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Note

Effect of water temperature on haemocyte responses in the disk abalone *Haliotis discus hannai* Ino, 1953

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ABSTRACT

Effects of gradual water temperature modulations on neutral red retention time (NRR time) of lysosomes in haemocytes and haemocyte counts were studied in the disk abalone *Haliotis discus hannai* Ino, 1953. Experimental animals were acclimated at 18ºC for a period of one week before exposing to gradual water temperature modulations. After acclimation, the water temperature was gradually increased/decreased @ 1ºC every other day until the target temperature of 10ºC/26ºC was reached. The temperatures were maintained for seven days before being brought back to 18ºC from 10ºC/26ºC (@ 1ºC per day). Haemolymph samples were collected 48 h after each temperature change. The haemocyte density at 26ºC was significantly higher ($p<0.05$) than that at 18ºC and 10ºC, and the lowest haemocyte density was obtained at 10ºC. The NRR time of the abalone held at 26ºC, 18ºC and 10ºC were 31.6±1.1min, 84.6±2.7 min and 41.3±1.7 min, respectively. The temperature changes between17ºC and 20ºC did not significantly affect the NRR time suggesting that this is the optimal water temperature range for maintaining lysosomal membrane integrity in this species.

Keywords: Disk ablone, *Haliotis discus hannai,* Haemocyte density, Haemocyte mortality, Lysosomal membrane stability, Neutral red retention time, Water temperature changes

Abalones are thermoconformers, and their body temperature changes according to environmental temperature (Hahn, 1989; Prosser, 1991). Evidences indicate that adaptation to gradual temperature changes in abalones is weak, and abalones have limited ability to withstand thermal shock (Paladino *et al.*, 1980; Jobling, 1981; Gilroy and Edwards, 1998). Narrow thermal ranges and relatively small differences in thermal preference have been considered as a kind of evolutionary adaptation (Gilchrist, 1995), however, such unique character may also be disadvantageous in aquaculture production of abalones. Unfavourable water temperatures can affect the growth and survival in various abalone species (Winstanley, 1972; Steinbeck *et al.*, 1992; Cheng *et al.*, 2004). For these reasons, knowledge on the responses of abalone to water temperature changes would help improving production performance of abalone and in avoiding mortalities in commercial production.

Haemocytes in molluscs play important roles as they produce mediators for stress and immune responses (Ottaviani and Franceschi, 1996; Ottaviani and Franceschi, 1997; Ottaviani *et al.*, 1997). Evidences indicate that haemocyte densities are sensitive to stresses and can be affected by infection, wounding and temperature fluctuations (Suresh and Mohandas, 1990; Mohandas *et al.*, 1992). Increase in haemocyte densities has been considered as the result of proliferation or movement of cells from tissues into the haemolymph, whereas decrease in haemocyte densities are thought to be a consequence of cell lysis or reduced cell movement from tissues to haemolymph (Pipe and Coles, 1995). Therefore, in the present study, haemocyte densities were used to evaluate the response of the disk abalone *Haliotis discus hannai* to changes in water temperature.

Neutral red is a cytotoxic weak base colourant, and has been used in assessing the lysosomal membrane integrities in molluscs (Fernley *et al.*, 2000; Wang *et al.*, 2006; Zhang *et al.*, 2006). Generally, the lysosomes in unstressed cells can retain neutral red dye for a longer period, and the retention time in stressed cell tends to be shorter (Lowe *et al.*, 1992; Lowe and Pipe, 1994). Therefore, the neutral red retention time (NRR time) and stress condition of the animals can be correlated (Harding *et al*., 2004). The NRR assay has been widely applied to measure the lysosome membrane stability in molluscs under different environmental stress conditions (Hauton *et al.*, 1998; Camus *et al.*, 2000; Fernley *et al.*, 2000).

The disk abalone *Haliotis discus hannai* belonging to the family Haliotidae, is endemic to the waters off Japan and eastern Asia. Because of the high flesh quality and unique flavour, disk abalone is very popular in Asian market. Aquaculture of this species has been successfully carried out. However, high mortality rate of disk abalone

in summer has significantly hindered the commercial production of this species. The present study was undertaken in order to understand the temperature related mortality in disk abalone and to explore the potential solution in terms of temperature impaction. Haemocyte density, haemocyte morality rate and NRR time were used as bioindicators to investigate the influence of water temperature on the immune responses in disk abalone *H. discus hannai*.

