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THE EFFECTS OF TRANSPORTATION ON IMMUNE MODULATION OF WILD AND OCEAN-RANCHED GREENLIP ABALONE (*Haliotis laevigata*)

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ABSTRACT

The effects of live transportation on the immune modulation of wild and ocean-ranched greenlip abalone (*Haliotis laevigata*) were evaluated. Samples of abalone were collected both in autumn and winter in Flinders Bay, Augusta, Western Australia, and land transported for 6 (six) hours. Several immunological parameters were assessed, including survival rate, total haemocyte count, osmoregulatory capacity, phagocytic rate, lactate level, and glucose level. The results indicated that the abalone samples collected in the two seasons showed different physiological responses; the winter samples were more responsive to transportation than the autumn samples. Transportation stress significantly increased total haemocyte count and osmoregulatory capacity of the winter samples, suggesting an immune stimulation. This stress also triggered an immune suppression, causing the phagocytic rate and lysosomal stability to significantly decrease after transportation. Lactate levels in the winter samples decreased significantly after transportation, possibly indicating the transition from a stressed to normal state, during the period of recovery. The constant level of glucose before and after transportation in both seasons showed that it was the least sensitive indicator used in this study. In general, there was no difference in the immune modulation parameters between wild and ranched abalone in either of the seasons sampled. However, in the autumn samples, there were significant differences ($P < 0.05$) in haemocyte count and osmoregulatory capacity of the wild and ranched samples collected from all sites. These differences did not form a consistent indicator trend between the sites from the two sample sources. Therefore, the results do not raise any immediate concern that ranched abalone were differently stressed to those from the wild. The results in present study could serve as useful data in developing the live transportation method of greenlip abalone.

KEYWORDS: ***greenlip abalone; transportation; sea ranching, immune***

INTRODUCTION

Abalone or sea snail is a premium seafood product that experiences increasing demand in recent years. High price and growing market of abalone worldwide have led to over-exploitation of its wild stocks in parts of the world (Campbell, 2000; Taniguchi *et al*., 2013; Troell *et al*., 2006). To counter the declining stocks and to increase production, there has been a worldwide growth in abalone aquaculture since the 90's. China is the biggest contributor to farmed abalone in global markets, followed by Korea and Chile. In Australia, the production of farmed abalone is only around 1 to 2% of China's annual production (Cook, 2014).

In Western Australia (WA), abalone demand is mostly fulfilled from wild capture fishery. That industry focuses on three species, namely brown lip (*Haliotis conicopora*), greenlip (*H. laevigata*), and Roe's abalone (*H. roei*) (DoFWA, 2013). Aquaculture of abalone in the state is still in its infancy. There is a land-based abalone hatchery in Bremer Bay on the south coast of WA, as well as an approximately 80 ha sea ranching site in Augusta (WAFIC, 2016).

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The development of abalone aquaculture in WA, especially for commercial sea ranching, is constrained by limited suitable sites and stringent environmental regulations (Partridge & Furey, 2002). Another challenge comes from post-harvest handling, particularly in live-transportation. Bubner *et al*. (2009) found that ice and oxygen supplementation during transportation of live abalone reduce the mortality rate. The effects of physical perturbations on stress and immune responses of abalone have been widely studied across a range of abalone species, for example in *H. iris* and *H. australis* (Baldwin *et al*., 1992; Behrens *et al*., 2002; Wells & Baldwin, 1995) *H. diversicolor supertexta* (Cheng *et al*., 2004), *H. rubra* (Dang *et al*., 2012) and in hybrid *H. laevigata* and *H. rubra* (Hooper *et al*., 2011). Hormones produced during stress exposure affect the immune functions indicating a consistent link between stress response and immune response (Adamo, 2012; Malham *et al.*, 2003).

This study aimed to investigate the effects of live transportation on the immune modulation of ranched and wild greenlip abalone (*H. laevigata*). Some general physiological parameters of abalone, such as total haemocyte count, phagocytic rate, lactate level, osmoregulatory capacity, and glucose level, were examined to assess the species' immune responses. This information can be used as a proxy for determining the health status of ranched abalone compared to wild stock during transportation.

MATERIALS AND METHODS

Experimental System & Location

Sixty live greenlip abalones with size varying between 61 and 135 mm shell length (SL) (Table 1), both ranched and wild stocked, were collected from Flinders Bay (Figure 1) .

