Fish and Shellfish Immunology 84 (2019) 485–490

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Sub-optimal or reduction in temperature and salinity decrease antioxidant activity and cellularity in the hemolymph of the Pacific abalone (*Haliotis discus hannai*)

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ARTICLE INFO

Keywords:
Pacific abalone
Haliotis discus hannai
Temperature
Salinity
Oxidative stress
Hemocyte response

ABSTRACT

This study investigated the oxidative stress and hemocyte responses of Pacific abalone exposed to various water temperatures (4, 6, 8, and 10 °C) and salinities (26, 30, and 34 psu) for 7 days, to identify their tolerance ranges of temperature and salinity. The survival rate of Pacific abalone ranged from 98.7 to 100% at 8 °C and 10 °C, but dropped to 25–55% at 4 °C at all levels of salinity. The levels of superoxide dismutase and glutathione in the hemolymph were significantly higher at 4 °C and 6 °C than in the controls in all salinity groups, indicating that these temperatures induced greater stress in the Pacific abalone. Total hemocyte count was lowest at 6 °C in the 26 psu group. The percentages of apoptotic and necrotic cells were higher in the 26 psu group than in the other salinity groups, and higher in the 4 °C and 6 °C groups than in the other temperature groups. These results indicate that the lowest tolerance to water temperature and salinity in the Pacific abalone was 8 °C and 30 psu, respectively.

1. Introduction

The Pacific abalone (*Haliotis discus hannai*) is considered a high value species in Korea with an annual production value of 347.5 billion Won (about 347.5 million USD), accounting for 56.5% of Korea's total farmed shellfish value [1]. Pacific abalone farms have recently replaced the traditional methods of inland aquaculture with sea cage faming to reduce production costs and increase production. It is known that water temperature in the range of 18–22 °C, salinity in the range of 32–33 psu, and dissolved oxygen in the range of 6–8 mg/L are optimal for the growth and the survival of the Pacific abalone [2]. However, sea cages have some disadvantages as they are directly exposed to the ocean environment; therefore, the aquatic organisms reared in the cages are likely to be directly affected by sudden changes in the ocean environment.

Changes in the ocean environment strongly affect physiological responses in aquatic organisms, which affects their survival, growth, and metabolism. In particular, water temperature and salinity are critical factors that affect the physiology and ecology of aquatic organisms; inappropriate water temperature or salinity outside the tolerance ranges cause stress in aquatic animal and this can lead to physiological dysfunction and finally to mortalities [3,4]. In fact, abalone farms in Korea experience mass mortalities every year caused by extreme cold water in winter and low salinity due to freshwater influx during the rainy season [5]. To mitigate these stresses, it is necessary to identify the tolerance ranges of Pacific abalone for water temperature and salinity that can be used to relocate abalone farms to sites with more favorable farming conditions or to sites that protect abalones from cold water during winter. Moving abalone farms is seen as a cost effective and efficient method of combating mortalities.

Changes in water temperature and salinity significantly affect the physiology of marine invertebrates [6] by changing the functioning of blood corpuscles or activating NADPH-oxidase. This generates ROS (reactive oxygen species), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) [7], and weakens the immune system [8]. The resulting loss of defense against diseases or environmental changes leads to mass mortalities. Common indicators of oxidative stress and hemocyte response in invertebrates include hemolymph antioxidant enzyme levels and hemocyte mortality [9–11]. Studies on these physiological indicators have been conducted for invertebrate species such as scallop *Chlamys farreri* [10], oyster *Crassostrea virginica* [12], Taiwan abalone *Haliotis diversicolor supertexta* [13],

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; THC, total hemocyte count

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https://doi.org/10.1016/j.fsi.2018.10.041
Received 15 December 2017; Received in revised form 11 October 2018; Accepted 15 October 2018
Available online 16 October 2018
1050-4648/

manila clam *Ruditapes philippinarum* [14], and sea cucumber *Apostichopus japonicus* [8]. However, little or no data are available for Pacific abalone. Water temperature and salinity can have both individual and combined effects on invertebrates [15,16]. Therefore, it is necessary to identify the tolerance range for the combination of these factors.

This study investigated the survival and stress responses (superoxide dismutase, SOD; glutathione; total hemocyte count, THC; hemocyte mortality) of Pacific abalone during exposure to various water temperatures and salinities to identify their tolerance ranges to these stresses.

