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Vinh T. Dang
Flinders University

Peter Speck
Flinders University

Kirsten Benkendorff
Southern Cross University

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TITLE PAGE

Influence of elevated temperatures on the immune response of abalone, *Haliotis rubra*

Vinh T. Dang[1], Peter Speck[1], Kirsten Benkendorff[2*]

[1] School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia

[2] School of Environmental Sciences and Management, Southern Cross University, PO Box 157 Lismore, NSW 2480

* Corresponding author: Kirsten Benkendorff

Ph: +61 2 6620 3755

kirsten.benkendorff@scu.edu.au

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ABSTRACT

Elevated water temperature can act as a stressor impacting the immune responses of molluscs, potentially increasing their susceptibility to microbial infections. Abalone are commercially important marine molluscs that have recently experienced disease outbreaks caused by a herpesvirus and *Vibrio* bacteria. Sampling of wild-caught *Haliotis rubra* showed a significant correlation between water temperature and both antiviral and antibacterial activity, with higher activity in summer than in winter months. However, antibacterial activity was compromised in favor of antiviral activity as the water temperatures peaked in summer. A controlled laboratory experiment was then used to investigate several immune responses of *H. rubra*, including total haemocyte count (THC), stimulated superoxide anion production (SO), antiviral activity against a model herpesvirus, herpes simplex virus type 1 and antibacterial activity against a representative pathogenic bacterium, *Vibrio anguillarum*, over one week after raising water temperature from 18 to 21 or 24 °C. THC and SO increased at day 1 and then dropped back to control levels by day 3 and 7. By comparison, the humoral immune parameters showed a delayed response with antibacterial and antiviral activity significantly increasing on day 3 and 7, respectively. Consistent with the field study, antibacterial activity became significantly depressed after prolonged exposure to elevated temperatures. A principal components analysis on the combined immune parameters showed a negative correlation between antiviral and antibacterial activity. SO was positively correlated to THC and neither of these cellular parameters were correlated to the humoral antimicrobial activity. Overall, this study indicates that abalone may have more resilience to viruses than bacterial pathogens under conditions of elevated temperature, such as those predicted under future climate change scenarios.

1. INTRODUCTION

Climate change is a major threat to the economic and ecological sustainability of marine fisheries and aquaculture. The average sea surface temperatures have increased by 0.6°C in the last 100 years and these changes are ongoing [1, 2]. Recently, climate change has been implicated in the increasing frequency and severity of disease outbreaks in the marine environment [3-7]. Notable examples of recent disease epidemics in marine molluscs include the northward expansion of oyster diseases [8, 9], escalating summer mortality in European and Australian bivalve and abalone aquaculture [10-14] and the recent detection of new oyster and abalone herpesviruses [15]. Climate change associated with El Niño and La Niña events has also been implicated in the prevalence of *Perkinsus* infections in oysters [16] and withering syndrome in abalone [17, 18]. Increased incidence and prevalence of marine disease is likely to have substantial ecological and economic costs, providing a compelling need to understand the complex interactions leading to disease outbreaks [1].

Disease outbreaks in marine molluscan populations are often associated with increases in water temperature [19-23]. The manifestation of disease involves an interaction between pathogen, environment and the physiological status of the molluscan host (Fig. 1). The incidence of disease increases under environmental conditions that cause stress to the host and/or increase pathogenicity and pathogen prevalence. Elevated temperature can increase the growth rate and virulence of microbial pathogens, including *Vibrio* spp. [4, 24]. Environmental stressors, such as elevated temperature, are also known to decrease molluscan host resistance to bacterial pathogens [25, 26]. For example, when exposed to elevated temperatures outside the preferred temperature range, abalone *Haliotis diversicolor supertexta* and *H. tuberculata* have shown

significantly increased total haemocyte counts (THC), superoxide anion levels (SO) and susceptibility to infection by *Vibrio parahaemolyticus* or *V. harveyi* [21-23, 27]. Viral abundance and virulence also appear to increase positively with water temperature [28]. However, to date, no studies have investigated the effects of temperature on antiviral defense mechanisms in marine molluscs.

The abalone innate immune system consists of cellular and humoral components [26]. Antimicrobial compounds acting as humoral effectors of molluscan immunity can be constitutively expressed and rapidly induced to provide an immediate response to invading microorganisms [29, 30]. Humoral immunity of abalone has been demonstrated using antibacterial activity assays against marine pathogenic bacteria, *V. harveyi* and *V. anguillarum* [31, 32] and an antiviral assay against herpes simplex virus type 1 (HSV-1) [31, 33] on cell-free haemolymph. Because antimicrobial factors in the haemolymph are often synthesized by haemocytes [26], humoral immunity could be partly dependent on the concentration and activity of haemocytes. Cellular immunity is centered on the activity of haemocytes, including the elimination of infectious agents by release of superoxide anion, phagocytosis of microbial pathogens and the recognition and elimination of infected cells [26, 34]. Antibacterial and antiviral levels in the haemolymph plasma, total haemocyte count and superoxide anion production in haemocytes have been used as representative humoral and cellular immune parameters of abalone in the current study.

Abalone are a major economic species in many countries including United State, Mexico, South Africa, Australia, New Zealand, Japan, China, Taiwan, Ireland, and Iceland [35-39]. *Haliotis*

rubra is a common cold water abalone species in south-eastern Australia, with a preferred temperature range of 8 to 17 °C [40, 41] and a critical thermal maximum reported at 26.9 °C [41]. With elevated temperatures, animals need to increase their metabolism to acquire an adequate energetic supply for respiration and general survival [42, 43]. We therefore hypothesize that less energy is available for an immediate immune response and the synthesis and release of antibacterial and antiviral factors will be compromised after temperature stress. Field sampling across seasons was used to correlate changes in antiviral activity against HSV-1 and antibacterial activity against *V. anguillarum* to natural changes in water temperature. A manipulative laboratory experiment was then used to further investigate THC, SO, antiviral and antibacterial activity to in response to short term elevated temperature. This combination of experiments provides an insight into the potential resilience of abalone to viral and bacterial pathogens under realistic seasonal and rapid (within a low tide cycle) temperature fluctuations and provides a model for predicting the longer term impacts of ocean climate change.