Due to the time limitation, the samples were only taken during autumn (April) and winter (July) seasons of 2016 from two wild sites (34°22.421 S, 115°10.214 E and 34°22.252 S, 115°29.935 E) and three ranching sites. Six individual healthy abalones were collected from each site in each season. Haemolymph was obtained from each abalone before and after transportation to assess total haemocyte count, phagocytic rate, osmolality, lactate, and glucose level to evaluate the transportation effects. This first haemolymph sample was taken immediately onshore, which was used as a control and represented the normal immune modulation of the abalone. Shortly after arrived at the Curtin Aquatic Research Laboratory, the abalones were transferred to a 70 L recirculated and aerated fiberglass tank and allowed to recover. Visual observations on their condition were made after ~12 hours. At the same time, haemolymph was taken from abalone from each site that had not previously had haemolymph extracted.

Transportation Method

Straight after being brought to the surface, the abalone samples were temporarily placed into labelled plastic bags and stored in a cool box with ice or ice bricks to maintain their condition. Once ashore, all abalone were packed into 40L polystyrene boxes. Crushed ice was placed at the bottom of the boxes covered with wet sponge sheets to avoid a direct contact with the abalone on top. This arrangement was done to manage a consistent temperature in the boxes and keep the abalone moist during transportation.

Haemolymph Withdrawal

There are a lot of stress indicators reliable to determine stress responses on abalone. Most of them are adopted from other invertebrates and focused on haemolymph as the immune system's centre (Day *et al*., 2010). In the present study, approximately 1 mL haemolymph was taken from the cephalic sinus of abalone using a 3 mL sterile syringe and a one-inch needle (25G). Haemolymph samples were placed in crushed ice after withdrawal to preserve their condition. All haemolymph samples were analyzed within 24 hours of being frozen. A 200 μL haemolymph sample from each abalone was separated and treated with 6% perchloric acid (PCA) for lactate assay.

Survival Rate

The survival rate of abalone was expressed as the percentage of survived abalone after transported and calculated using the following formula:

$$\text{Survival rate } \% = 100 \text{ x } \frac{\text{Number of survived abalone}}{\text{Initial number of abalone}}$$

Table 1. Mean SL (mm) of collected Abalone from five site locations

Mean size (mm SL)				
Ranched Site 1	Ranched Site 2	Ranched Site 3	Wild Site 1	Wild Site 2
80.5 ± 3.78	83.33 ± 3.73	89.08 ± 2.78	109.67 ± 5.84	104.00 ± 3.24