2. Materials and methods

2.1. Experimental organisms and conditions

Pacific abalones (mean ± SD shell length 7.5 ± 0.4 cm; mean ± SD total weight 49.0 ± 11.3 g) were purchased from an aquaculture farm located in Tongyeong, Gyeongsangnam-do (May 2014, 14.5 ± 1.1 °C, 34.3 ± 0.6 psu) and transported to the rearing facility at National Institute of Fisheries Science (NIFS), where they were placed in a flow-through circular FRP tank (2 tons) for 14 days to acclimate. During acclimation, the animals were maintained at a water temperature of approximately 15 ± 1 °C and salinity of 34.0 ± 0.5 psu and fed dried laminaria once a day to full satiation.

In total, 12 combinations of treatments (four water temperatures of 4, 6, 8, and 10 °C and three salinities of 26, 30, and 34 psu) were used to investigate stress responses of Pacific abalone to water temperature and salinity. To conduct the experiment, 36 recirculating acrylic tanks (200 L) adjusted to the different salinity treatment conditions were each stocked with 30 abalones (i.e. abalones were directly exposed to each experimental salinity). Then, the water temperature was lowered at a rate of 2 °C/hr using a cooler. In each treatment combination, the water temperature and salinity were consistently maintained throughout the experimental period within an error range of ± 0.4 °C and ± 0.2 psu, respectively. A similar-sized tank maintained at natural sea conditions of 15 °C and 34 psu was also stocked with 30 abalones as the control. Three replicates of both the treatment and control groups were established and the experiment lasted for 7 days. During the experiment, the light:dark cycle was maintained at 12 L:12 D and the dissolved oxygen was maintained at over 90% of the saturated concentration using full aeration. Dead animals were counted daily during the experiment to calculate the survival rate.

2.2. Collecting hemolymph

At the end of the experiment, we collected hemolymph from 15 live abalones randomly selected from each tank. The hemolymph was collected from the cephalic arterial sinus at 1 cm below the mouth using a 3-mL (26 G) syringe. Part of the collected hemolymph was immediately used to analyze THC and hemocyte mortality and the remainder was centrifuged (14,000 × *g*, 4 °C, 10 min) and then stored at −80 °C for later analysis of antioxidant enzymes.

2.3. Analyzing antioxidant enzyme

SOD activity was measured by SOD Assay Kit (Cayman Chemical Company, USA) following the manufacturer's instruction. The glutathione content in the hemolymph was measured by Glutathione Assay Kit (Cayman Chemical Company, USA) following the manufacturer's instruction.

2.4. Flow cytometry

The THC and hemocyte mortality in the hemolymph was analyzed using flow cytometry (Gallios flow cytometry, Beckman Coulter, USA). THC was measured according to the method of Hong et al. [17]. Two-hundred microliters of hemolymph was fixed using 200 μL of 3% formalin and then SYBR green l (Sigma, USA) was added. The mixture was then left at room temperature for a 90-min reaction. THC was measured from hemocytes stained by SYBR green l using the FL-1 detector of the flow cytometer.

Hemocyte mortality was measured using the PE Annexin V Apoptosis Detection Kit (BD Pharmingen™, USA), which double-stains hemocytes using PE Annexin V and 7-Amino-Actinomycin (7-AAD). Cell staining was performed according to manufacturer protocol. The stained cells were analyzed using flow cytometry within 1 h to distinguish live cells (not stained), early apoptotic cells (stained by Annexin V), late apoptotic cells (stained by Annexin V and 7-AAD) and necrotic cells (stained by 7-AAD).

2.5. Statistical analysis

Statistical analyses were performed using the software program SPSS (version 17.0). The results are presented as the mean ± standard deviation and the significance value ($p < 0.05$) was tested using one-way ANOVA (significant difference between water temperature groups at the same salinity), two-way ANOVA (significant differences between water temperature and salinity groups, and interaction between salinity and temperature) or Duncan's multiple range test. Significant differences between treatment and controls groups were tested using Student's *t*-tests ($p < 0.05$).

3. Results

3.1. Survival rate

At 34 psu salinity, the survival rate at the end of the experiment was 100% in the 10 °C and 8 °C groups, 91.5 ± 2.1% in the 6 °C group, and 55.0 ± 7.1% in the 4 °C group. At 30 psu, the final survival rate was over 95% in the 10 °C and 8 °C groups, 83.5 ± 9.2% in the 6 °C group, and 30.0 ± 4.2% in the 4 °C group. At 26 psu, 100% of the experimental abalones survived in the 10 °C and 8 °C groups, but the survival rate decreased to 80.0 ± 0.0% in the 6 °C group, and to 25.0 ± 2.8% in the 4 °C group, which was the lowest among all the salinity groups (Fig. 1).