2. MATERIALS AND METHODS

2.1 Field sampled abalone

H. rubra were sampled for haemolymph at O'Sullivan Beach, South Australia, every month from August 2009 (austral winter) to February 2010 (summer). For each time point, at least 20 abalone were collected for haemolymph sampling. Water temperature was also recorded at each sampling time point using a hand-held thermometer.

2.2 Laboratory temperature challenge

The laboratory experiment was intended to compliment the field study by examining rapid changes in water temperature, which do in fact naturally occur on a day to day basis in natural habitats and in shallow water tanks on abalone farms in South Australia. Wild abalone can experience significant changes in temperature over diurnal tidal cycles, especially for those in the shallow intertidal rock pools (exceeding 6 °C increases during summer low tides). Abalone, *H. rubra* (n = 90), were collected in May 2010 from the intertidal zone of O'Sullivan Beach, South Australia, then acclimated in aquaria at Flinders University for two weeks in filtered seawater with continuous aeration at 18 °C (to match the mean temperature in the field at the time of collection). The abalone were determined to be healthy, with no visible lesions and they maintained a firm grip on the tank surface. The abalone of similar size, 8-9 cm in shell length, were kept in 9 PVC tanks (50 liter capacity, 10 abalone in each) containing 45 L per tank of sea water. Abalone holding tanks were connected to a 50 liter sump tank in groups of three, allowing water to flow continuously at the same rate of about 2 L min^{-1}. During the acclimation period, abalone were fed three times per week with fresh *Ulva lactuca*, collected from O'Sullivan Beach.

H. rubra were subjected to indoor temperature challenge in triplicate tanks (Fig. 2). Water temperature was kept at 18 °C in one sump tank (unchallenged group), while it was raised to 21 °C or 24 °C in the other sump tanks (temperature challenged groups) at the rate of 1 degree h^{-1} by using portable 300 watt glass heaters (Aqua One). Haemolymph was sampled from six replicate abalone from the unchallenged and challenged groups (two abalone from each of three tanks per treatment) at day 1, 3 and 7 (Fig. 2). Haemolymph (3 ml) was withdrawn from the anterior sinus of abalone using a pre-cooled sterile syringe with 25G needle and kept on ice.

2.3 Haemolymph parameter measurements

H. rubra haemolymph collected from the field from August 2009 to February 2010 was assessed for antiviral and antimicrobial activity. *H. rubra* haemolymph from the laboratory temperature challenge experiment was measured for total haemocyte, intracellular superoxide anion, antiviral activity against HSV-1 and antibacterial activity against *V. anguillarum*.

2.3.1 Total haemocyte count (THC)

Fresh haemolymph (50 µl) was fixed in 10% formalin in PBS solution (100 µl) in pre-cooled centrifuge tubes on ice to prevent haemocytes from aggregating or clumping. All haemolymph samples were briefly vortexed before being placed on an improved Neubauer hemocytometer (Weber, England) in duplicate for counting the number of haemocytes under a microscope (Olympus CX40). THC is expressed as cells x 10^4 per ml.

2.3.2 Superoxide anion production

Superoxide anion level (SO) of haemocytes, was quantified using reduction of nitroblue tetrazolium (NBT) to formazan, as described by Cheng et al. [23]. Fresh haemolymph (150 µl) was kept on ice before being placed in triplicate into wells of a 96-well microtitre plate for 20–25 min at room temperature to obtain a cell monolayer. PBS was used as negative control to measure the background breakdown of NBT. After attachment, the supernatant was discarded, and 100 µl of sodium alginate (0.2 mg ml^{-1} in PBS) was added to activate SO production within the haemocytes [e.g. 23, 32, 44, 45]. After incubation at 26 °C for 30 min, sodium alginate was discarded and haemocytes were stained with 100 ml NBT solution (0.3 %) for 30 min at 26 °C. The NBT solution was removed and haemocytes were fixed with 100 µl methanol (100 %),

washed three times with 100 µl methanol (70 %, in PBS) and air dried. The formazan was dissolved by addition of 120 µl KOH (2 M) and 140 µl dimethyl sulphoxide (DMSO). Absorbance was measured at 630 nm using a microplate reader (FLUOstar Omega). Superoxide anion level was expressed as treatment absorbance - negative control absorbance.

2.3.3 Antiviral assay

Phylogenetic analysis of DNA polymerase genes from abalone and oyster herpesviruses suggests that the abalone and oyster herpesviruses are within the family *Malacoherpesviridae* and distantly related to other members of the *Herpesviridae* [15, 46, 47]. Due to the lack of a cell line for culturing abalone herpesvirus, a heterologous model using Vero cells and herpes simplex virus type 1 (HSV-1) was chosen for investigating the effect of increased temperature on abalone antiviral activity, as this has been successfully used to assess antiviral activity in previous studies on molluscs [31-33, 48, 49]. A well-characterized strain, SC16 [50, 51] of wild-type herpes simplex virus type 1 (HSV-1) was obtained from the Institute of Medical and Veterinary Science, Adelaide. Culture of Vero cells and HSV-1 and the plaque reduction assay to measure antiviral activity of abalone haemolymph against HSV-1, were carried according to our previous studies [31, 33]. Haemolymph plasma was obtained by centrifuging crude haemolymph (3,000 rpm, 10 min, 4 °C) then the cell free plasma layer was pipetted off the top, leaving behind the cell pellet. The cell free plasma was then stored at -80 °C until assayed. Haemolymph plasma at 6 % (v/v) was used throughout to compare antiviral activity (EC_{50} = 6.23 %, v/v i.e. the effective concentration required to inhibit HSV-1 plaque formation by 50%).