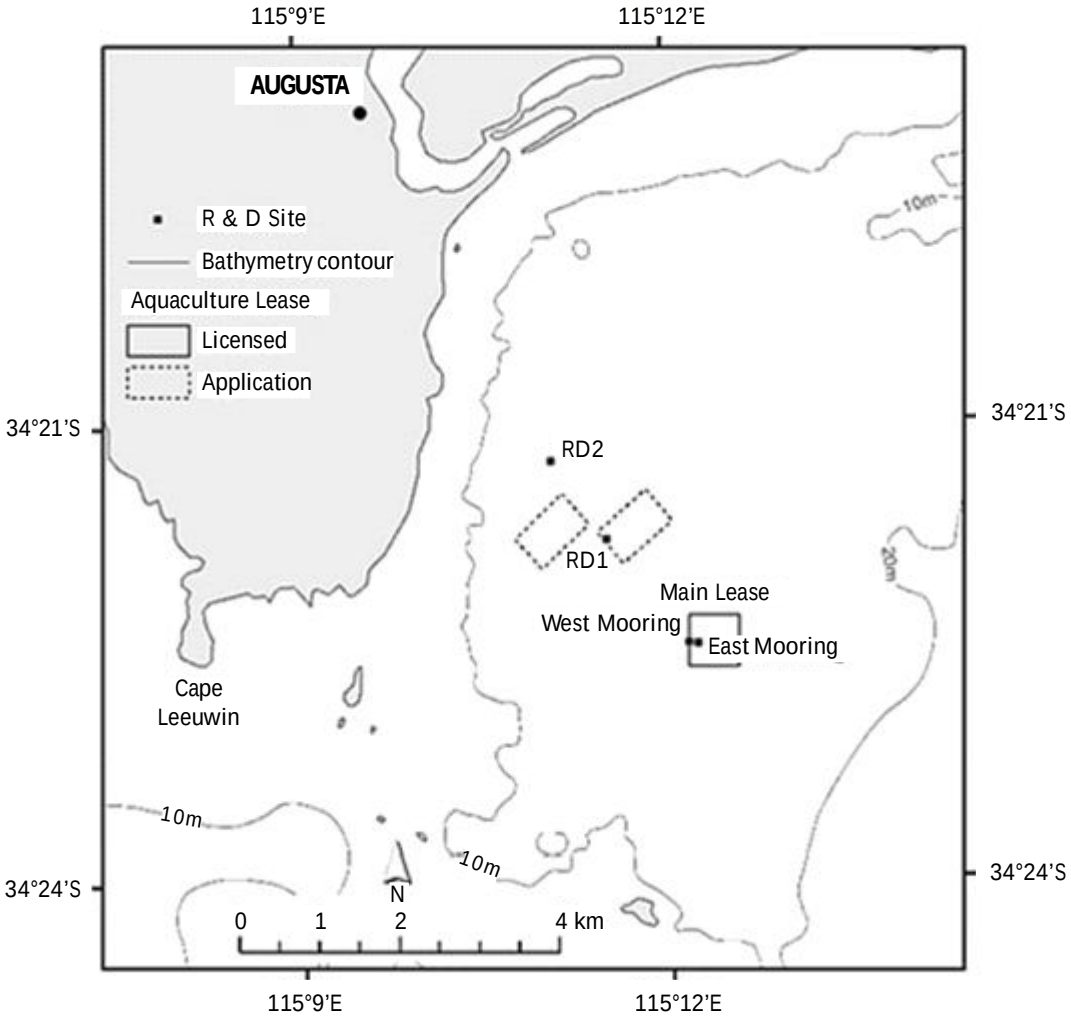

Figure 1. Map showing greenlip abalone (*H. laevigata*) sampling sites at the Ocean Grown Abalone Pty Ltd sea ranch in Flinders Bay, Augusta.

Total Haemocyte Count

The total haemocyte count has been used widely in the immune responses of abalone (Dang *et al*., 2012; Day *et al*., 2010; Hooper *et al*., 2011; Hooper *et al*., 2014). Total haemocyte count (THC) was measured using the method described by Day *et al*. (2010), with a modification in terms of the use of cold-stored haemolymph. A small amount of haemolymph was taken using a micropipette and transferred into a haemacytometer (Reichert, USA). The number of observed haemocytes in both grids were counted under 100 x magnification of a light microscope (Motic, China). The mean values from both grids were used to calculate the total haemocyte counts. The results were expressed as the number of haemocytes (5 x 10^4 cell/mL) and measured using the following equation:

$$THC = \left(\frac{\text{Mean no cells x dilution factor (1) x 1000}}{\text{Volume of the grid (0.1mm}^3\text{)}} \right)$$

Osmoregulatory Capacity (OC)

A 30 μL sample of cold-stored haemolymph was placed into a 0.5 mL Eppendorf tube. The osmolality of haemolymph was measured using a freezing point osmometer (Osmomat 030-D, Gonotec, Germany). The results were presented as Osmol/kg. Osmoregulatory capacity was determined by the difference in serum osmolality and the medium (seawater) osmolality.

Phagocytic Rate (PR)

The ability of hemocyte to engulf pathogenic agents or other foreign materials can be measured using phagocytosis slide-based assay. The assay uses zymosan (yeast cell) as the agent (Chen *et al*., 2005). The effects of transportation on the phagocytic rate of abalone were measured using the established protocol with some modifications described by Cheng *et al*. (2004). A 40 μl sample of haemolymph was smeared onto two Poly-LLysine® glass slides and in-

cubated for a minimum of 10 minutes, to allow adherence of the haemolymph to the glass. Excess haemolymph was poured off the slides before adding 40 μL of Zymosan solution (0.031 g Zymosan + 25 mL of sterile-filtered seawater). The slides were re-incubated for a further 30 minutes before being fixed for 20 min with seawater formalin (10% formaldehyde in filtered seawater). The haemocytes and zymosan were stained with May Grunwald or Giemsa. Under a light microscope using 100x magnifications, the number of phagocytic haemocytes is representated by the number of haemocytes which engulf zymosan particles (Chen *et al*., 2005; Wang *et al*., 2004). The results were expressed as the percentage of phagocytosis. Phagocytic rate (PR) was determined using the following equation:

$$PR\ \% = 100\ x \left(\frac{\text{Number of phagocytic haemocytes}}{\text{Total adhered haemocytes}} \right)$$