3.2. Antioxidant enzyme

The SOD level was significantly higher in the 26 psu groups than in the 30 and 34 psu groups. At 30 and 34 psu, the SOD level was higher in the 4 °C and 6 °C groups than in the 8 °C and 10 °C groups. At 26 psu, SOD was not detected in the 4 °C group. The SOD level was significantly higher than in the control in the 4 °C and 6 °C groups at 30 and 34 psu, and at all temperatures at 26 psu (Fig. 2).

The glutathione level was the highest in the 26 psu groups, and was significantly higher in the 4 °C and 6 °C groups than in the 8 °C or 10 °C groups, or the control group (Fig. 2). SOD and glutathione levels were affected by both salinity and water temperature, and by the interaction between the two factors (Table 1).

3.3. THC

At the end of the experiment, THC was lowest in the 26 psu groups, and significantly lower in the 4 and 6 °C groups than in the 8 °C or 10 °C groups, or the control group. THC was also affected by both salinity and water temperature (Fig. 3).

3.4. Hemocyte mortality

The hemocyte mortalities for different combinations of salinity and water temperature are shown in Fig. 4. The ratio of live cells was significantly lower in the 26 psu groups than in the 30 or 34 psu groups,

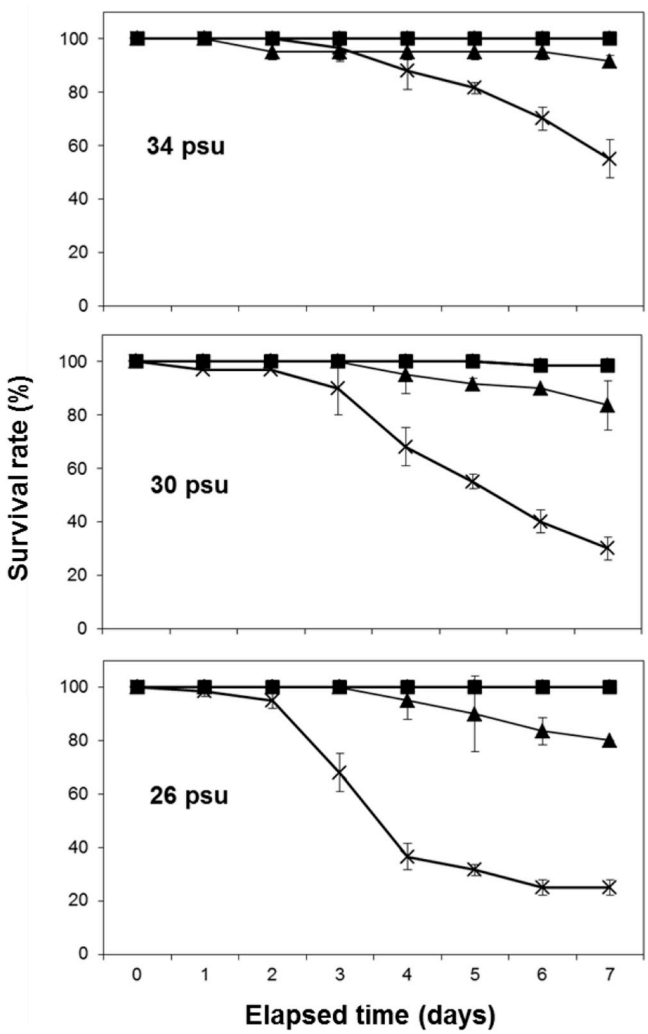

Fig. 1. Change in survival rate of Pacific abalone (*Haliotis discus hannai*) exposed to various temperatures (4, 6, 8, 10°C) and salinities (26, 30, 34 psu) for 7 days. —◆—: 10 °C, —■—: 8 °C, —▲—: 6 °C, —×—: 4 °C.