2.3.4 Antibacterial assay

V. anguillarum is a common pathogen of marine molluscs including abalone. Stock cultures of *V. anguillarum* were obtained from the Fish Health Unit, Department of Primary Industries, Tasmania and held at -80 ºC in 10% glycerol, nutrient broth until use. This bacterium was cultured in sterile nutrient broth (1 g NaCl, 2 g yeast extract and 1 g peptone per 100 ml distilled H_2O) overnight at 37 ºC on an orbital mixer shaker (Ratek) at 200 rpm. The cultures were diluted to OD600nm = 0.1 on a spectrophotometer (Metertech, UV/VIS SP8001) and returned to exponential growth phase (OD600nm = 0.18 - 0.2) prior to use in antimicrobial assays. Antibacterial activity in the cell-free haemolymph plasma was measured using MTS assay against *V. anguillarum*, as described previously [31, 32]. The MTS assay is based on reduction of MTS tetrazolium to a red formazan product by dehydrogenase enzymes from live cells. 90 µl of hemolymph plasma and 10 µl of *V. anguillarum* in exponential growth culture were added into a 96-well plate in triplicate. Negative controls had 90 µl hemolymph in 10 µl nutrient broth and positive controls had 10 µl of *V. anguillarum* in 90 µl of nutrient broth. After 30 min incubation, 20 µl of CellTitre 96® Aqueous One Solution (Promega, NSW, AUS) was added to each well. The plates were then incubated at 37 °C for a further 2 hrs or until development of the red formazan product in positive controls. Different incubation temperatures including 18, 24, 30 and 37 °C were also tested and showed the same trends in antibacterial activity (Supplementary Figure 1). Absorbance was measured at 492 nm using a 96-well plate reader (FluoStar Omega). Antibacterial activity (%) was calculated from 100 - (treatment absorbance - negative control absorbance) / positive control absorbance x100.

2.4 Statistical analysis

To investigate how abalone antimicrobial activity varies in the field in relation to water temperature, a correlation between water temperature and antiviral or antibacterial activity was tested using Pearson Correlation in PASW/SPSS statistics 18. Univariate PERMANOVA analyses using Primer V6 + PERMANOVA (Plymouth Marine Lab) were performed to identify how each immune parameter (THC, superoxide anion level, antiviral activity and antibacterial activity) was individually influenced by temperature and/or length of exposure in laboratory challenge experiment. The four immune parameters were normalized to the same scale [52] before conducting multivariate analysis for all four immune parameters combined. A principal components analysis was run using the multivariate data with vector overlay to investigate how each immune parameter influenced the grouping of the data.

3. RESULTS

3.1 Field survey

Water temperature was lowest in August and September, (13 and 12.5 °C respectively), and reached 26.5 °C in February (Fig. 3). Generally, antiviral activity increased across months consistently with an increase in water temperature (Fig. 3). There was a strong correlation between water temperature and antiviral activity, with 85% of the variation in antiviral activity across the seven months explained by water temperature (Pearson correlation, $r=0.92$, $p=0.003$). Antiviral activity was lowest in September 2009 (mean of 46.04 %) and highest (63.76 %) in February 2010 (Fig. 3). At each monthly sampling point, there was high variation in antiviral activity (up to 42-86 %) with a trend towards more variation in higher temperature months (November- February, Fig. 3).

Antibacterial activity against *V. anguillarum* showed more variation across months, which was not directly explained by increase in water temperature (Fig. 3). Nevertheless, 61% of the variation in antibacterial activity was still explained by water temperature (Pearson correlation, r=0.78, p=0.04). Antibacterial activity peaked in December 2009 (42.31 %) then decreased slightly in January (38.12 %) and February 2010 (36.54 %), despite increases in water temperature (Fig. 3). Antiviral and antibacterial activities across monthly sampling points were not significantly correlated (Pearson correlation, r=0.62, p=0.14).

3.2 Laboratory temperature challenge experiment

There was no mortality of abalone in the temperature challenge experiment over 7 days. The effect of temperature across days was tested on each of the individual immune parameters. By day 1, THC was elevated in the temperature-challenged groups, and these haemocyte numbers remained variable with prolonged exposure at 21 °C, but appeared to drop back to the level of controls by day 7 in the 24 °C group (Fig. 4a). Univariate analyses revealed significant difference in the THC of *H. rubra* according to temperature, but there was no significant effect of day or an interaction between these factors (Table 1). Pair-wise tests between temperatures across all days revealed that THC in both of the temperature-challenged groups was significantly higher than in control group (18 vs 21 °C, t=3.79, p=0.003; 18 vs 24 °C, t=2.18, p=0.037). Intracellular superoxide anion levels in haemocytes were elevated in temperature treated groups compared to the controls. In the 24 °C group, SO anions peaked on day 1 then dropped back to the level of controls by day 7, whereas at 21 °C, SO anion levels appeared to rise more slowly across the 7 day period (Fig. 4b). However, univariate PERMANOVA found no significant difference in superoxide anion levels according to temperature or day (Table 1).

On average, antiviral activity was higher in both temperature-challenged groups compared to the control groups and reached a maximum of 72.5% inhibition of HSV-1 plaque formation at day 7 in the 24 °C group (Fig. 4c). There was a significant interaction between temperature and day (Table 1), however, most of the variation was due to temperature (coefficients of variation for temperature and day were 54.4 and 8.5, respectively) (Fig. 4c). Pair-wise analyses for temperature across days revealed a significant difference in antiviral activity on day 1 between 18 vs 21 °C ($t=4.54$, $p=0.001$) and 18 vs 24 °C ($t=1.96$, $p=0.05$) and on day 7 between 18 vs 24 °C ($t=4.37$, $p=0.01$). There was no significant difference between different days within the controls or 21 °C challenged abalone. The antiviral activity of abalone in the 24 °C group was significantly higher on day 7 than on day 1 ($t=2.78$, $p=0.025$).

On day 3, antibacterial activity tended to be elevated in the temperature-treated groups, whilst by day 7, antibacterial activity was depressed relative to 18 °C controls (Fig. 4d). Antibacterial activity of *H. rubra* haemolymph against *V. anguillarum* showed more variability between days than temperature treatments (estimates of variation for temperature and day were 4.43 and 113.53, respectively), and the interaction between temperature and day was significant (Table 1). Pair-wise analyses for temperature across days revealed significantly higher antibacterial activity in the 21 °C challenged group than in control group on day 3 ($t=2.17$, $p=0.048$) and significantly lower activity in the 24 °C challenged group than in control group on day 7 ($t=2.27$, $p=0.043$). According to pair-wise analyses for day within the control group at 18 °C, antibacterial activity was similar across all days ($p>0.05$). At 21 °C, antibacterial activity was significantly higher on day 3 than on day 7 ($t=3.63$, $p=0.01$), but was not significantly different between day 1 vs 7

(t=1.84, p=0.1) and day 1 vs 3 (t=2.35, p=0.079). At 24 °C, antibacterial activity was significantly higher on day 1 and 3 than on day 7 (t=3.7, p=0.02 and t=3.59, p=0.002, respectively).

