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Immune response to temperature stress in three bivalve species: Pacific oyster *Crassostrea gigas*, Mediterranean mussel *Mytilus galloprovincialis* and mud cockle *Katelysia rhytiphora*

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ABSTRACT

Summer mortality of some bivalve species is often associated with the change of environmental temperature. This study compares the response of immunological parameters to temperature change in three marine bivalves: Pacific oyster *Crassostrea gigas*, Mediterranean mussel *Mytilus galloprovincialis* and mud cockle *Katelysia rhytiphora*. Each species was exposed to three temperatures, 15 °C, 20 °C and 25 °C for 14 days. The total haemocyte count (THC), phagocytosis, reactive oxygen species (ROS) and the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) were used as indicators to measure the response of each species to different temperatures. The highest temperature (25 °C) significantly increased the THC and phagocysis of haemocytes in all species. The SOD and CAT activities in the haemocytes of *M. galloprovincialis* and *K. rhytiphora* rapidly increased with temperature elevation, concomitantly with the increase of ROS ions. In contrast, the increases of ROS and SOD in *C. gigas* only occurred from 20 °C to 25 °C, suggesting that this intertidal species is more adaptive to different temperature levels. This study indicates that the activities of antioxidant enzymes can reflect the immune response of marine bivalves to thermal stress. Intertidal species such as Pacific oysters have a greater tolerance to thermal stress than subtidal species (e.g. Mediterranean mussel) and demersal species buried in sand (e.g. cockle).

1. Introduction

Shellfish farming is an important aquaculture industry in Australia, with the Pacific oyster *Crassostrea gigas* alone contributing a yearly revenue of > AUD60 million [1]. Mediterranean mussel *Mytilus galloprovincialis* production is currently > 1400 tonnes, worth AUD2.9 million [2], and the mud cockle *Katelysia rhytiphora* is an emerging aquaculture species. These bivalves are all filter feeders, inhabiting coastal waters and estuarine areas that are subject to a wide range of stressors including seasonal temperature change, pathogens and pollution [3–5]. In bivalve farming, seasonal temperature change has a significant impact on growth, reproduction and mortality [6–8]. In many farming regions, summer mortality of molluscs is often associated with temperature increase [9] and reproductive cycles [10]. Summer mortality events are a consequence of complex interactions between pathogens and environmental factors. The increase of water temperature may lead to bacterial proliferation in the water and bacterial accumulation in the tissues, leading to stress [11], disease and mortality [12].

Immune system function in shellfish has been a research focus in the past two decades. Molluscs, such as oysters, mussels and cockles, have evolved mechanisms that rely heavily on their innate immune system to defend against infection from exogenous pathogens [13]. It consists of both humoral and cell-mediated immunity systems to recognise and respond to pathogens in a generic way. Innate immunity is activated by the chemical properties of the antigen and comes into play immediately after antigens infect. When microbial infection occurs, the organism will then use enzymes to trigger immune responses [12,14,15].

The immune capacity can be measured by the response of immunological and pathological parameters [16,17]. In bivalves, the ability to counteract environmental stress and bacterial infections is mediated by plasma proteins, glycoproteins and circulation of haemocytes [11]. The haemocytes are responsible for recognition, phagocytosis and elimination by enzymatic or oxidative degradation of exogenous organisms. This process is accomplished by phagocytic

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haemocytes within the blood and haemolymph of the organism [11,18]. The response of immunocytes in invertebrates includes generation of reactive oxygen species (ROS), which is induced by the stress of environmental factors [19] and exposure to pathogens. This process involves reduction of oxygen to a superoxide anion (O_2^-), which generates various highly reactive oxygen species such as hydrogen peroxide, singlet oxygen or hydroxyl radicals [20]. Excess of these components can damage cellular structure and protein function [21]. However, the effect can be minimised by the physiological antibody defence mechanisms regulated by enzymes such as superoxide dismutase (SOD) and catalase (CAT). These enzymes can catalyse the conversion of hydrogen peroxide into less-reactive gaseous oxygen and water [22] and strengthen the defence mechanism for species relying on the innate immunity system [23]. Extracellular SOD also exists in the plasma of oysters and the purified SOD from the haemolymph of Pacific oysters has the function to reduce ROS by binding with lipopolysaccharides in *Escherichia coli* bacteria [44].