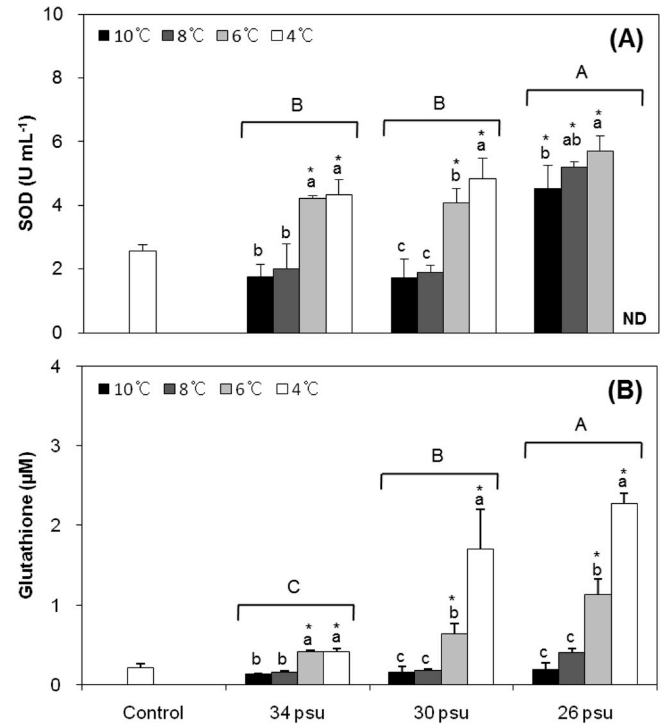

Fig. 2. SOD (A) and glutathione (B) activity in Pacific abalone (*Haliotis discus hannai*) exposed to various temperatures (4, 6, 8, 10°C) and salinities (26, 30, 34 psu) at day 7. Values are presented as mean ± S.D. For each condition, n = 15. Different capital letters indicate significant difference between salinity and small letters between temperature ($p < 0.05$). *Significant difference between control and experimental group ($p < 0.05$). ND: Not detected.

Table 1
P-values from two-way ANOVA of SOD and glutathione activity in Pacific abalone (*Haliotis discus hannai*) exposed to various temperatures (4, 6, 8, 10 °C) and salinities (26, 30, 34 psu) at day 7.

Parameter	Salinity	Temperature	Salinity × Temperature
SOD	0.006	< 0.001	< 0.001
Glutathione	< 0.001	< 0.001	< 0.001

and the ratio was significantly lower in the 4 °C and 6 °C groups than in the 8 °C or 10 °C, or control groups for all salinity levels.

The ratio of apoptotic cells was significantly higher in the 26 psu groups than in the 30 or 34 psu groups. At 30 and 34 psu, the ratio was significantly higher in the 4 °C and 6 °C groups than in the 8 °C or 10 °C, or control group. At 26 psu, the ratio was significantly higher in all the temperature groups than in the control.

The ratio of necrotic cells was significantly higher in the 26 psu groups than in the 30 or 34 psu groups. The ratio was highest at 4 °C in all salinity groups. At 34 psu, the ratio was significantly higher in the 4 and 6 °C groups than in the control. At 30 and 26 psu, all the treatment groups showed a significantly higher ratio than the control, except for the 8 °C group at 26 psu. Live, apoptotic, and necrotic cells were affected by salinity and water temperature, and by the interaction between the two factors (Table 2).

4. Discussion

Temperature or salinity below the tolerance range of an organism weakens its physiological function and health, and under prolonged periods, cause mass mortality. *Haliotis diversicolor supertexta* juveniles can tolerate low water temperatures ranging from 3.5 to 14.0 °C [18], and *Haliotis sieboldii* juveniles have an optimum temperature range of 10–25 °C [19]. The present study found that the survival rate of Pacific

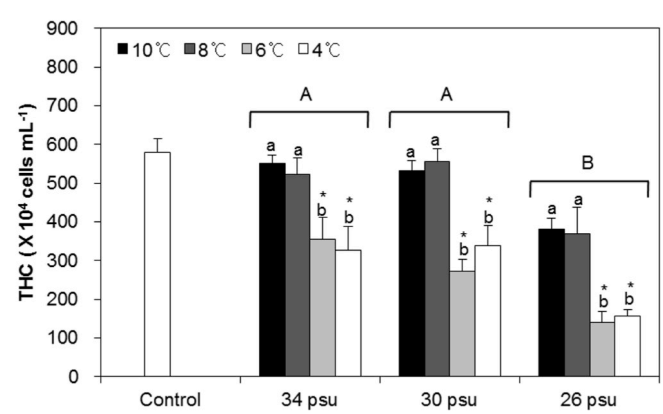

Fig. 3. THC in Pacific abalone (*Haliotis discus hannai*) exposed to various temperatures (4, 6, 8, 10°C) and salinities (26, 30, 34 psu) at day 7. Values are presented as mean ± S.D. For each condition, n = 15. Different capital letters indicate significant difference between salinity and small letters between temperature ($p < 0.05$). *P*-value from two-way ANOVA (Salinity: < 0.001; temperature: < 0.001; salinity × temperature: < 0.744). *Significant difference between control and experimental group ($p < 0.05$).