Principal coordinates analysis revealed that when the four immune parameters are combined, data from the control abalone at 18 ^{o} C clusters much more tightly than 21 and 24 ^{o} C groups, with the greatest variability in the abalone treated at 24°C (Fig. 5). The first two eigenvectors explain 64.6 % of the variability in the immune data (Fig. 5). Vector overlay of immune parameters (r>0.2) reveals that the data separates along the first eigenvector (X axis) primarily due to differences in THC and SO, whereas antibacterial and antiviral activity drive the separation of data points along the second eigenvector (Y axis). Antibacterial and antiviral activity appears to be inversely related, and these humoral parameters show no correlation to the cell-mediated immune parameters of THC and SO. By using multivariate PERMANOVA, we found that the combined immune responses of abalone were significantly affected by both temperature and length of exposure and there was an interaction between these two factors (Table 1).

4. DISCUSSION

The innate immune system of abalone has evolved to cope with a wide range of microbial pathogens, including viruses and bacteria. Our field study indicates that antimicrobial activity in the hemolymph of abalone generally increases over the summer period as the water temperatures increase. However, as the temperatures peak in summer, antibacterial activity decreases and the immune response appears to favor antiviral activity. Viral abundance has been observed to

increase with water temperature in different oceanic regions, where increase of water temperature by only a few degrees was associated with a doubling of viral abundance [28]. Increase in temperature also leads to higher host metabolism, which has been linked to higher rates of virus production [28, 53, 54]. The elevated antiviral activity with increasing temperature, observed in the field study and further supported by our laboratory study, is possibly an adaptation to higher viral abundances in warmer conditions. However, this effect of temperature on antiviral activity cannot be generalized between molluscan species. For example, antiviral activity against HSV-1 in Pacific oysters, *C. gigas*, has been reported to show an inverse pattern, with low activity in summer (<40 %) compared to winter months (90-100 %) [48]. Indeed high temperature is likely to be an important factor contributing to the occurrence of herpesvirus outbreaks, which are typically reported in summer months in abalone [55] and other molluscs species such as oyster *C. gigas* [56-59], scallop *Pecten maximus* [60] and clam *Ruditapes philippinarum* [61].

In addition to a greater threat of viruses at higher temperature, there is also greater risk of bacterial infection [4, 24]. Although we observed some correlation between antibacterial activity and temperature in the field study, unlike antiviral activity, the antibacterial activity dropped off in January and February 2010 when the water temperature approached the critical thermal maximum of *H. rubra* (26.9 °C). Consistent with this, in the laboratory experiment, the antibacterial activity appears to decrease after prolonged (7 day) exposure to elevated temperatures. Such compromised antibacterial response under high temperature stress may explain the high “summer mortality” reported in abalone populations [19-23]. Summer mortality has a major economic impact on Australian and European abalone fisheries and has primarily

been linked to pathogenic *Vibrio* species [20-22, 62, 63]. In the European abalone *H. tuberculata*, it has been demonstrated that growth of *V. harveyi* was triggered by temperature, resulting in 90% mortality at 19 °C in comparison to no mortalities at 17 °C [22]. Travers *et al.* [21] further showed that a 1 °C difference of temperature leads to an increased mortality rate after exposing abalone to pathogenic bacteria. *H. diversicolor supertexta* are also more susceptible to *V. parahaemolyticus* at high temperature, 28-32 °C, than at 20-24°C [23]. Therefore, abalone in general appear to be more susceptible to bacterial infection at elevated temperatures, which could be due to exhaustion of humoral antibacterial factors with prolonged heat stress.

The water temperature at O'Sullivans Beach, SA, was measured above 17 °C, that is above the preferred temperature range of *H. rubra*, for three summer months, with the highest recorded temperature at 26.5 °C in February 2010, near the lethal thermal limit for this species. The waters around Australia have been predicted to warm 1-3 ºC by 2070, with greatest warming in southern and south-eastern Australia [64-66]. As a result, there will be an increase in the amount of time that abalone spend above their optimal temperature. With the longer term chronic temperature stress predicted to accompany ocean warming, antibacterial activity is likely to be compromised, leaving abalone more vulnerable to infections. Since the optimal temperature for *H. rubra* survival has been reported to range from 8 to 17 °C [40, 41], the low antibacterial activity in winter months with temperatures below 18 °C does not appear to contribute to mortality in this species. It is very likely that moderate expression of antibacterial factors in winter months is due to lower pathogen abundance in colder months [4, 24].

Immunity depends on a complex interaction between cell-mediated and humoral factors, all of which can be impacted by environmental stressors [26, 67-69]. Consequently in our manipulative laboratory study, THC and intracellular superoxide anion were selected as haemocyte responses and antibacterial activity against *V. anguillarum* and antiviral activity against HSV-1 were used as indicators of humoral immunity. All four immune parameters showed an initial elevation in response to elevated temperature, but the humoral antimicrobial activity varied according to length of exposure to high temperature. Consistent with the field study, antiviral activity increased over time at the higher temperatures, whereas antibacterial activity became depressed after seven days exposure to elevated heat stress. Using multivariate analysis, this study reveals significant differences in the combined immune parameters of abalone subjected to elevated temperature. Principle components analysis revealed a correlation between the two cellular parameters, whereas antibacterial and antiviral activities appear to be negatively correlated, indicating possible trade-offs in the humoral immune system of heat stressed abalone.