The experimental animals (mean body weight 14.1±0.3 g) were obtained from a commercial farm located in Shandong Province, P. R. China, and were transferred to the wet lab in Qingdao Agriculture University, Qingdao. Upon arrival, the abalones were tagged individually using plastic tags adhered to individual shell with an identification number and acclimated in 18°C water for 7 days prior to initiation of the experiment. The salinity was maintained at 32‰. During the acclimation and experimental period, the abalones were held in 400 l tanks and fed with a formulated diet (Haida, P. R. China).

Two experiments were conducted to assess the effect of gradual water temperature change on the haemocytes. In the first experiment, experimental animals acclimated at 18°C were divided into three groups with three replicates in each group. The first two groups were subjected to gradual increase/decrease in water temperature, while the third group was maintained at 18°C as control. The temperature was changed at the rate of 1°C every other day until the final temperature of 10°C/26°C was reached. In the second experiment also, experimental animals were divided into three groups with three replicates in each group. The first two groups were acclimated at 10°C or 26°C initially, and then subjected to gradual increase or decrease in water temperature @ 1°C per day until 18°C was reached. The third group was maintained at 18°C as control.

In each experiment, hemolymph was sampled 1 h prior to water temperature changed to the next level. Once the target temperatures were reached, the abalones were maintained for a further period of seven days, with hemolymph samples being taken at every 48 h. Haemolymph samples were also collected from control animals at each sampling time, from the 18ºC tanks directly.

The haemocyte density was determined according to the method described by Suresh *et al*. (1994) with slight modification. After acclimation at 10°C, 18°C or 26°C for 7 days, 0.1 ml haemolymph per individual was collected from the cephalic arterial sinus at the anterior end of the foot using a sterile syringe. After collection, the haemolymph was immediately placed in a 1 ml siliconised Eppendorf tube containing 0.2 ml of 0.38% sodium citrate (Sigma) in 0.45 µm filtered seawater (FSW), pH 7.5, to prevent clotting. Haemocyte density was then determined using a haemocytometer and expressed as the number of haemocytes per milliliter haemolymph. At each target temperature, eight animals were assessed. Haemocyte mortalities were also examined at each target temperatures. A total of 0.1 ml haemolymph from each animal was placed in a 1 ml siliconised Eppendorf tube containing 0.2 ml of 0.1% trypan blue (Sigma) solution and gently mixed. After 5 min, 0.1 ml hemolymph was placed on a slide and examined using a compound microscope under x600 magnification. Two hundred haemocytes per slide were examined, and the haemocytes stained in blue were recorded as dead. The percentage of mortality was then calculated individually. A total of eight abalones were examined at each target temperature.

Neutral red retention assay used in this study was based on the methods developed by Hauton *et al.* (1998). Neutral red stock solution was prepared by dissolving 2.28 mg of neutral red powder (Sigma) in 10 ml of dimethyl sulphoxide (DMSO). Working solution was prepared by diluting 17 µl of the stock solution with 2 ml of FSW. Heamolymph was collected individually following the same method as described in the previous section and placed in a siliconised Eppendorf tube containing 0.2 ml of FSW and then mixed gently. A total of 30 µl of the mixed sample was placed on a microscope slide. The slide was then placed in a 10°C light-proof humidity chamber to allow the cells to adhere to the slide. After 10 min, the slide was removed from the chamber, the excess hemolymph was removed, 20 µl of neutral red working solution (10°C) was added to the cell layer and incubated in the chamber for another 15 min. A cover slide was then placed onto the slide, and the hemocytes were examined using a compound microscope at x600 times magnification. The slide was examined at 10 min interval for the first 60 min and subsequently at every 15 min. A total of 30 granulocytes were examined at each time interval. Once >50% of the haemocytes lost neutral red dye from their lysosomes, the time was recorded as the NRR time.

The value of each variable was expressed as mean±SD and compared by one-way ANOVA (PASW Statistics 18.0, Chicago, USA). All the data were tested on their normality and showed normal distribution. A probability level of $p<0.05$ was defined as statistically significant.