Water temperature fluctuation can significantly change immune functions in molluscs [11,13,23]. Despite the adverse effect of thermal stress on molluscan survival on farms, little is known on the physiological and immunological responses of commonly farmed molluscan species to temperature variation. Summer mortality events are more commonly reported on the sedentary Pacific oysters, but our knowledge is limited on other molluscan species for their susceptibility to increasing temperatures.

This study aims to understand the response of three bivalve species, Pacific oysters, Mediterranean mussels and mud cockles, within their respective habitats of the intertidal, sedentary and underground zones, to changes in ambient water temperatures experienced in temperate southern Australia. The effect of temperature on the defence system was examined by measuring immunological parameters and antioxidant enzymes (SOD and CAT) relevant to stress response in aquatic animals. The results of this study provide an insight into the understanding of the immune response of different molluscan species to temperature variation and identify possible strategies to reduce economic loss due to unexpected mortality of commercially important marine bivalves in southern Australia.

2. Materials and methods

2.1. Animal collection and management

Ninety Pacific oysters (*C. gigas,* mean shell length = 64.9 ± 2.5 mm), 90 Mediterranean mussels (*M. galloprovincialis,* mean shell length = 59.1 ± 1.8 mm), and 180 mud cockles (*K. rhythiphora,* mean shell length = 42.9 ± 1.1 mm) were collected from Coffin Bay, South Australia. Animals were dry-transported to the marine laboratory at Flinders University in foam boxes within 48 h of collection. The animals were acclimatised in a flow-through system for 2 weeks at 20–21 °C. During the acclimatisation and experimental periods, animals were fed daily *ad libitum* with mixed species of microalgae (*Isochrysis galbana*, *Pavlova lutheri* and *Chaetoceros muelleri*). Dead animals were removed from the tanks and replaced with similar sized animals and 50% of seawater in the tank was replaced once every 24 h through continuous water flow. The experiment was conducted in 25-L aquaria with aeration in triplicate. Dissolved oxygen (DO), pH, and salinity were monitored daily and ranged from 7.8 to 8.3 mg L^{-1}, 8.0–8.4 and 35.5–37.5‰, respectively. The nitrate, nitrite and ammonia levels were measured every second day using the Aquaspex test kit (Aquaspex Water Testing Product, Australia) and maintained at < 0.05 mg L^{-1} during the experimental period.

2.2. Experimental temperatures

Prior to the temperature trial, all animals were acclimatised to the experimental conditions by increasing or decreasing the water temperature from 20 °C progressively (2 °C/day) to achieve a low (15 °C), medium (20 °C) or high (25 °C) temperature. The water temperature in the experimental tanks was maintained at a constant value using a thermostat device. All species were analysed after being maintained at their respective temperatures for 14 days in triplicate. Each tank contained 10 animals of each species.

2.3. Haemolymph collection

Haemolymph (500 µl per individual) was collected from the posterior adductor muscle using a 16-gauge needle with 3 ml disposable syringe. The volume of haemolymph from five individuals was pooled as one sample for each replicate, centrifuged at 1000 × *g* for 10 min, and stored in liquid nitrogen. Fresh haemolymph (200 µl) of pooled haemolymph from five individuals was used to determine total haemocyte count (THC) and phagocytic rate. For each enzyme assay, 500 µl of haemolymph was pipetted off, transferred to a new tube, immediately on ice and kept at −80 °C until analysis.

2.4. Total haemocyte count (THC)

The THC was determined according to the method described by Chen et al. [24] with a slight modification of mixing 50 µl of haemolymph with 100 µl of 6% formalin (35 ppt saline solution). A drop of mixture was placed on a haemocytometer and the number of haemocytes was counted on a phase contrast microscope (CK40, Olympus).

