Change in water temperature on the immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*

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Abstract

Taiwan abalones, *Haliotis diversicolor supertexta*, held in 30% seawater at 28 °C, were injected with TSB-grown *Vibrio parahaemolyticus* (1.6 × 10⁵ cfu abalone⁻¹) and then transferred to 20, 24, 28 and 32 °C. All abalones transferred to 32 °C died by 72 h. The mortality of *V. parahaemolyticus*-injected abalone held at 20 and 24 °C was significantly lower over 24–96 h, compared to animals held at 28 and 32 °C. In a separate experiment designed to measure immune function, abalones held in 30% seawater at 28 °C and then transferred to 20, 24, 28 and 32 °C were examined for total haemocyte count, phenoloxidase activity, respiratory burst, and phagocytic activity to *V. parahaemolyticus* after 24, 72 and 120 h. The phenoloxidase activity and phagocytic activity decreased significantly, whereas respiratory burst increased significantly in abalone transferred to 32 °C. It is concluded that transfer of abalone from 28 °C to 32 °C reduced their innate immunity and resistance against *V. parahaemolyticus* infection.

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Keywords: *Haliotis diversicolor supertexta; Vibrio parahaemolyticus; Temperature; Challenge; Phenoloxidase activity; Respiratory burst; Phagocytic activity*

1. Introduction

Temperature and salinity are the primary physical factors affecting the life of molluscs. It is reported that salinity in the range of 30–35%, and temperature in the range of 24–30 °C are optimal for the growth of the Taiwan abalone *Haliotis diversicolor supertexta* [1]. *H. diversicolor supertexta* reared at 35%, and at 20, 25 and 30 °C survive temperatures in the range of 3.5–32.7, 5.3–33.3 and 10.6–35.2 °C, respectively, when water temperature is decreased or increased gradually [2].

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**Haliotis diversicolor supertexta** have been mostly cultured in land-based concrete farms adjacent to the sea coast. Seasonal variation of water temperature in fish farms is from 15 to 32 °C. Cultured *H. diversicolor supertexta* have suffered mass mortality due to vibriosis since 1998 in Taiwan. The bacteria, *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, isolated from the haemolymph of moribund abalone, have been demonstrated to cause outbreaks of vibriosis associated with warm water [3,4].

In molluscs, agranular haemocytes (hyalinocytes) and granular haemocytes (granulocytes) are considered as two distinct cell types [5]. They are involved in phagocytosis, an important process of eliminating microorganisms or foreign particles [6]. Haemocytes are involved not only in coagulation but also in the production of melanin via the prophenoloxidase system [7]. Several kinds of reactive oxygen intermediates (ROIs) are produced during phagocytosis. These include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), singlet oxygen (1O$_2$) and hydroxyl radical (OH') [8]. The release of superoxide anion is known as the respiratory burst, and it plays an important role in antibacterial activity.

Physico-chemical changes like temperature and salinity, and environmental pollutants have been reported to affect the circulating haemocytes and phagocytosis in several species of molluscs including the red abalone *Haliotis rufescens*, the black abalone *Haliotis cracherodii* and *H. diversicolor supertexta* [9–13]. Change in salinity has been reported to affect the immune parameters of *H. diversicolor supertexta*, and lead to its susceptibility to *Vibrio* infection [13]. It is assumed that changes in temperature may also affect immune parameters of *H. diversicolor supertexta*, leading to increased susceptibility to *Vibrio* infection. This study was aimed at determining (1) the effect of temperature on the resistance of *H. diversicolor supertexta* to *V. parahaemolyticus*, and (2) the effect of temperature on the immune parameters of *H. diversicolor supertexta*. For the latter purpose, total haemocyte count (THC), phenoloxidase activity, respiratory burst activity (release of superoxide anion), and phagocytic activity of *H. diversicolor supertexta* to *V. parahaemolyticus* were examined.

### 2. Materials and methods

#### 2.1. Haliotis diversicolor supertexta

*Haliotis diversicolor supertexta* obtained from a commercial farm in Kaohsiung were shipped to the laboratory in Pingtung, and acclimated at 30 °C for 2 weeks at room temperature. The shell length of abalone ranged from 3.7 to 4.2 cm with an average of 3.91 ± 0.35 cm. The body weight of the abalones ranged from 5.1 to 7.7 g with an average of 6.35 ± 0.38 g. During the acclimation period, animals were fed marine alga *Gracilaria tenuistipitata* daily.