The haemocyte density of the abalones held at 26°C (8.07×10^6 cells ml^{-1}) were significantly higher than those held at 18°C (3.55×10^6 cells ml^{-1}) and 10°C (3.05×10^6 cells ml^{-1}) ($p<0.05$, Fig. 1), and no significant difference was found between 18 and 10°C ($p>0.05$, Fig. 1). Similar results have also been reported in other molluscs and crustaceans

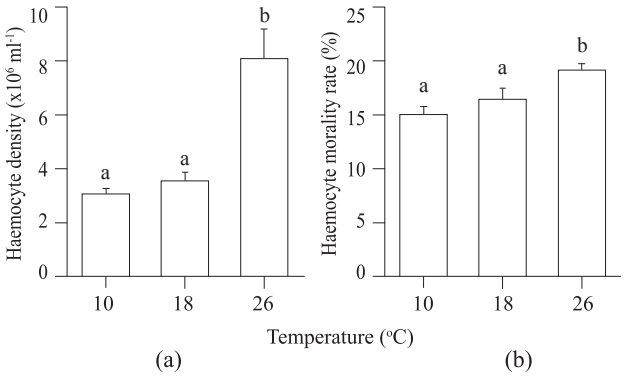

Fig. 1. Haemocyte density (a) and haemocyte mortality (b) rates of disk abalone *Haliotis discus hannai* at different temperatures

(Truscott and White, 1990; Paillard *et al.*, 2004; Monari *et al.*, 2007). The haemocyte density in both molluscs and crustaceans tends to fluctuate with environmental temperature and higher environmental temperature normally results in higher haemocyte density (Truscott and White, 1990; Le Moullac and Haffner, 2000). Galloway and Depledge (2001) opined that haemocytes can move from tissue to haemolymph to adapt to environmental stress when water temperature is increased, and haemocytes can also be transferred/reserved to tissue when the water temperature is reduced. In the present study, haemocyte mortality rate at 26°C was significantly higher than the results obtained at 18 and 10°C ($p<0.05$, Fig. 1). As invertebrates rely primarily on innate immune defences implemented through a non-lymphoid system (Galloway and Depledge, 2001), and haemocytes are involved in most of the physiological and pathological functions (Cheng, 1981; Bayne, 1983; Fisher, 1986). Reduction in haemocytes density and increase in haemocytes mortality may suggest reduced immunosurveillance in stressed animals.

In the present study, the NRR time of abalones held at 26, 18 and 10°C were 31.6±1.1, 84.6±2.7 and 41.3±1.7 min, respectively. In the first experiment, the NRR time did not change significantly ($p>0.05$) when water temperature changed from 18 to 17°C (Fig. 2a) and from 18 to 21°C (Fig. 2b). When the water temperature reduced from 18 to 10°C, the NRR time dropped significantly from 84.6±2.7 to 41.3±1.7 min ($p<0.05$). When water temperature increased from 18 to 26°C, significant NRR time drop was noticed between 22 and 23°C ($p<0.05$, Fig. 2b). When water temperature reached 24°C, the NRR time decreased to 33.7±1.7 min and then remained at same level until the experiment was completed.

In the second experiment, when the water temperature was gradually increased from 10 to 18°C, the NRR time increased with increase of temperature (Fig. 2c). When the temperature reached 17°C, the NRR time reached a level that was not significantly different from the control ($p>0.05$, Fig. 2c). When the water temperature decreased from 25 to 18°C, the NRR time increased with decrease in temperature, and the NRR time was not significantly different between the control and treated group when the temperature reached 20°C ($p>0.05$, Fig. 2d). Subsequently, the NRR time remained at similar level until the experiment was completed.

Based on the results obtained in this study, the temperature range between 17 and 20°C was found optimal for maintaining lysosomal membrane stability in disk abalone *H. discus hannai*. This range is comparable with the finding reported by Zhang *et al*. (2005). In this study, the lowest NRR times were recorded at 10 and 26°C, suggesting a significant impact on the lysosomal stability under these temperatures. However, no mortalities were recorded at 10 and 26°C, indicating that *H. discus hannai* could stand gradual water temperature changes at these temperature levels.