2.5. Phagocytic activity

The phagocytic rate was measured using a modified method [25]. Briefly, a yeast solution for phagocytosis assay was prepared by autoclaving 2.5% baker's yeast *Saccharomyces cerevisiae* (Tandaco, Cerebos Foods, Seven Hills, NSW, Australia) in 4% Congo red (Sigma) in filtered seawater (FSW). The stained yeast cells were centrifuged at 1500 × *g* for 10 min, washed three times with FSW and re-suspended in FSW (0.2 µl) at 1 × 10^7 cells mL^{-1}. Fresh haemolymph (150 µl) was added to a 40 µl yeast suspension at room temperature, lightly vortexed and then settled for 20 min in the dark. At the end, tubes were vortexed and two drops (~50 µl) were placed onto a glass slide with a coverslip. Phagocytic rate was determined in triplicate as percentage of phagocytic haemocytes in 100 haemocytes under a microscope at 400 × magnification.

2.6. Quantification of reactive oxygen species (ROS)

ROS values were determined using an OxiSelect™ in vitro ROS/RNS assay kit (Green Fluorescence, STA-347; Cell Biolabs, Inc., San Diego, USA). The ROS free radical content in an unknown sample was measured fluorometrically by comparing with the hydrogen peroxide standard curve. A 50-µl plasma sample was transferred to a 96-well plate for fluorescence measurement. Next, 50 µl of catalyst was added to all wells and incubated for 5 min at room temperature. Subsequently, 100 µl of 2,7-dichlorodihydrofluorescein solution was added to each well and incubated at room temperature for further 15–45 min in dark. The plate reaction was then read at 480-nm excitation/530-nm emission on a FLUOstar Omega microplate reader (BMG Labtech, German).

2.7. Superoxide dismutase (SOD) activity assay

SOD activity was determined with a superoxide dismutase assay kit (706002; Cayman Chemical, USA) to evaluate the ability of the xanthine/xanthine oxidase system for generating superoxide anions. In brief, 200 µl of the diluted radical detector and 10 µl of plasma were mixed and added on a 96-well microtiter plate. Next, 20 µl pre-diluted xanthine oxidase solution was added into each well and shaken for a few seconds to mix and cover the plate. The plate was then incubated for 30 min at room temperature. The absorbance was read at

440–460 nm on a FLUOstar Omega microplate reader (BMG Labtech, German).

2.8. Catalase (CAT) activity assay

Catalase activity was determined using a catalase assay kit (707002; Cayman Chemical, USA). In the current study, 30 µl methanol, 20 µl sample, and 20 µl diluted hydrogen peroxide were mixed thoroughly on a 96-well microtiter plate and incubated on a shaker for 20 min at room temperature. Next, 30 µl of the potassium hydroxide was added into each well and mixed thoroughly to terminate the reaction and then followed by 30 µl catalase purpald (Chromogen). The plate was covered and incubated for 10 min at room temperature on a shaker. Catalase potassium periodate (10 µl) was then added to each well, covered and incubated for 5 min at room temperature on a shaker. The plate was read at 540 nm absorbance on the CLARIOstar Omega plate reader (BMG Labtech, German). The catalase activity was measured using the catalase activity assay standard curve.

2.9. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Data were tested for normality and homogeneity using Kolmogorov-Smirnov and Levene's test. The normality distributed data were analysed using two-way analysis of variance (two-way ANOVA) to test the effect of temperature and molluscan species on all dependent variables. When significant interactions between main factors were observed, pairwise comparisons were used to determine significant differences between treatment combinations. If the interaction between the temperature and species was not significant, the main effect was considered and the post-hoc Tukey's HSD was used for multiple comparisons. The level of significant difference was set at $P < 0.05$. All data were analysed using the statistical package IBM SPSS Statistics 22.