Despite the fact that humoral antimicrobial compounds are primarily synthesized and released by haemocytes [29, 30], there was no apparent correlation between total haemocyte count and antimicrobial activity. Furthermore, the lack of correlation between SO and antimicrobial activity in the PCO analysis confirms that humoral antimicrobial activity is not simply due to the presence of reactive oxygen species. In our study, there were no significant differences in SO anion levels, although similar to THC, SO peaked on day 1 in the 24 °C treatment. By comparison, in *H. diversicolor supertexta* SO was found to increase significantly after day 3 or 5 under elevated temperature stress [23], suggesting specific immune responses may vary according to species and the situation, despite a general elevation in haemocyte numbers. The

effect of temperature was immediately evident on THC, with significant increases within the first day, followed by an apparent recovery three days after temperature challenge. This may be due to an influx of circulating haemocytes from peripheral tissues, as has been previously suggested for stressed abalone [26]. Some of these haemocytes may then be involved in extracellular killing resulting in elevated humoral activity, with a coincident drop in THC by day 3. Humoral antibacterial activity against *V. anguillarum* preceded antiviral activity against HSV-1, peaking at day 3 and day 7, respectively. Antibacterial activity subsequently showed a significant drop on day 7, suggesting that the antibacterial factors are not being replenished within a few days. Rather the abalone appeared to switch to the release of more antiviral factors after prolonged heat stress. The lack of correlation between antiviral and antibacterial activity (against HSV-1 and *V. harveyi*, respectively) has been previously reported across a number of farmed and wild populations of *H. laevigata* in South Australia [31], suggesting different compounds are involved in defense against bacteria and viruses in abalone. Future studies aimed at investigating differential gene expression in haemocytes from abalone under elevated temperature conditions would provide further insight into the cell signaling and specific mechanisms responsible for these short term changes in humoral antimicrobial activity.

THC is highly variable across individuals, both within and between the temperature treatment groups. Within the first day of elevated heat exposure, the number of circulating haemocytes increased, but then returned to baseline levels by day 7. These THC results are consistent with a study by Cheng *et al* in *H. diversicolor supertexta*, where THC increased one day after being transferred from 28 to 32 °C, followed by a small decrease in THC on day 3 and 5 [23]. Increased THC as a result of increased water temperature has been observed in controlled

laboratory experiments for other molluscan species, for example the clams *Chamelea gallina*, *Mactra veneriformis* and *Ruditapes philippinarum* [70-73], as well as the oyster *C. virginica* [74]. Similar effect of temperature on THC has been also found in previous field studies on molluscan species, such as the clam *Venerupis philippinarum* [75], the mussel *Mytilus galloprovincialis [76]* and the oyster *C. virginica* [77]. Other invertebrate species, including the crustaceans *Panulirus interruptus* (lobster) and *Litopenaeus stylirostris* (blue shrimp), also experience an increase in THC in accordance with a rise of water temperature [78, 79], suggesting that this is a general invertebrate immune response to heat stress. Increased THC at higher temperatures could be, in part, attributed to metabolic activity, as higher respiration rates have been reported for a range of invertebrates at higher temperatures [42, 43]. Nevertheless, some exceptions have been reported for the effects of heat stress on THC in freshwater species (e.g. the prawn *Macrobrachium rosenbergii* [80] and the snail *Lymnaea stagnalis* [81]), suggesting there are complex species interactions between host immunity and water temperature in different environments. Since haemocytes are involved not only in immune responses, but also in non-immune functions such as tissue and shell repair, nutrition, transport and excretion [82-84], variation in the concentration of haemocytes could be influenced by the physiological condition and metabolic activity of specific individuals.

Under prolonged heat stress, available energy is redirected to support essential metabolic functions such as respiration [85], resulting in compromised abalone antibacterial activity. Reduced antibacterial activity under prolonged elevated temperature has also been reported from laboratory and field studies on the Pacific oyster *C. gigas* [13]. However unlike in these studies on oysters, our previous study reported no effect of reproduction on abalone humoral immunity,

evidenced by no significant difference in the antibacterial activity of pre and post spawning abalone [31]. Nevertheless, it is likely that other metabolic stressors, including spawning and poor nutrition would add to the impact of elevated temperature and further compromise the immune system of abalone. Previous studies on abalone and oyster immunity have reported synergistic effects of heat stress with reproduction [11, 13, 21], starvation [12] and simulated bacterial challenge [26]. Consequently, these studies highlight serious implications for the ability of molluscs to defend themselves against pathogenic bacteria under natural environmental conditions, where they are simultaneously encounter multiple stressors. The combination of elevated temperature and other stressors that cause immune-depression, along with changing oceanic conditions that favour pathogen growth [4, 24] is likely to explain the increasing frequency and intensity of disease in marine mollusc populations on a global scale [5, 86].

In conclusion, this study provides the first evidence for an effect of high temperature on abalone antiviral activity and confirms the effects on other cellular and humoral immune effectors, as well as highlighting a time-lag difference in the responses of these immune components. Consequently, predictions about disease resilience in light of ocean warming cannot be generalized across all types of pathogens. Comparison with previous studies implies that different immune responses to elevated temperature can occur in different species, further complicating the ability to predict patterns of disease susceptibility. Nevertheless, a consensus appears to be that molluscs typically suffer some immune depression with prolonged exposure to elevated water temperatures, increasing the likelihood that epidemic disease due to bacterial infection will occur with continued ocean warming. Further studies on antiviral activity and

susceptibility of molluscs to viral infection under temperature stress would complement the current studies on *Vibriosis* in molluscs.

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Table

Table 1. The effect of temperature and length of exposure (fixed factors) on individual immune parameter (univariate PERMAONVA) or combined four different immune parameters of *H. rubra*. The level of significant differences was set at p=0.05.

Parameter	Factor	PERMANOVA		
		df	Pseudo-F	p
Total haemocyte count	Temperature	2	7.04	0.002*
	Length of exposure (LE)	2	0.16	0.85
	Temperature x LE	4	1.6	0.21
Superoxide anion	Temperature	2	2.93	0.06
	Length of exposure (LE)	2	0.03	0.97
	Temperature x LE	4	1.76	0.13
Antiviral activity	Temperature	2	12.8	0.001*
	Length of exposure (LE)	2	2.84	0.07
	Temperature x LE	4	2.92	0.03*
Antibacterial activity	Temperature	2	1.3	0.28
	Length of exposure (LE)	2	8.63	0.002*
	Temperature x LE	4	3.38	0.02*
Combined four different immune parameters	Temperature	2	5.39	0.001*
	Length of exposure (LE)	2	3.12	0.007*
	Temperature x LE	4	1.92	0.022*

* Significant effect of temperature, length of exposure or their interaction on individual immune parameter or combined different immune parameters of abalone.