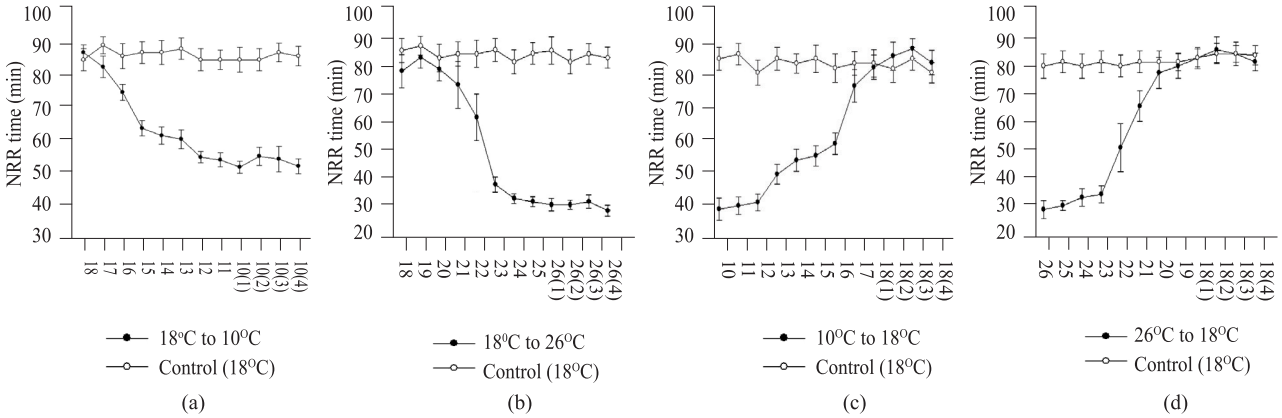

Fig. 2. Neutral red retention time (NRR time) of disk abalone *Haliotis discus hannai* in response to water temperature changes, a: reduction in water temperature from 18 to 10°C; b: increase in water temperature from 18 to 26°C; c: increase in water temperature from 10 to 18°C; d: reduction in water temperature from 26 to 18°C

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References

Bayne, C. J. 1983. Molluscan immunobiology. In: Saleuddin, A. S. M. and Wilbur, K. M. (Eds.), *The mollusca 5, Physiology, Part 2.* London, Academic Press, p. 407-486.

Camus, L., Grosvik, B. E., Borseth, J. F., Jones, M. B. and Depledge, M. H. 2000. Stability of lysosomal and cell membranes in haemocytes of the common mussel (*Mytilus edulis*): effect of low temperatures. *Mar. Envir. Res.,* 50: 325-329.

Cheng, T. C. 1981. Bivalves In: Ratcliffe, N. A. and Rowley, A. F. (Eds.), *Invertebrate blood cells.* New York, Academic Press, p. 231-300.

Cheng, W., Hsiao, I. S. and Chen, J. C. 2004. Change in water temperature on the immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. *Fish Shellfish Immunol.,* 17: 235-243.

Fernley, P. W., Moore, M. N., Lowe, D. M., Donkin, P. and Evans, S. 2000. Impact of the *Sea Empress* oil spill on lysosomal stability in mussel blood cells. *Mar. Envir. Res.,* 50: 451-455.

Fisher, W. S. 1986. Structure and function of oyster haemocytes In: Brehelin, M. (Ed.), *Immunity in invertebrates.* Heidelberg: Springer-Verlag, Berlin, p. 25-35.

Galloway, T. S. and Depledge, M. H. 2001. Immunotoxicity in invertebrates: measurement and ecotoxicological relevance. *Ecotoxicology,* 10: 5-23.

Gilchrist, G. W. 1995. Specialists and generalists in changing environments. I. Fitness landscapes of thermal sensitivity. *Am. Natural.,* 142: 796-818.

Gilroy, A. and Edwards, S. J. 1998. Optimum temperature for growth of Australian abalone: preferred temperature and critical thermal maximum for blacklip abalone, *Haliotis rubra* (Leach), and greenlip abalone, *Haliotis laevigata* (Leach). *Aquac. Res.,* 29: 481-485.

Hahn, K. O. 1989. Abalone aquaculture in Japan. In: Hahn, K. O. (Ed), *The culture of abalone and other marine gastropods.* CRC Press, Boca Raton, p. 185-194.

Harding, J. M., Couturier, C., Parsons, G. J. and Ross, N. W. 2004. Evaluation of the neutral red assay as a stress response indicator in cultivated mussels (*Mytilus spp.*) in relation to post-harvest processing activities and storage conditions. *Aquaculture,* 231: 315-326.

Hauton, C., Hawkins, L. E. and Hutchinson, S. 1998. The use of neutral red retention assay to examine the effects of temperature and salinity on haemocytes of the European flat oyster *Ostrea edulis* (L). *Comp. Biochem. Physiol.,* 119 B: 619-623.

Jobling, M. 1981. Temperature tolerance and the final preferendum-rapid methods for the assessment of optimum growth temperatures. *J. Fish Biol.,* 19: 439-455.