#### 2.2. Vibrio parahaemolyticus

*Vibrio parahaemolyticus* was isolated from diseased abalones with withering syndrome [3]. It was cultured on 50 ml tryptic soy broth supplemented with 3% NaCl (TSB, Difco) for 24 h at 30 °C as a stock culture for tests. The stock cultures were centrifuged at 7155 × g for 15 min at 4 °C. The supernatant fluid was removed and the bacterial pellet was re-suspended in saline solution (0.85% NaCl) at 8 × 10$^6$ and 5 × 10$^8$ cfu ml$^{-1}$ as stock bacterial suspension for injection challenge and phagocytic activity test.

#### 2.3. Effect of temperature on the susceptibility of *H. diversicolor supertexta* to *V. parahaemolyticus*

Each abalone was injected into the pallial sinus with 20 μl of bacterial suspension (8 × 10$^6$ cfu ml$^{-1}$) following the method described previously [12,14]. Challenge tests at a dose of 1.6 × 10$^5$ cfu abalone$^{-1}$ were conducted in triplicates. The test and control groups comprised ten abalones each. After injection, abalones
were kept in 15 l PVC tanks (ten abalones in each) containing 10 l of aerated seawater at 20, 24, 28 and 32 °C. They were fed G. tenuistipitata, and observed for 120 h. Abalones injected with an equal volume of sterile saline solution and kept at 28 °C served as the unchallenged controls (Table 1). The LT_{50} (median lethal temperature) for H. diversicolor supertexta was determined as described by Trevors and Lusty [15].

2.4. Effect of temperature on the immune parameters of H. diversicolor supertexta

For haemocyte counts and enzyme activity assays, H. diversicolor supertexta acclimated to 30°C seawater at 28 °C were transferred to 20, 24, 28 and 32 °C in eight replicates of 20 l PVC tanks (one abalone each). There were 16 treatments (four temperatures plus four exposure times of 0, 24, 72 and 120 h). Abalones were sampled individually at the beginning of the test and at 24, 72 and 120 h. Haemolymph (100 μl) was withdrawn from the pallial sinus of each abalone with a 1 ml sterile syringe (25 gauge) containing 100 μl PBS (phosphate-buffered saline) (0.01 M, osmolality 980 mOsm kg⁻¹, pH 7.4). A drop of diluted haemolymph was placed on a haemocytometer, and THC (total haemocyte count) was measured using an inverted phase contrast microscope (Leica DMIL, Leica Microsystems Wetzlar, Germany).

Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) [14]. Briefly, 100 μl of diluted haemolymph in PBS was deposited in triplicate in 96-well microplates, and then centrifuged at 300×g for 15 min. The supernatant fluid was discarded, and 100 μl of sodium alginate (0.5 mg ml⁻¹ in PBS) (used as an activator) were added for 30 min at 26–27 °C [16]. Fifty microlitres of L-DOPA (3 mg ml⁻¹ in PBS) were then added, and the optical density at 490 nm was measured 10 min later using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA). The control solution, which consisted of 100 μl of haemolymph, 100 μl PBS (to replace the sodium alginate) and 50 μl of L-DOPA, was used to measure the background phenoloxidase activity in all test conditions. The optical density values corresponding to the background phenoloxidase activity were in the range of 0.04 to 0.05. The phenoloxidase activity of abalones for all test conditions was expressed as dopachrome formation in 50 μl of haemolymph.

The respiratory burst activity of haemocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion, as described previously [17]. Briefly, 100 μl of diluted haemolymph in PBS solution was deposited in triplicate in microplates previously coated with 100 μl poly-L-lysine solution (0.2%). Microplates were centrifuged at 300×g for 15 min. Plasma was removed and 100 μl sodium alginate (0.2 mg ml⁻¹ in PBS) was added and allowed to react for 30 min at 26–27 °C. Sodium alginate was discarded and the haemocytes were stained with 100 μl NBT solution (0.3%) for 30 min at room temperature. The NBT solution was removed and the haemocytes were fixed with 100% methanol, and washed three times with 100 μl 70% methanol and air-dried. The formazan was dissolved by the addition of 120 μl 2 M KOH and 140 μl dimethyl sulphoxide (DMSO). The optical density at 630 nm was