3. Results

3.1. Survival of each bivalve species

The survival of *C. gigas*, *M. galloprovincialis and K. rhytiphora* was 97.78%, 85.56%, and 78.34% respectively at the end of experiment across all temperature treatments. There was no mortality in *C. gigas* at 15 °C and 20 °C and in *M. galloprovincialis* at 15 °C. The lowest survival (51.66%) was observed in *K. rhytiphora* at 25 °C. The average survival for *K. rhytiphora* at 15 °C and 20 °C was 88% and 95% respectively. In *C. gigas* mortality only occurred at 25 °C, with two dead animals during the experimental period. The average survival of *M. galloprovincialis* at 20 °C and 25 °C was 87% and 80% respectively (Fig. 1).

3.2. Total haemocyte count (THC)

An increase in THC was observed as water temperature increased (Fig. 2a). At the end of the experiment, the THC was significantly affected by temperature (2-way 3 × 3 ANOVA; $P < 0.001$). The animals of all species held at 15 °C had significantly lower THC than those at 20 °C and 25 °C ($P < 0.001$). Furthermore, the highest THC was observed in the animals at 25 °C in all species. Post hoc comparisons indicated that there was significantly different THC levels between *M. galloprovincialis* and *K. rhytiphora* at 20 °C ($P = 0.044$). There was no interactive effect between species and temperature on THC ($P = 0.466$).

3.3. Phagocytic activity

The phagocytic activity in haemocytes (Fig. 2b) significantly varied among temperature treatments regardless of species ($P < 0.001$) and was also significantly different between species regardless of temperature ($P < 0.001$). There was no interaction between temperature and species in phagocytic activity ($P = 0.179$). Post hoc comparison detected that phagocytic activity significantly increased when temperature increased from 15 °C to 20 °C–25 °C in all three species, respectively ($P < 0.001$). At all temperatures tested, phagocytic activity in *C. gigas* was significantly higher than in *M. galloprovincialis* and *K. rhytiphora*, but no significant difference between *M. galloprovincialis* and *K. rhytiphora* ($P = 0.057$) was observed.

3.4. Reactive oxygen species (ROS)

As an index of oxidative stress, the ROS activity in haemocytes (Fig. 3a) significantly increased with temperature escalation ($P < 0.001$) and varied among species ($P = 0.006$). However, there was no significant interaction between temperature and species on ROS production ($P = 0.337$). Post hoc comparisons indicated when temperature increased from 15 °C to 20 °C–25 °C, the ROS activity significantly increased by each temperature increment in all species ($P < 0.001$), except *C. gigas* between 15 °C and 20 °C ($P = 0.123$). The ROS activity in *C. gigas* and *M. galloprovincialis* was significantly higher than in *K. rhytiphora* regardless of temperature ($P < 0.05$), but there was no significant difference between *C. gigas* and *M. galloprovincialis* ($P < 0.05$) at all temperatures.

3.5. Superoxide dismutase (SOD) activity

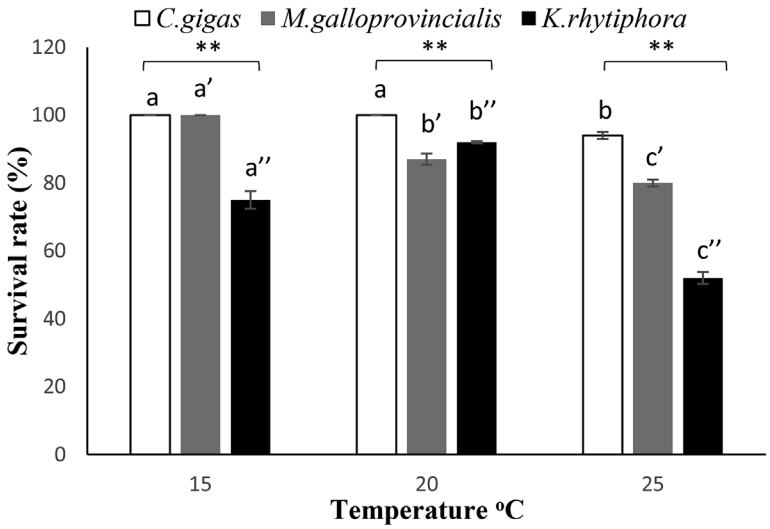
In haemolymph fluid, the production of SOD enzyme showed a significant increase with the increase of temperature from 15 °C to 20 °C–25 °C regardless of species ($P < 0.05$, Fig. 2b), with no significant difference between species ($P = 0.134$). No significant interaction was found between temperature and species on the SOD activity ($P = 0.995$). A post hoc comparison revealed that the SOD activities between 15 °C to 20 °C did not significantly differ ($P = 0.45$) in *C. gigas,* which was congruent with a lesser generation of ROS in *C. gigas* from 15 °C to 20 °C.