Le Moullac, G. and Haffner, P. 2000. Environmental factors affecting immune responses in Crustacea. *Aquaculture,* 191: 121-131.

Lowe, D. M., Moore, M. N. and Evans, B. M. 1992. Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. *Mar. Ecol. Pro. Ser.,* 91: 135-140.

Lowe, D. M. and Pipe, R. K. 1994. Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an *in vitro* study. *Aquat. Tox.,* 30: 357-365.

Mohandas, A., Adema, C. M., Knaap, W. P. W. and Sminia, T. 1992. The effect of haemolymph extraction on distribution of lysosomal enzymes *Lymnaea stagnalis*; haemocytes: a cytochemical study. *Comp. Haemato. Int.,* 2: 61-67.

Monari, M., Matozzo, V., Foschi, J., Cattani, O., Serrazanetti, G. P. and Marin, M. G. 2007. Effects of high temperatures on functional responses of haemocytes in the clam *Chamelea gallina*. *Fish Shellfish Immunol.,* 22: 98-114.

Ottaviani, E., Caselgrandi, E. and Kletsas, D. 1997. Effect of PDGF and TGF-B on the release of biogenic amines from invertebrate immunocytes and their possible role in the stress response. *FEBS Letter,* 403: 236-238.

Ottaviani, E. and Franceschi, C. 1996. The neuroimmunology of stress from invertebrates to man. *Prog. Neurobiol.,* 48: 421-440.

Ottaviani, E. and Franceschi, C. 1997. The invertebrate phagocytic immunocyte: clue to a common evolution of immune and neuroendocrine systems. *Immunol. Today,* 18: 169-173.

Paillard, C., Allam, B. and Oubella, R. 2004. Effect of temperature on defence parameters in Manila clam *Ruditapes philippinarum* challenged with *Vibrio tapetis*. *Dis. Aqu. Org.,* 59: 249-262.

Paladino, V., Spotilla, J. R., Schubauer, J. P. and Kowalski, K. T. 1980. The critical thermal maximum: a technique used to elucidate physiological stress and stress and adaptation in fishes. *Rev. Canadian Biol.,* 39: 115-122.

Pipe, R. K. and Coles, J. A. 1995. Environmental contaminants influencing immunefunction in marine bivalve molluscs. *Fish Shellfish Immunol.,* 5: 581-595.

Prosser, C. L. 1991. Temperature In: Prosser, C. L. (Ed.), *Comparative animal physiology, environmental and metabolic animal physiology,* Wiley-Liss, New York, p. 109-165.

Steinbeck, J. R., Groff, J. M., Friedman, C. S., McDowell, T. and Hedrick, R. P. 1992. Investigations into a mortality among populations of the California black abalone, *Haliotis cracherodii*, on the central coast of California, USA. In: Shepherd, S. A., Mia J. Tegner and Guzman del Proo, S. A. (Eds.), *Abalone of the world: biology, fisheries and culture.* Wiley-Blackwell, 624 pp.

Suresh, K. and Mohandas, A. 1990. Effect of sublethal concentrations of copper on haemocyte number in bivalves. *J. Inver. Pat.,* 55: 325-331.

Suresh, P. G., Reju, M. K. and Mohandas, A. 1994. Factors influencing total haemocyte counts in freshwater gastropods. *Comp. Haemat. Int.,* 4: 17-24.

Truscott, R. and White, K. N. 1990. The influence of metal and temperature stress on the immune system of crabs. *Funct. Ecol.,* 4: 445-461.

Wang, T., Li, X., Kriston, B., Song, L., Steven, C. and Zhao, W. 2006. Effects of water temperature on the lysosomal membrane stability in haemocytes of blacklip abalone, *Haliotis rubra* (Leach). *J. Shellfish Res.,* 25: 935-940.

Winstanley, R. H. 1972. Abalone and rock lobster mortality and abnormal water temperatures. *Tasmanian Fish. Res.,* 6: 21.

Zhang, M., Wang, Z. and Gao, X. 2005. Comparison on adaptability of abalone *Haliotis discus hannai* Ino to temperatures at different growing stages. *J. Fish. Sci. China,* 12: 720-725.

Zhang, Z., Li, X., Vandepeer, W. and Zhao, W. 2006. Effects of water temperature and air exposure on lysosomal membrane stability of haemocytes in Pacific oyster *Crassostrea gigas* (Thunberg). *Aquaculture,* 256: 502-509.

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