FIGURES

Fig.1

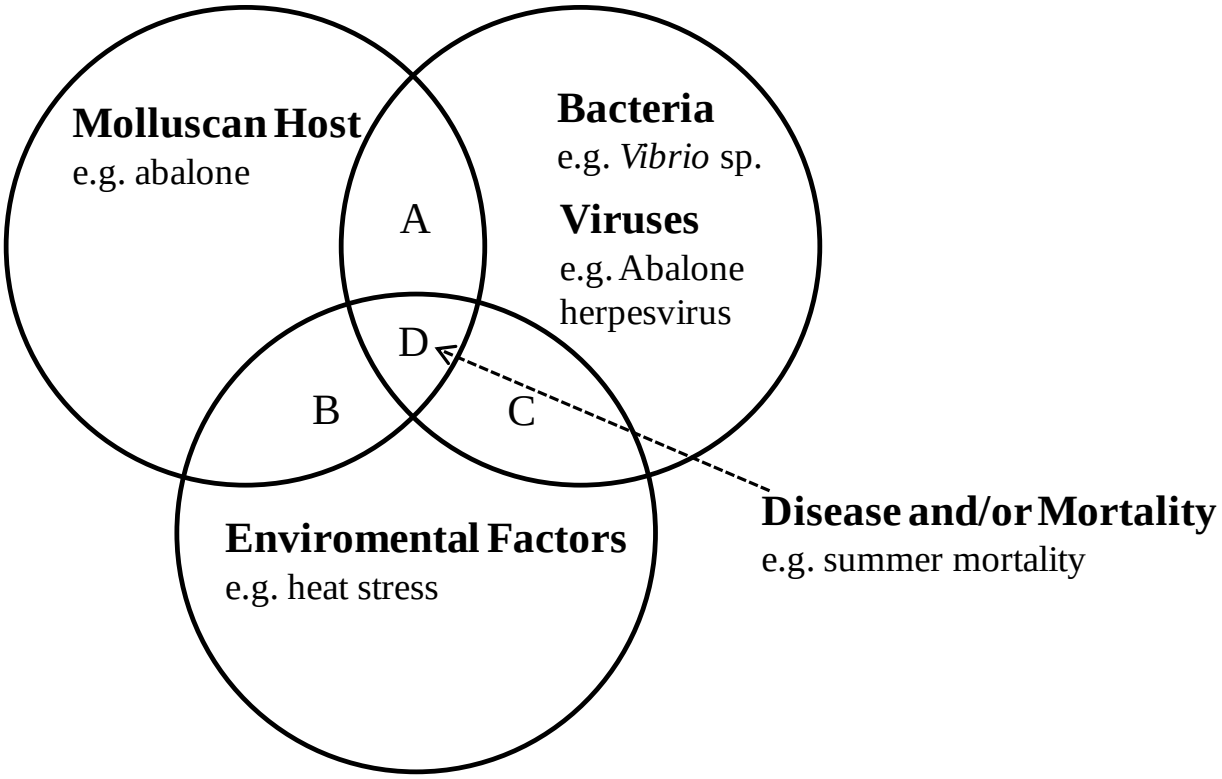


Fig. 1. The "holy triad" of disease causality: interactions between molluscan host, environmental factors and pathogens. A) Subclinical stage of infection where the molluscan host does not show disease signs. B) Immune-suppression by environmental stressors. C) Increased abundance and virulence of pathogens driven by environmental factors. D) Disease and/or mortality due to immune-suppression by environmental stressors in the presence of pathogen(s) [adapted from 87].

Fig.2

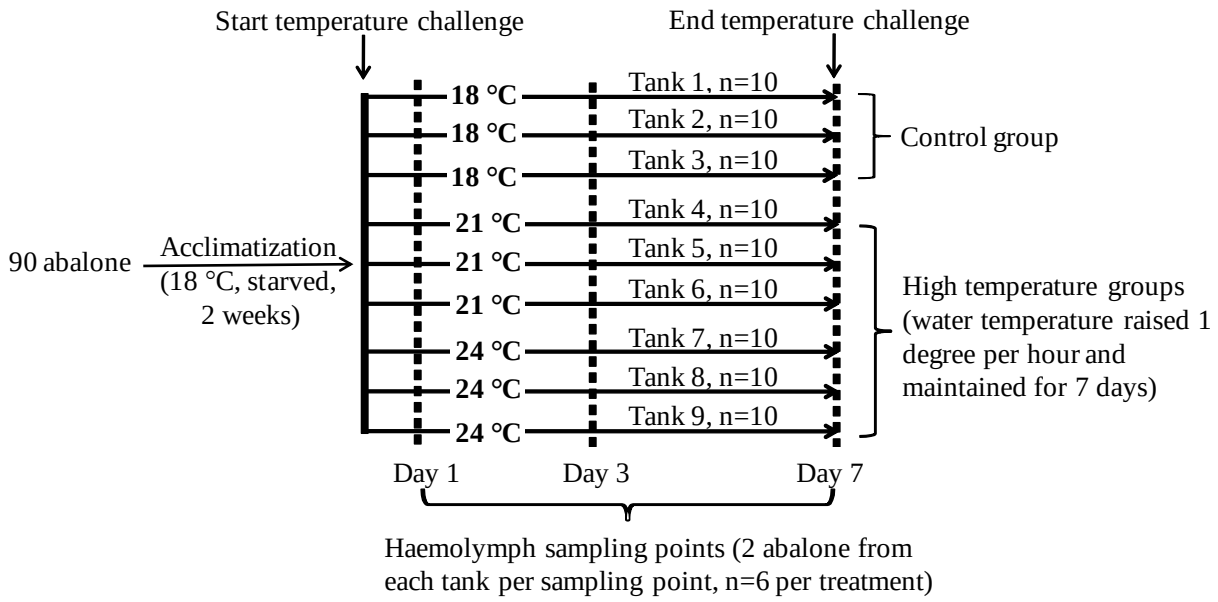


Fig. 2. Summary of laboratory temperature challenge experiment. 90 abalone were randomly divided into 9 tanks (n = 10 per tank) and three replicate tanks were allocated to each temperature treatment group (18, 21 and 24 °C). From each tank, 2 abalone were sampled for haemolymph at days 1, 3 and 7 (total of 6 individual samples per temperature treatment per day). Fresh haemolymph was measured for total haemocyte count and intracellular superoxide anion. The remaining haemolymph was centrifuged (3,000 rpm, 10 min, 4 °C) to obtain cell-free plasma, which were then stored at -80 °C for antibacterial and antiviral assay.