3.6. Catalase (CAT) activity

The CAT activity significantly increased with increasing temperature in all species (two-way ANOVA, $P < 0.001$) (Fig. 3c) and was significantly different between species ($P < 0.001$). There was no significant interactive effect between temperature and species ($P = 0.133$). Post hoc comparison revealed that catalase activity significantly increased from 15 °C to 20 °C ($P < 0.001$) and from 20 °C to 25 °C ($P < 0.001$) regardless of species. Conversely, there was no significant difference of catalase activity between 20 °C and 25 °C in *C. gigas* ($P = 0.075$). The catalase activity in *C. gigas* and *M. galloprovincialis* was significantly higher than that in *K. rhytiphora* ($P < 0.001$) at all temperature levels. However, there was no significant difference between *C. gigas* and *M. galloprovincialis* at any temperature ($P > 0.05$).

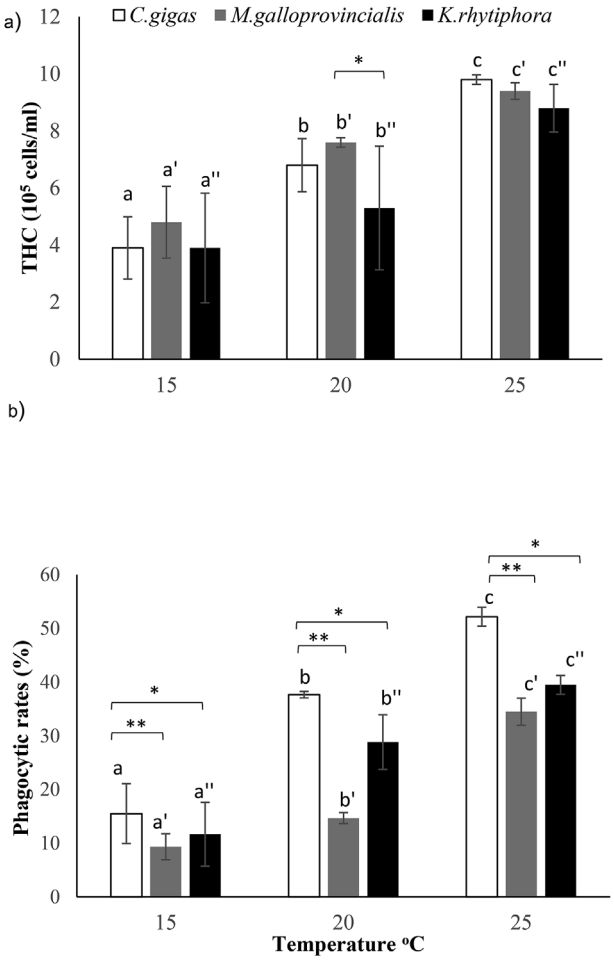
4. Discussion

Mortality in epidemic outbreaks is the most important factor leading to low production in shellfish aquaculture. In marine environments, temperature can have a strong effect on the function of immune defence systems in molluscs [11,13,23]. Indeed, temperature also alters the rate of biological, chemical and enzymatic reactions [26]. In the present study, temperature affected the survival of all molluscan species with highest mortality occurrence at the highest temperature of 25 °C. The *K. rhytiphora* is the most affected species by the elevation of temperature. Over the last decade, there has been growing awareness that temperature is related to disease incidence or mass mortality in marine bivalves due to immunosuppression [23,27,28]. This comparative immune study of marine bivalves from different thermal conditions has