Lactate

Lactate can accumulate in haemolymph or muscles during transportation or in hypoxic conditions. Lactate level was measured by taking 200 μL sample of haemolymph and treated in 6% PCA. The sample then was centrifuged at 5,000 rpm at 4°C for 10 minutes (5804R Centrifuge Eppendorf, Hamburg-Germany). The supernatant was rinsed with 5 mol/L K_2CO_3 to neutralize the PCA (for one hour under chilled conditions). PCA was then removed from the supernatant by re-centrifuging it at 5,000 rpm at 4°C for 10 minutes. The ready-to-assay supernatant was directly used as described by (Behrens *et al*., 2002). The assay was undertaken using a commercial reagent set kit (Pointe Scientific L7596). A 12 μL sample of haemolymph and 600 μL of reagent 1 were mixed and incubated at 25°C for 30 seconds. In the next step, a 400 μL of reagent 2 was added into the mixture and the sample was incubated at 25°C for 5 minutes. Finally, the mixture was measured for the absorbance at 546 nm using an Ultraviolet-Visible (UV-Vis) Spectrophotometry (Shimadzu, Japan), and the results were presented as mmol/L of lactate.

Glucose

The glucose level of abalone haemolymph was examined using liquid glucose (oxidase) reagent set (Pointe Scientific, Inc). A 1.0 mL quantity of working reagent was incubated at 25°C for approximately 5 minutes, followed by the addition of 0.01 mL of haemolymph. The mixture was then incubated at 25°C for approximately 10 minutes before measuring the absorbance at 500 nm using UV-Vis Spectrophotometer. The results were presented as mmol/L of glucose.

Statistical Analysis

Statistical analysis was performed using IBM SPSS statistics software version 22.0 at a probability level of 0.05. The mean difference of each immune response indicator before and after transportation in each sampling period was analysed using independent t-tests. The differences in immune response between sources (ranching and wild sites) were also analysed using independent t-tests. Differences in immune response between sites were analyzed using one-way ANOVA and Least Significant Difference post hoc tests. In the case of non-normal data distribution, transformation and, if necessary, non-parametric statistical analyses (Mann Whitney-U and Kruskal Wallis tests) were also conducted.

RESULTS AND DISCUSSION

This study demonstrates that physical disturbance during transportation, particularly aerial exposure, might contribute to inducing stress to the abalone. However, transportation-induced stress over the relatively short (< 6 hours) period of travel in this study, is at the level that can be physiologically managed by the animals. Visual observations showed that all abalone (100%) from both the autumn and winter samplings had survived transportation to the laboratory. However, some were in poor condition characterized by limp muscles and weak attachment to the tank wall.

The effect of transportation on THC was different between autumn and winter. An independent t-test indicated that there was no difference in THC before and after transportation in autumn ($P > 0.05$). However, different THCs were observed in winter ($P < 0.05$). THC was significantly increased after transportation in winter, which was from 483.067 ± 33.833 to 644.8 ± 61.185 (5 x 10^4 cell/mL) (Table 2). The significant increase in THC in the winter samples is a common stress response reported for abalone as a response to physical perturbations and pathogenic infections (Dang *et al*., 2013; Hooper *et al*., 2011).

When abalones are heat-stressed, the haemocyte count increases briefly before returns back to normal levels as the haemocytes infiltrate into the connective tissues (Hooper *et al*., 2011). It is hypothesised that the open circulatory system in abalone may allow the migration of haemocytes from tissues to the circulatory system during stress exposure, the same way it occurs in bivalves (Cajaraville *et al*., 1996).