Fig.3

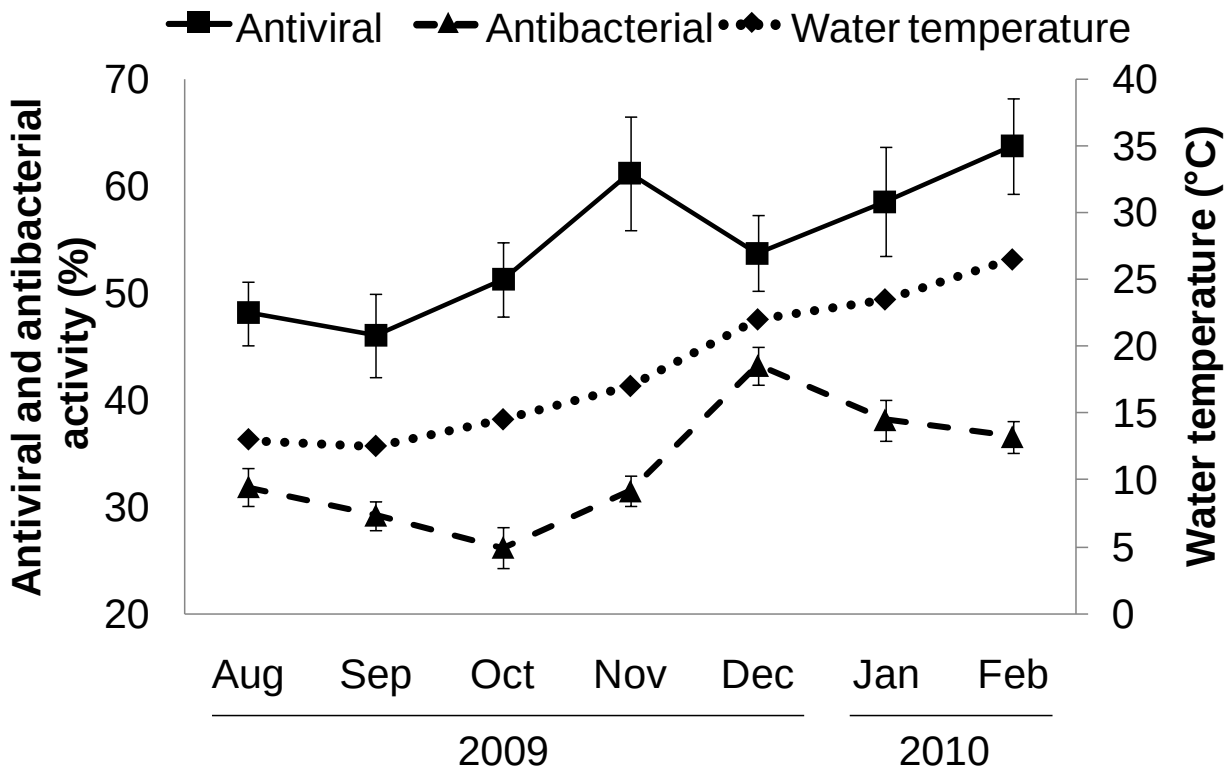


Fig. 3. The relationship between antiviral and antibacterial activity and water temperature. Abalone *H. rubra* were collected from the same site, O'Sullivan Beach, South Australia from August 2009 (winter) to February 2010 (summer) ($n \geq 20$ per month).