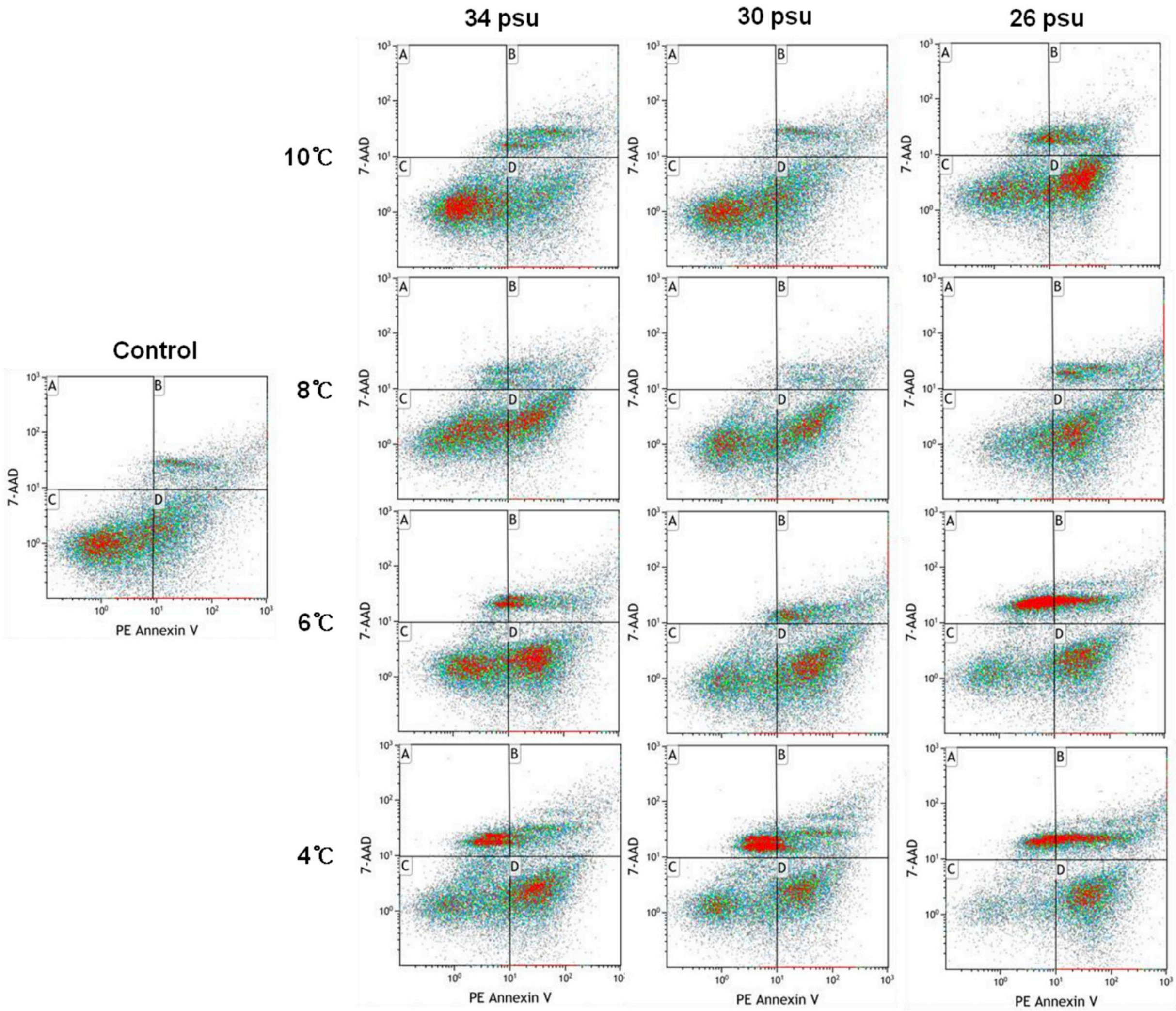

Fig. 4. Flow cytometric determination of live cells, apoptotic cells and necrotic cells in hemocyte populations of Pacific abalone (*Haliotis discus hannai*) exposed to various temperatures (4, 6, 8, 10°C) and salinities (26, 30, 34 psu) at day 7. A: necrosis; B: late apoptosis; C: live; D: early apoptosis. Representative results of 15 abalone individuals.