### Table 1

Susceptibility of Haliotis diversicolor supertexta to Vibrio parahaemolyticus at different temperatures at 30°C

<table>
<thead>
<tr>
<th>Bacterial dose (cfu abalone⁻¹)</th>
<th>Temperature (°C)</th>
<th>Cumulative mortality (%)</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.6×10⁵</td>
<td>20</td>
<td>0.00±0.00ᵇ</td>
<td>10.00±5.77ᶜ</td>
<td>23.33±3.33ᵇ</td>
<td>33.33±3.33ᶜ</td>
<td>36.67±6.67ᶜ</td>
<td>36.67±6.67ᶜ</td>
<td></td>
</tr>
<tr>
<td>1.6×10³</td>
<td>24</td>
<td>0.00±0.00ᵇ</td>
<td>16.67±6.67ᵇ</td>
<td>23.33±3.33ᵇ</td>
<td>30.00±5.77ᵇ</td>
<td>33.33±6.67ᶜ</td>
<td>33.33±6.67ᶜ</td>
<td></td>
</tr>
<tr>
<td>1.6×10⁵</td>
<td>28</td>
<td>3.33±3.33ᵇ</td>
<td>26.67±6.67ᵇ</td>
<td>33.33±6.67ᵇ</td>
<td>53.33±3.33ᵇ</td>
<td>60.00±5.77ᵇ</td>
<td>60.00±5.77ᵇ</td>
<td></td>
</tr>
<tr>
<td>1.6×10³</td>
<td>32</td>
<td>10.00±5.77ᵃ‡</td>
<td>33.33±3.33ᵃ‡</td>
<td>56.67±3.33ᵃ‡</td>
<td>76.67±3.33ᵃ‡</td>
<td>100.00±0.00ᵃ‡</td>
<td>100.00±0.00ᵃ‡</td>
<td></td>
</tr>
</tbody>
</table>

Data in the same column with different superscript letters are significantly different (P < 0.05) among treatments. Values are mean±SE (n = 30 abalones in each case).
measured in triplicate using a microplate reader (Model VERSAmax, Molecular Devices). Respiratory burst was expressed as NBT reduction in 50 μl of haemolymph.

2.5. Effect of temperature on the phagocytic activity

For the phagocytic activity test, the experimental parameters were the same as those described above. Abalones after 0, 24, 72 and 120 h transfer to 20, 24, 28 and 32 °C were injected in the pallial sinus with 20 μl bacterial suspension (5×10⁸ cfu ml⁻¹ in 0.85% NaCl), resulting in 1×10⁷ cfu abalone⁻¹. After injection, the abalones were held in their respective test solutions for 3 h at 30°C. Two hundred microlitres of haemolymph were collected from the pallial sinus, and mixed with 200 μl of PBS (pH 7.4, 980 mOsm kg⁻¹). Phagocytic activity was measured. With two abalones in each of three replicates, this gave six measurements per parameter for each treatment.

Phagocytic activity was measured following the method described by Weeks-Perkins et al. [18]. Briefly, haemocytes contained in 200 μl of the diluted haemolymph sample were fixed with 200 μl of 0.1% paraformaldehyde for 30 min at 4 °C, and then centrifuged at 800×g at 4 °C. The precipitated haemocytes were washed with 0.4 ml of sterile PBS. Fifty microlitres of the suspension was sampled and spread on a glass slide, and then centrifuged at 113×g for 3 min using a cytospin centrifuge (Model Cytospin 3, Shandon, UK). The slide was air-dried, stained with Diff-Quick stain, and then observed under a light microscope. Two hundred haemocytes were counted. Phagocytic activity was expressed as percentage phagocytosis as follows: Percentage phagocytosis = ((phagocytic haemocytes)/(total haemocytes))×100.

2.6. Statistical analysis

A multiple comparison (Tukey) test was conducted to compare the significance of differences among treatments using the SAS computer software (SAS Institute Inc., Cary, NC, USA). Percent data (susceptibility study) were normalised using arcsine transformation before analysis. Differences were considered significant when P < 0.05.

3. Results

3.1. Effect of temperature on the susceptibility of H. diversicolor supertexta to V. parahaemolyticus

All the unchallenged control abalones survived. In contrast, death began to occur at 6 h in the challenged abalone when held at temperatures of 28 and 32 °C. All abalones transferred to 32 °C died within 72 h. Over 24–120 h, the cumulative mortality for the abalones held at 28 °C was significantly higher than for the abalones held at 20 and 24 °C (Table 1). The 24 and 48 h LT₅₀ water temperature was calculated to be 31.1 and 27.3 °C, respectively.