Fig.4

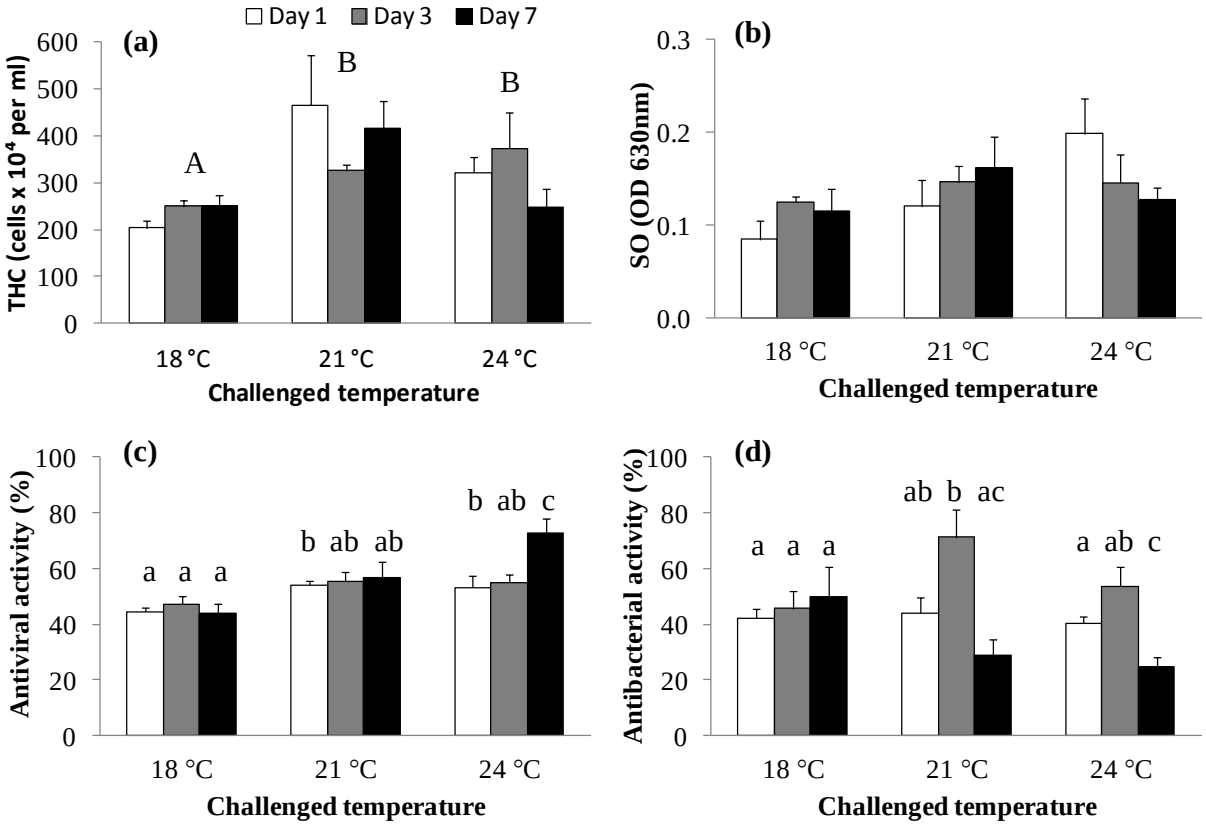


Fig. 4. Effect of challenge temperature and length of exposure on a) total haemocyte count (THC, cells x 10^4 per ml), b) superoxide anion (SO, OD 630nm), c) antiviral activity (%) against HSV-1 and d) antibacterial activity (%) against *Vibrio anguillarium*. Each immune parameter was measured from six replicate abalone. Different capital letters indicate significant differences ($p<0.05$) between temperature groups across all days for THC (a) where there was no significant interaction. However, for antiviral (c) and antibacterial activity (d), univariate PERMANOVA revealed a significant interaction between day and temperature. Therefore, different small letters indicate significant differences between temperatures within the relevant days and between days within temperature groups.

Fig. 5

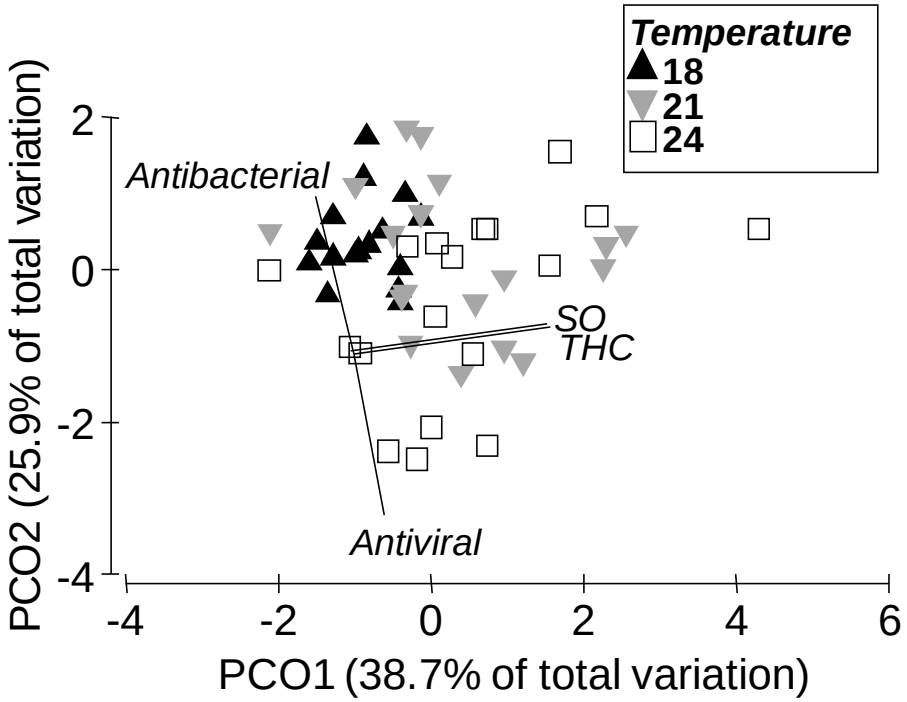
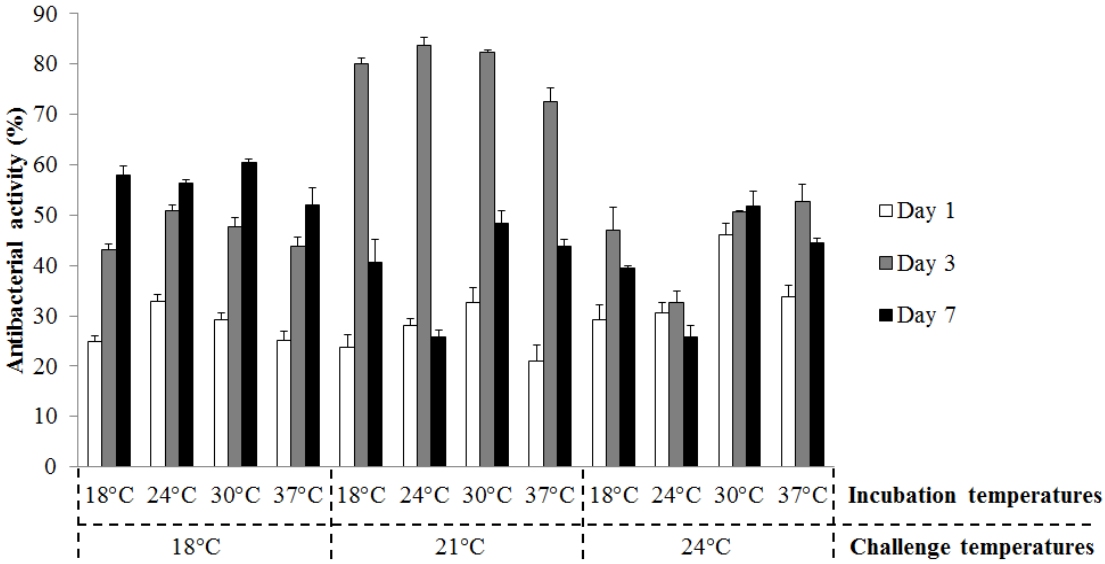


Fig. 5. Principal coordinates plot showing the grouping of abalone according to temperature based on the first two eigenvectors that contribute the most to the variability in abalone immune parameters. Vectors overlayed for the four immune parameters show that the data points separate according to THC and SO along the first eigenvector (X axis), whereas antibacterial and antiviral activity drive the separation of data points along the second eigenvector (Y axis). THC and SO vary in the same way, whereas antibacterial and antiviral activity are inversely related.



Supplementary Figure 1: Effect of incubation temperature on antibacterial activity in antibacterial assay of abalone haemolymph against *Vibrio anguillarium*. Haemolymph pooled from 6 individuals of the same temperature treatment group and the same day were tested for antibacterial activity using 4 different incubations temperatures 18, 24, 30 and 37 °C. The overall trends in the results were the same irrespective of incubation temperature.