In autumn, there were significant differences in THC between sites ($P < 0.05$) and between sources ($P < 0.05$). In autumn, there were significant differences in THC between sites ($P < 0.05$) (Figure 2) and

Table 2. Total haemocyte count (5 x 10^4 cells/mL); phagocytic rate (%); lactate level (mmol/L); Osmoregulatory capacity (osmol/kg); and glucose level (mmol/L) in the haemolymph of greenlip abalone before and after transportation in autumn and winter

Indicators	Autumn samples		Winter samples	
	Before transport	After transport	Before transport	After transport
THC	491.5 ± 151.3[a]	271.467 ± 54.9[a]	483.067 ± 33.8[a]	644.8 ± 61.2[b]
PR	38.067 ± 3.0[a]	17.667 ± 1.4[b]	42.333 ± 2.4[a]	24.467 ± 1.8[b]
Lactate	0.386 ± 0.0[a]	0.352 ± 0.0[a]	0.393 ± 0.0[a]	0.294 ± 0.0[b]
OC	0.128 ± 0.0[a]	0.129 ± 0.0[a]	-0.035 ± 0.0[a]	0.046 ± 0.0[b]
Glucose	11.819 ± 0.6[a]	14.956 ± 1.6[a]	5.453 ± 0.2[a]	6.007 ± 0.4[b]

*) The same superscript letter in the same row (sample group) indicates not significantly different (P > 0.05)

between sources (P < 0.05) (Table 3). The THCs of abalone from Wild Site 2 was significantly higher than Ranching Sites 2 and 3, but it was similar to Ranching Sites 1 and Wild Site 1 (Figure 2). In contrast, there were no significant differences in THCs of the winter-collected abalone either between sites (P > 0.05) or between sources (P > 0.05) (Figure 2). In this study, the haemocyte count in the winter samples might experience a decrease during transportation. However, the haemocyte count increase beyond the pre-treatment following 12 hours of water immersion. That pattern is similar to that described by Mahlam *et al*. (2003) for *H. tuberculata* when shaken under experimental conditions (Malham *et al.*, 2003).

One possible reason is that abalones are likely in a better health condition during winter leading to better immunity response mechanisms than that of in autumn. During the winter season, storms triggered by winter cold fronts passing across the country are causing big swells resulting in abundant food in the form of drifted algae available to abalone. The excess food also leads to high growth in the winter months (Melville-Smith *et al*., 2013). This explanation for seasonal differences in stress response is similar to a pattern that has been noted and reported on in bivalves (Santarem *et al*., 1992). Cajaraville *et al*. (1996) reported that fluctuations in temperature, salinity, and food availability in different seasons are likely the causes of variation in physiological responses in bivalves.

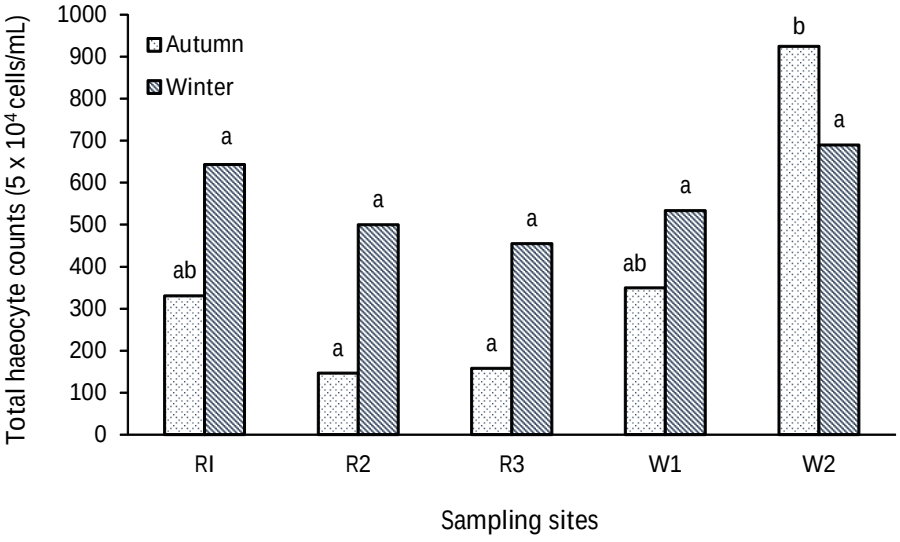

Figure 2. Different amounts of circulating haemocytes (5 x 10^4 cell/mL) in the haemolymph of ranched and wild-caught greenlip abalone measured after transportation during different seasons. The same letter on top of the bar within the same seasonal group indicates no significant difference.

