90* analyses were collected after 24 h of thermal exposure, expression levels might have previously peaked and subsided, necessitating smaller sampling intervals in future.

In both *hsp70* and *hsp90*, changes in gene expression related to heat-stress showed high individual variability among the geoduck. This is particularly so when *P. zelandica* was exposed to 25 °C. This level of variability suggests that *P. zelandica* may have the ability to acclimate to increased temperature as a result of the upregulation in the gene expression of heat shock proteins. Such variability has been demonstrated in other marine invertebrates, such as the urchin *Evechinus chloroticus*, and can indicate that some individuals are naturally preconditioned to tolerate heat-shock compared to others (Delorme et al., 2020). This variability is an important mechanism for the population in the face of ocean warming as it will allow resilient individuals or genotypes to be selected for future conditions. This can also be valuable for future aquaculture ventures involving *P. zelandica* as it can act as a screening tool for future selective breeding to generate resilient *P. zelandica* stock.

5. Conclusions

The novel findings from this study demonstrate that *P. zelandica* has a heightened response to thermal changes. As the temperature increased from 17 to 25 °C, there were changes in behaviour, haemocytes, and expression of *hsp70* and *hsp90*. Behaviourally, there was an increase in the number of animals emerging from the sediment with increasing temperatures. However, it is not known if the juveniles within the current experiment would have been able to rebury when the conditions became favourable again, thus representing an existential threat. Internally within the haemolymph, there was an increase in the proportions of granulocytes and cellular aggregations and a decrease in the proportions of small hyalinocytes in animals at 25 °C. At the molecular level, there was an increase in the expression of HSP70 within the haemolymph, indicating that *P. zelandica* was actively trying to prevent damage to proteins and tissues. The current study contributes to our understanding of how juvenile *P. zelandica* respond to thermal stress, providing underpinning knowledge for fishery management decisions and aquaculture planning phases, in an increasingly marine heatwave-affected environment.

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Ethical approval

This research was conducted ethically, and all experiments performed in accordance with the relevant institutional and national guidelines for the care and use of laboratory animals.

Declaration of Competing Interest

The Authors have no conflicts of interest to declare.

Data availability

Data were collected by the authors who take responsibility for their integrity and accuracy of analysis. Data presented in this paper will be made available on request.

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