Treatment	*P*
Temperature	<0.001
Species	<0.001
Temp x Species	<0.001

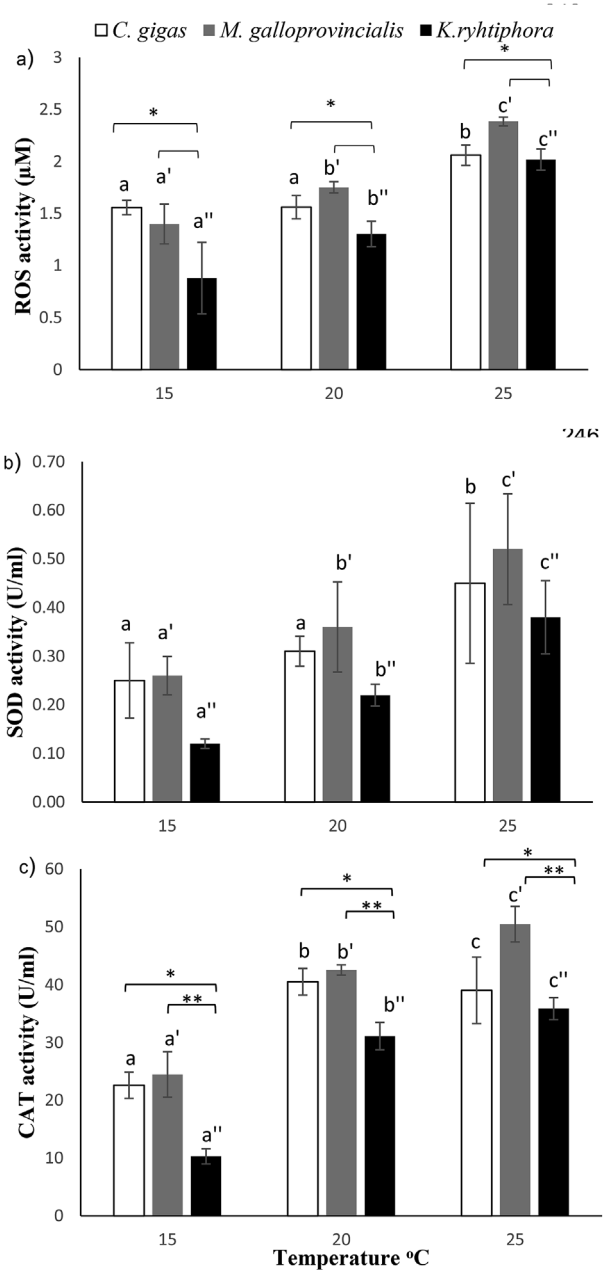
Fig. 1. Survival of animals (*Crassostrea gigas*, *Mytilus galloprovincialis* and *Katelysia rhytiphora*) after 14 days exposed to temperature treatments (15 °C, 20 °C or 25 °C). Different sets of letters (a, b and c for oysters; a', b' and c' for mussels and a", b" and c" for cockles at three temperatures, respectively) indicate significant difference between temperatures within a species. The hatching bracket between 2 bars represents significant difference between species at the same temperature. The single asterisk (*) on each bar represents significant difference at $P < 0.05$, and double asterisks (**) are for a difference at $P < 0.001$. The same letter with different apostrophes (e.g., a, a' and a") is not for comparison between species at a same temperature. Error bars represent standard deviation.



Treatment	*P*
Temperature	<0.001
Species	0.051
Temp x Species	0.466

Treatment	*P*
Temperature	<0.001
Species	<0.001
Temp x Species	0.179

Fig. 2. Water temperature changes affect a) total haemocyte counts (THC); b) phagocytic rates for three molluscan after 14 days exposed to temperature treatments (15 °C, 20 °C or 25 °C). Symbols and signs refer to Fig. 1.