The effect of transportation on phagocytic activities between the abalones collected in autumn and winter were similar. In both groups, an independent t-test indicated that the PR decreased significantly after transportation ($P<0.05$). The ability of haemocytes to phagocytose zymosan decreased from approximately 38% to 18% and from 42% to 24% in autumn and winter, respectively. Previous studies have indicated that physical stresses induce the release of stress hormones (catecholamines), such as noradrenaline (NA) and dopamine into haemolymph. This process can elevate the susceptibility of juvenile oyster (*Crassostrea gigas*) (Lacoste *et al*., 2001) and abalone *H. tuberculate* (Malham *et al.*, 2003) to pathogens. In the current study, the release of NA during transportation may have affected the immune responses of abalone by inhibiting the ability of haemocytes to phagocytose zymosan as a pathogenic agent.

During both autumn and winter samplings, the phagocytic rate after transportation decreased significantly, suggesting that this indicator is a sensitive tool to measure immune response in abalone. This implies that the detrimental effects of transportation on the immunomodulation of abalone is universal.

The lactate levels in the haemolymph samples of abalones collected in autumn were not affected by transportation procedures ($P>0.05$). However, the decrease in lactate levels in winter from 0.393 down to 0.294 mmol/L after transportation was shown by an independent t-test to be significantly different ($P<0.05$) (Table 4). The differences in lactate levels between sites (autumn, $P>0.05$; winter, $P>0.05$) and between sources (autumn, $P>0.05$; winter, $P>0.05$) were not significant for either sampling period of autumn or winter (Table 4). In the present study, the lactate levels in the haemolymph measured 12 hours after recovery were significantly below the control levels in the winter samples, but above the control levels in the auntumn samples. This reduction may indicate the transitional state of the abalone from a stressed to normal condition during recovery time.

The decrease of lactate levels in haemolymph due to stress conditions was also observed in a crab species, *Cancer pagurus* (Barrento *et al*., 2012). In that study, the lactate levels returned to control values within 72 hours after recovery initiated. Another study on the same species by Lorenzon *et al*. (2008) recorded the lactate levels taking 96 hours to return to pre-stress levels. Slower anaerobic metabolisms during transportation may cause a low level of metabolites, including lactate due to low temperatures, as indicated by Barrento *et al.* (2012).

The osmoregulatory capacity of autumn samples was not affected by transportation ($P>0.05$). However, the OC means of winter samples increased significantly from 0.035 to 0.046 Osmol/kg ($P<0.05$) (Table 2). The OCs of winter samples in all sites were similar ($P>0.05$), while autumn samples showed significant differences between sites ($P<0.05$). The significant differences between sites were depicted in Table 2. There was no significant difference in OCs between the ranch and wild-sourced abalones in both sampling periods of autumn and winter ($P>0.05$) (Table 4). To the best of our knowledge, the use of osmoregulatory capacity (OC) in this study as a transport-induced stress indicator for abalone is considered the first time. Previously, haemolymph osmolality was used to demonstrate the cadmium effect on osmoregulation of Chinese mitten crab (Silvestre *et al.,* 2005). OC has also been used to demonstrate stress responses of abalone and crustaceans in inland salinity water (Fotedar *et al.*, 2008; Prangnell & Fotedar, 2006; Tantulo & Fotedar, 2006). The increase of OCs after transportation in winter samples indicated the ability of abalones to osmoregulate their haemolymph during transport. In contrast, the OCs levels in autumn samples suggested that the abalo-

Table 3. Total haemocyte count (5 x 10^4 cell/mL); phagocytic rate (%); neutral red retention (min); lactate level (mmol/L); Osmoregulatory capacity (osmol/kg); glucose level (mmol/L) in the haemolymph of greenlip abalone between sources in two different sample group

Indicators	Autumn samples		Winter samples	
	Ranched abalone	Wild abalone	Ranched abalone	Wild abalone
THC	211.30 ± 39.3^a	636.75 ± 174.7^b	532.38 ± 41.8^a	611.25 ± 69.8^a
PR	29.611 ± 3.3^a	25.250 ± 3.8^a	34.111 ± 3.1^a	32.333 ± 3.1^a
Lactate oxidase	0.385 ± 0.0^a	0.344 ± 0.0^a	0.357 ± 0.0^a	0.325 ± 0.0^a
OC	0.127 ± 0.0^a	0.132 ± 0.0^a	-0.0002 ± 0.0^a	0.015 ± 0.0^a
Glucose level	13.703 ± 1.2^a	12.915 ± 1.2^a	5.628 ± 0.3^a	5.883 ± 0.3^a