3.2. Effect of temperature on the immune parameters of H. diversicolor supertexta

There were no significant differences in THC for the abalones kept at 28 °C at the different sampling times. The mean (±S.E.) THC varied from 253.4±33.9×10⁴ to 274.4±23.7×10⁴ cells ml⁻¹. After 72 and 120 h, the THC increased significantly by 33% and 34% for the abalones transferred to 20 and 32 °C, respectively (Table 2).

Phenoloxidase activity decreased significantly for the abalones transferred to 24 and 32 °C, whereas phenoloxidase activity increased significantly for those transferred to 20 °C after 24, 72 and 120 h (Fig. 1A). The respiratory burst decreased significantly for the abalones transferred to 20 °C after 24, 72
and 120 h, whereas the respiratory burst increased significantly for those transferred to 32 °C after 72 and 120 h (Fig. 1B).

At time 0 h, the phagocytic activity was 17.7%. After 24 h, phagocytic activity decreased significantly to 9.7%, 12.5% and 8.5% for the abalones transferred to 20, 24 and 32 °C, respectively, as compared to the activity of those at 28 °C (19.3%). After 72 h, phagocytic activity decreased significantly to 9.5% and 5.3% for the abalones transferred to 20 and 32 °C (Fig. 2).

4. Discussion

Changes in physico-chemical parameters like temperature, salinity, and pH have been reported to increase the susceptibility of the giant freshwater prawn *Macrobrachium rosenbergii* against *Lactococcus garvieae* [19]. *M. rosenbergii* was more susceptible to *L. garvieae* when reared in 33 °C than when reared in 27 and 30 °C water [19]. *H. diversicolor supertexta* was more susceptible to *V. parahaemolyticus* when the animals were transferred to 20 and 25 °C from 30 °C in 48 h [13]. In the present study, it was found that *H. diversicolor supertexta* was more susceptible to *V. parahaemolyticus* when the animals were transferred to 32 °C from 28 °C over 12 h. It may be concluded that change in temperature and salinity can trigger a disease outbreak by affecting the defence mechanism(s) of the host: the susceptibility of *H. diversicolor supertexta* to *V. parahaemolyticus* is enhanced by high temperature (32 °C) and low salinity (20 and 25 °C) [2]. Chen and Chen [2] reported that *H. diversicolor supertexta* which had been maintained in 30 °C and at 30 °C, and transferred to a series of higher temperatures, exhibited a 24 h LT<sub>50</sub> of 32.2 °C, which was higher than the 24 h LT<sub>50</sub> of the present study (31.1 °C).

In the present study, increases in mortality in the high temperature groups (32 °C) after bacterial challenge may be a function of bacterial physiology as much as immunomodulation. The pathogen was cultured at its optimal temperature, and its multiplication and toxin production might be reduced at lower temperatures (20 and 24 °C). This could be important in the disease response of molluscan hosts that are incapable of regulating their body temperatures.

The total number of circulating haemocytes and phenoloxidase activity could be affected by physico-chemical changes and environmental toxicants including cadmium, copper, and phenol in several species of molluscs [10,20,21]. The lobster *Panulirus interruptus* reared at 4 °C had a significantly lower THC and phenoloxidase activity as compared to the lobster reared at 14 and 19 °C [22]. *Macrobrachium rosenbergii* reared at 20 °C had a significantly lower THC and phenoloxidase activity as compared to prawns reared at 27 and 30 °C [23].

A decrease in water temperature also caused a decrease in THC of the blue shrimp *Litopenaeus stylirostris* reared at 18 °C, as compared to the shrimp reared at 27 °C [24], in the THC of the shore crab *Carcinus maenas* reared at 10 °C, as compared to the crab reared at 20 °C [25], and in the phenoloxidase activity of *C. maenas* reared at 10 °C, as compared to the crab reared at 20 °C [26].