abalone (*H. discus hannai*) reached 98.7–100% at 8 °C and 10 °C, irrespective of the salinity level. The survival rate declined to 80.0–91.5% at 6 °C and was as low as 25–55% at 4 °C. *Haliotis diversicolor supertexta* can tolerate salinities ranging from 14 to 33 psu [20], while *H. asinine* can tolerate salinities ranging from 32.5 to 20.5 psu [21]. All *H. discus hannai* survived at a salinity of 37 psu, but all were dead after 6 days at 19.8 psu and within 24 h at 12.8 psu or less [22], indicating very low survival at salinity levels below 20 psu. Therefore, water temperatures and salinities below 6 °C and 26 psu, respectively, are not tolerable for Pacific abalone.

Aquatic organisms show a higher level of antioxidant enzyme activity under stress, and the antioxidant enzyme level in blood is used as a stress indicator [23,24]. For example, the South African abalone (*Haliotis midae*) has higher glutathione peroxidase activity under hyperoxia than under normal conditions [25], and the Pacific abalone exhibits higher SOD levels at water temperatures over 25 °C [26].

This study showed that Pacific abalone had higher levels of SOD and glutathione at 4 °C and 6 °C than the controls, irrespective of the salinity level, indicating that Pacific abalone were stressed at these water temperatures. The abalones also exhibited higher levels of SOD and glutathione at 26 psu than at other salinity levels, and were under greater stress when low salinity was combined with low water temperature.

Generally, greater stress produces higher levels of antioxidant enzymes in the blood; thus, it was expected that the most extreme experimental condition of 26 psu/4 °C would produce the highest level of SOD; however, no SOD was detected in abalones exposed to this combination. This might be explained by a physiological disorder caused by the extreme stress. In the Japanese pearl oyster (*Pinctada fucata*), populations (SOD activity 38.8 U mL^{-1}) affected by mass mortality exhibited lower SOD than healthy populations (SOD activity 97.7 U mL^{-1}) [27]. Furthermore, scallops (*Chlamys farreri*) exposed to different air temperatures of 5, 17, and 25 °C, showed the highest mortality of hemocytes after 6 h at 25 °C, but showed the lowest level of SOD activity [10]. *Haliotis midae* also showed significantly lower SOD activity in hypoxic conditions than in normal conditions [25].

In mollusks such as Pacific abalone, hemocytes play a critical role in physiological functions and immune responses. Hemocytes move between the hemolymph and tissues and are involved in nutrient digestion and transportation, detoxification, and repair of damaged shells and tissues [28].

Water temperature and salinity affect the THC in aquatic organisms [29,30], which is an important immunological indicator for mollusks that are affected by stress factors such as pathological bacteria, environmental changes, and heavy metals [31]. In the striped Venus clam (*Chamelea gallina*), THC increases with increasing bacteria in the hemolymph as water temperature rises from 25 °C to 30 °C [32]. THC increases in American oyster (*Crassostrea virginica*) when they are

Table 2
Percentage of live cells, apoptotic cells and necrotic cells in hemocyte populations of Pacific abalone (*Haliotis discus hannai*) exposed to various temperatures (4, 6, 8, 10 °C) and salinities (26, 30, 34 psu) at day 7.