Treatment	P
Temperature	<0.001
Species	0.006
Temp x Species	0.337

Treatment	P
Temperature	0.009
Species	0.134
Temp x Species	0.995

Treatment	P
Temperature	<0.001
Species	<0.001
Temp x Species	0.133

Fig. 3. Average of a) reactive oxygen species (ROS); b) superoxide dismutase (SOD) and c) catalase (CAT) activities in haemocytes for three molluscan species after 14 days exposed to temperature treatments (15 °C, 20 °C or 25 °C). Symbols and signs refer to Fig. 1.

illustrated that water temperature has a significant impact on immune systems, both at cellular and antioxidant levels. However, the nature of each immune parameter differed among the three species, suggesting a considerable degree of inter-species variation in the relative importance of individual parameters to the overall immune response. Moreover, the induction of oxidative stress in all species tested were minimised by active anti-oxidative enzymes (SOD and CAT) in response to temperature elevation during a short-term exposure.

4.1. Cellular response to different temperatures

In this study, the temperature change significantly increased the value of total haemocyte count (THC) in all species. The THC in *C. gigas*, *M. galloprovincialis* or *K. rhytiphora* was sensitive to temperature stress and increased from 15 °C to 20 °C or from 20 °C to 25 °C after a period of 14 days exposure, indicating that variations of temperature could affect the functional responses of mollusc haemocytes. The results were also in agreement with other studies where haemocyte counts in *C. gigas*, *M. galloprovincialis*, and *Ruditapes phillipinarum* are positively correlated with increasing water temperature [29–31]. It is suggested that the increased THC in organisms at the different temperature levels could be a result of cell mobilisation or cell proliferation from tissues into the haemolymph circulation [23,32]. In any event, haemocytes are important and known to be involved in wound healing to avoid loss of haemolymph and interference of microorganisms upon injury. In addition, haemocytes also secrete antimicrobial metabolites for bacterial recognition and killing [11]. It is clear that temperature is a crucial factor to regulate THC levels. This coincides with previous studies that temperature can influence haemocyte activities of mollusc species [13,18,28,32].

Temperature change affects other important immune functions, such as inhibition of phagocytic activity [28,32–34]. In the current

study, the phagocytic activity of haemocytes increased significantly over the thermal stress application, which shows a similar pattern to the changes of THC in *C. gigas*, *M. galloprovincialis*, and *K. rhytiphora*. Similar observations were reported previously in *M. galloprovincialis* where the capability of haemocytes to engulf foreign particles is lower at 10 °C than at 20 °C and 30 °C [31]. Likewise, Monari [23] has reported that the clam *Chamelea gallina,* exposed to 30 °C water temperature, suffered a significant inhibition to phagocytic activity and Hegaret et al. [18] demonstrated a significant decrease in phagocytic activity in *Crassostrea virginica* kept at 28 °C for 7 days. In a similar experiment, Chu and La Peyre [34] indicate that phagocytosis reduces in oysters at 25 °C compared to oysters held at 10 °C and 20 °C for 68 days. Above a certain threshold, the temperature-induced reduction in enzymatic processes for phagocytic activities leads to increased cells damage [27,29].

Thermal stress induces the formation of ROS in haemocytes, and other small toxic molecules including superoxide radical (O_2^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and singlet oxygen ($^1O_2$), which are involved in internal defence to eliminate non-self particles [19]. The production of ROS was previously reported in the haemocytes of bivalve molluscs, including oysters [20,35], mussels [36], scallops [32], and clams [28]. All of these studies demonstrate that higher temperatures strongly influence several metabolic, physiological and immune parameters. In the present study, the increased level of ROS varied among species. The *M. galloprovincialis* and *K. rhytiphora* were significantly stressed by the increasing or decreasing temperature. Despite an increase of ROS level in *C. gigas* throughout temperature treatments from 20 °C to 25 °C, no mortality was observed, suggesting a greater resilience to temperature stress. Gagnaire et al. [29] also demonstrate that only extreme temperature conditions (40 °C, 50 °C, and 60 °C) significantly affect the function of *C. gigas* haemocyte activities. Additionally, this phenomenon has also been observed in *C. gigas* under heavy metal stress, where only high concentrations of mercury chloride are able to affect and kill haemocyte cells after 4 h of in vitro exposure [37].