<table>
<thead>
<tr>
<th>Sampling time (h)</th>
<th>Temperature (°C)</th>
<th>THC (×10&lt;sup&gt;4&lt;/sup&gt; ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Phenoloxidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>253.4 ± 33.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.0 ± 6.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>253.4 ± 33.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>279.4 ± 11.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>336.3 ± 9.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>286.2 ± 34.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>339.5 ± 31.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>316.5 ± 28.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data in the same column with different superscript letters (a, b) are significantly different (P < 0.05) among time periods (h), and data in the same row with different letters (x, y) are significantly different (P < 0.05) among different temperature levels. Values are mean ± SE (n = 8).
activity of *H. diversicolor supertexta* when transferred to 24 °C from 28 °C in the present study. In contrast, a decrease in water temperature caused an increase in phenoloxidase activity of the blue shrimp *L. stylirostris*, reared at 18 °C, as compared to the shrimp reared at 27 °C [24], and caused increases in both THC and phenoloxidase activity of *H. diversicolor supertexta* when transferred to 20 °C from 28 °C in the present study.

Several scientists reported decreases in both THC and phenoloxidase activity when animals reared in an optimal temperature condition were transferred to high temperature in a short time period. For example, Vargas-Albores et al. [26] reported that the yellowleg shrimp *Farfantepenaeus californiensis* reared at 32 °C had a significantly lower phenoloxidase activity, as compared to the shrimp reared at 18 °C. Smith and
Chisholm [27] reported that the shore crab *C. maenas* reared at 13 and 19 °C had significantly lower THC and phenoloxidase activity as compared to the crab reared at 6 °C. Cheng and Chen [23] reported that the freshwater prawn *M. rosenbergii* reared at 33 °C had significantly lower THC and phenoloxidase activity as compared to the prawn reared at 27 and 30 °C. In the present study, a decrease in phenoloxidase activity was also observed in *H. diversicolor supertexta* when transferred to 32 °C from 28 °C over 24 h.

Lacoste et al. [28] observed a stress-induced increase of circulating haemocyte count in the Pacific oyster *Crassostrea gigas* following 15 min of mechanical disturbance. The fact that the THC increased for the *H. diversicolor supertexta* transferred to 20 °C is considered a consequence of a stress response. Further research is needed to clarify whether the increase in THC results from proliferation of the cells, or movement of cells from tissues into the circulation [11].

Previous studies on *M. rosenbergii* indicated that phenoloxidase activity, phagocytic activity and clearance efficiency to *L. garvieae* were significantly higher for the animals reared at 27 and 30 °C than those reared at 20 and 33 °C [23,29]. In the present study, phenoloxidase activity increased together with an increase in THC, whereas respiratory burst decreased together with a decrease in phagocytic activity for *H. diversicolor supertexta* transferred to 20 °C. Phenoloxidase is stored in the secretory granules of the semi-granular and granular haemocytes, whereas agranular haemocytes are involved in phagocytosis and the release of superoxide anion and other ROIs [5]. It is expected that differential haemocyte count may differ for the abalone under different temperature stresses.

From the present experiments, it is not possible to distinguish whether the increase in superoxide anion resulted from increased activity of NADPH oxidase, responsible for the production of superoxide anion, or from decreased activity of superoxide dismutase (SOD) responsible for scavenging the superoxide anion. Further research is needed to examine the activities of enzymes like SOD, catalase and peroxidase [30] for the abalone at different temperatures.

Phagocytosis can be affected by environmental parameters in invertebrates [6]. For example, an elevated temperature has been reported to increase phagocytosis [31] and haemocyte activity (the ability of haemocyte to adhere the fluorescent beads) in the American oyster *Crassostrea virginica* [32].
et al. [33] reported that phagocytosis of the American lobster *Homarus americanus* was higher at 20 °C than that at 22 °C. Smith and Chisholm [27] reported that antibacterial activity of the shore crab *C. maenas* against *Planococcus citreus* was significantly higher at 6 °C as compared to the crab at 13 and 19 °C, which correlated well with higher THC and phenoloxidase activity. In the present study, phagocytic activity of *V. parahaemolyticus* decreased for *H. diversicolor supertexta* when transferred to 32 °C in 24 h, which correlated well with the decrease in phenoloxidase activity and increase in susceptibility to *V. parahaemolyticus*.

In conclusion, *H. diversicolor supertexta* transferred from 28 °C to 32 °C showed a higher susceptibility to *V. parahaemolyticus*, which together with lower phenoloxidase activity and phagocytic activity, indicates a reduction in immune ability.

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