n = 15	Temperature (°C)	Live cells (%)	Apoptotic cells (%)	Necrotic cells (%)
Control (34 psu, 15°C)		48.3 ± 5.4	49.7 ± 4.3	0.2 ± 0.1
34 psu	10	48.6 ± 11.3^{a}	51.0 ± 11.3^{b}	0.3 ± 0.2b
	8	48.2 ± 7.4^{a}	51.5 ± 7.4^{b}	0.2 ± 0.0b
	6	34.6 ± 2.6^{b*}	61.6 ± 3.0^{b*}	3.7 ± 0.8b*
	4	1.5 ± 0.2^{c*}	71.6 ± 6.7^{a*}	26.8 ± 6.5a*
30 psu	10	47.7 ± 6.2^{a}	50.8 ± 5.6^{c}	1.5 ± 0.7b*
	8	43.9 ± 4.9^{a}	54.4 ± 5.1^{c}	1.6 ± 0.5b*
	6	33.0 ± 6.0^{b*}	65.0 ± 5.9^{b*}	2.0 ± 0.6b*
	4	1.1 ± 0.1^{c*}	75.3 ± 9.0^{a*}	23.3 ± 8.8a*
26 psu	10	35.2 ± 5.9^{a*}	63.8 ± 5.6^{b*}	1.0 ± 0.5c*
	8	21.7 ± 7.5^{b*}	78.0 ± 7.7^{a*}	0.2 ± 0.0c
	6	1.3 ± 0.2^{c*}	71.5 ± 5.1^{ab*}	27.2 ± 5.2b*
	4	0.3 ± 0.1^{c*}	66.0 ± 3.9^{b*}	33.6 ± 4.0^{a*}
P-value from two-way ANOVA				
Salinity	< 0.001	0.008		< 0.001
Temperature	< 0.001	< 0.001		< 0.001
Salinity × Temperature	< 0.001	< 0.001		< 0.001

Values are presented as mean ± S.D. n, number of analyzed sample. Different small letters indicate significant difference between temperatures ($p < 0.05$).
*Significant difference between control and experimental group ($p < 0.05$).

exposed to copper [12], while THC decreases in mussel (*Mytilus edulis*) that are exposed to manganese [33]. In addition, *Chl. farreri* [10] and *H. diversicolor supertexta* [13] show reduced THC in hypoxic conditions.

The present study revealed that Pacific abalone had lower THC at 26 psu, and at temperatures of 4 °C and 6 °C. A previous study showed that *M. edulis* also had lower THC at lower salinity, indicating a similar response to that of the Pacific abalone [13]. Donaghy et al. [34] showed that mollusks lost metabolic activity and had lower THC at lower water temperatures and had less prey in winter compared to spring and summer. Considering that THC varies between species, stress factors, and seasons, further studies are required to clarify this response.

The health of shellfish can be evaluated using the condition index, histological change, hemolymph composition, free amino acids in tissues, and mortality of hemocytes [11,35,36]. The mortality of hemocytes includes apoptosis and necrosis, which are caused by heavy metals, bacterial infection, and extreme stress factors (large changes in salinity and water temperature) [11,37,38]. *Chlamys farreri* showed higher mortality of hemocytes under stress caused by hypoxia [10], while Pacific oyster (*Crassostrea gigas*) also shows high mortality of hemocytes under water temperature and salinity stress. *Perna viridis* also exhibited higher mortality of hemocytes under salinity stress [39]. In the present study, Pacific abalone showed greater numbers of apoptotic and necrotic cells than the control at water temperatures of 4 °C and 6 °C, and at a salinity of 26 psu, compared to 30 and 34 psu. These results indicate that the conditions of 4 °C and 6 °C and 25 psu are not suitable for the physiology of Pacific abalone. Hemocyte viability in shellfish varies with season [40]. Previously, it has been reported that exposure to extreme high or low water temperatures beyond the tolerance range triggers hemocyte apoptosis in shellfish and reduces hemocyte viability [41]. In the mussel *Mytilus galloprovincialis*, hemocyte viability was also significantly lower as temperature increased in spring than in other seasons [40]. Although in the present study we did not measure hemocyte viability in the Pacific abalone at high water temperatures, we suggest that hemocyte viability in the Pacific abalone is significantly lower in winter than in other seasons as they showed significantly lower hemocyte viability below 6 °C in our experiment.

Although the mechanism of cell apoptosis is not clearly understood, previous authors [42] have suggested that oxygen free radicals affect the mitochondrial membrane potential and induce apoptosis, which exposes phosphatidylserine. In the present study, the apoptosis of hemocytes in the Pacific abalone significantly increased at lower salinities, showing a similar pattern to the levels of SOD and glutathione activity. Thus, we concluded that stress-associated increases in oxygen free radicals contributed to apoptosis in the Pacific abalone. The physiologically lowest tolerance of Pacific abalone inhabiting the waters of Tongyeong is 8 °C for water temperature and 30 psu for salinity.

Acknowledgments

This work was supported by a grant from the National Institute of Fisheries Science (R2018012), Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Golden Seed Project, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA), Ministry of Oceans and Fisheries (MOF), Rural Development Administration (RDA), and Korea Forest Service (KFS) (213008-05-2-SB110).

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