4.2. Response of antioxidant enzymes to different temperatures

Antioxidant defences are composed of three general groups including fat-soluble vitamins, water-soluble reducing agents, and enzymes including superoxide dismutase and catalase [38]. One of the unique characteristic of these enzymes is their inducibility as an adaptation to the environmental change when marine invertebrates are under oxidative stress [38,39]. In this study, temperature increases enhanced ROS production, thereby increasing the risk of oxidative damage. The radical formation and damage to tissues is balanced by an array of main cellular antioxidants, superoxide dismutase ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$) and catalase ($2H_2O_2 \rightarrow 2H_2O + O_2$) to neutralise ROS before starting the radical reaction chains. The SOD activity in these marine bivalves increased significantly from a low temperature at 15 °C to a high temperature at 25 °C, suggesting that the enzyme has protective responses to catalyse reactive free radicals. We observed a higher SOD activity in *M. galloprovincialis* and *K. rhytiphora* compared to *C. gigas*. The change in environmental conditions of the habitat can lead to a functional response of antioxidant enzymes [40]. The off-shore and underground species are exposed to a less fluctuation of environmental condition compared to the intertidal species, such as *C. gigas,* that can adapt to elevated temperatures. However, differences in SOD between temperature treatments were not significant between *M. galloprovincialis* and *K. rhytiphora*. This is possibly because SOD activity could instantaneously counteract with the higher concentration of ROS in *M. galloprovincialis* and *K. rhytiphora* and result in no significant variation between these two species. The different of SOD level by *K. rhytiphora* supports the observation by Monari et al. [24] that the SOD activities in both haemocytes and cell-free haemolymph of clams *Chamelea gallina* after 7 days of experiment are higher in animals at 25 °C and 30 °C than those at 20 °C. Similarly, in the mussel *Perna viridis* the level of SOD activity increases significantly with the increase of body toxicant concentrations when the animals are transferred from relatively clean sites to various polluted sites [38]. These studies demonstrate that marine bivalves could often experience intensified oxidative stress and their mortality in estuarine habitats could partly depend on their ability to activate the antioxidant defence mechanism. In this study, the mortality of each species could not be well explained by the change of immunological parameters measured, suggesting that the cause of mortality is complex and goes beyond the change of these immunological parameters measured in this study.

When marine bivalves are exposed to H_2O_2 they can activate catalase to counteract stress. However, antioxidant activity may not necessarily increase as a result of environmental stress [23,41]. The activity of CAT showed high variation compared to the SOD responses in all three-molluscan species. The high CAT activity observed in the haemocytes of *M. galloprovincialis* and *K. rhytiphora* under temperature stress, suggests that the oxidative stress is prone to peroxide radicals. This result agrees with the elevated CAT activities after exposed to stressors in other molluscan species such as *Mytilus edulis*, *M. galloprovincialis* [42,44] and *Crassostrea* sp [43]. In contrast, the CAT activity in *C. gigas* was not significantly different among temperature treatments. The CAT enzyme level increased from 15 °C to 20 °C, but decreased from 20 °C to 25 °C. The weak response may be associated with the exposure to stress, which can be explained by the induction of antioxidant mechanism. In this case, *C. gigas* may be able to acclimate better to elevated temperatures as they dominate the region where fluctuating environmental conditions prevail.

In conclusion, the present study demonstrates that water temperature change affects heamocytic function and leads to oxidative stress, reducing immunosurveillance in these three marine bivalves. The major differences in immune dynamics are related to the habitat of these three species in the wild. Animals in the intertidal zone need to cope with more extreme temperatures compared to those in the deeper water. The *K. rhytiphora,* appears to be more sensitive to temperature changes than other species as indicated by haemocyte activities. However, further investigation is needed to understand the relationship between immune response to thermal stress and the mortality of bivalves to pathogen infection under thermal stress. Conversely, the intertidal species *C. gigas* can tolerate extreme thermal stress allowing it to densely dominate the intertidal and subtidal zones. This study improves our knowledge on temperature-induced immune modulation of marine bivalves in the scenario of possible global warming in future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.017.

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