The same superscript letter in the same sample group (row) indicates no significant difference

Table 4. Mean ± SE of phagocytic rate (%); lactate level (mmol/L); Osmoregulatory capacity (osmol/kg); glucose level (mmol/L) in the haemolymph of greenlip abalone (*H. laevigata*) between sites in two different sample groups

Indicators	Sites				
	Ranch site 1	**Ranch site 2**	**Ranch site 3**	**Wild site 1**	**Wild site 2**
Autumn samples					
THC	25.167 ± 4.1^{a}	31.833 ± 5.3^{a}	31.833 ± 7.9^{a}	25.833 ± 5.9^{a}	24.667 ± 5.2^{a}
PR	0.370 ± 0.0^{a}	0.388 ± 0.0^{a}	0.397 ± 0.0^{a}	0.359 ± 0.0^{a}	0.330 ± 0.0^{a}
Lactate oxidase	0.117± 0.0^{a}	0.142± 0.0^{c}	0.122± 0.0^{ab}	0.124±0.0^{abc}	0.139±0.0^{bc}
OC	15.211 ± 2.4^{a}	15.221 ± 2.4^{a}	10.675 ± 1.1^{a}	13.826 ± 2.3^{a}	12.005 ± 0.9^{a}
Winter samples					
THC	28.667 ± 3.9^{a}	35.0 ± 5.6^{a}	38.667 ± 6.4^{a}	32.333 ± 5.3^{a}	32.333 ± 3.7^{a}
PR	0.351 ± 0.0^{a}	0.402 ± 0.0^{a}	0.316 ± 0.0^{a}	0.330 ± 0.0^{a}	0.319 ± 0.0^{a}
Lactate oxidase	0.019 ± 0.0^{a}	-0.014 ± 0.0^{a}	-0.006 ± 0.0^{a}	0.0163 ± 0.0^{a}	0.013 ± 0.0^{a}
OC	5.883 ± 0.7^{a}	6.45 ± 0.5^{a}	4.55 ± 0.2^{a}	5.683 ± 0.4^{a}	6.083 ± 0.4^{a}

The same superscript letter in the same sample group (row) indicates no significant difference

nes had less ability to maintain their osmoregulation during transit. This finding confirms that winter samples had a relatively better defense mechanism due to the optimum winter environmental condition. This osmoregulation ability of winter samples is relatively the same among sites, both ranch and wild-sourced abalones. However, autumn samples had uneven ability in osmoregulation. The abalones in ranching site 1 showed less ability in osmoregulation while abalone in ranching site 2 showed the best osmoregulation ability within the seasonal group.

Glucose plays an important role in glycogen metabolisms in mollusks (Rossi & Da Silva, 1993). As an energy source, it allows the animals to maintain many physiological functions, including immunity (Day *et al*., 2010). In the present study, there was no effect of transportation on haemolymph glucose level in autumn and winter samples ($P > 0.05$). In addition, a similar pattern was observed between sites ($P > 0.05$) and between sources of both autumn and winter samples ($P > 0.05$). The glucose levels of autumn samples ranged from approximately 12 to 15 mmol/L, which were higher than those in winter samples (approximately 5 to 6 mmol/L). During stress conditions, organisms metabolises glucose in anaerobic condition (O'Omolo *et al*., 2003). The study of O'Omolo *et al*. (2003) postulated a significant reduction of glycogen concentration during transport simulation and exercise of *H. midae*. In the present study, the constant glucose level before and after transportation for either sampling periods suggests that this variable is less sensitive to determine abalone's immune response than the other indicators used in the study.

CONCLUSIONS

In conclusion, transportation could affect the immune functions of greenlip abalone (*H. laevigata*) in the form of immuno-stimulation and immune suppression. This study demonstrates that physical disturbance during transportation, particularly aerial exposure, contributes to some types of induced stress on live abalone. However, the extent of the transportation effects differs at different times of the year. The inconsistent trend results between the sites from the two sample sources occurred in this study. Therefore, any immediate concern should not point that ranched abalone at the study site were any differently stressed to those in the wild. We recommend further studies that consider multiple samplings within each season throughout a year. Such an arrangement might fully reveal the effects of handling stress on the immune modulation of green abalone in Flinders Bay, Augusta.

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