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Inland alkaline brackish water aquaculture of juvenile razor clam: Survival, growth, physiology and immune responses

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ABSTRACT

In this study, we investigated rearing and breeding razor clam (*Sinonovacula constricta*) in inland alkaline brackish water. During 45 days of the experiment in candidate target inland area brackish water (TBW), survival, growth, enzyme activity and immune responses were analysed. The results showed that shell length and weight were significantly inhibited in the TBW group (p <0.001). Although the cumulative mortality was 81.479 ± 7.028 ($p < 0.001$) at 15 days, it increased slowly to 92.915 ± 2.271 ($p < 0.001$) at 45 days. The enzyme activities of Na^+/K^+-ATPase, aspartate aminotransferase and superoxide dismutase peaked at 15 days, and juvenile *S. constricta* (JSC) may engaged in partial anaerobic metabolism or damage to gill tissue, which may explain the high mortality rate at this time. Additionally, the phagocytic ability of haemocytes was inhibited ($p < 0.001$), but the metabolic activity was enhanced ($p < 0.001$). This implies that 15 days was the peak of TBW stress, and stress gradually decreased by days 30 and 45. TBW affected metabolism, osmotic regulation, and immune responses. There was an independent ionic interaction perhaps effect on JSC, primarily through Na^+/K^+ rate. Approximately 7 % of animals adapted successfully to TBW after 45 days. In summary, *S. constricta* has a great potential in further anti-TBW conditions selective breeding research.

1. Introduction

Inland waters are usually separated into inland alkaline brackish water and freshwater. Saline alkali soil and inland alkaline brackish water are present in more than 100 countries (Shi, 2009). However, their use in crop cultivation and aquaculture is associated with extremely low yields (Qadir et al., 2001; Wang et al., 2011). Thailand was the first country to successfully breed *Litopenaeus vannamei*, and this highly tolerant species is now the most widely cultivated crustacean in the world (Roy et al., 2010). Although cultivation of molluscs such as *Mytilus edulis* (Dinh and Fotedar, 2016), *Haliotis laevigata* (Doupé et al., 2003), *Trochus niloticus* (Lee, 1997), *Crassostrea gigas* and *Sacco strea-glomerata* (Ingram et al., 2002) in inland alkaline brackish water has been reported, this is a relatively underexplored area.

China has abundant inland alkaline brackish water resources which ion composition and content fluctuate greatly depending on the region (Hui et al., 1997; Shentu et al., 2000). Salinity is the primary factor limiting the survival of economically important marine invertebrates in inland alkaline brackish water (Wang et al., 2011). Inland alkaline brackish water in the northwest of China is associated with high carbonate alkalinity (C_A) and pH values, and the main ionic components and proportions are quite different from normal seawater (Hui et al., 1997; Wang et al., 2015). The total alkalinity of carbonate-type waters is mainly due to CO_3^{2-} and HCO_3- ions (Liu et al., 2016). CO_3^{2-}, HCO_3-, OH- (pH), Na^+, K^+, Ca^{2+}, Mg^{2+} and SO_4^{2-} are the main ions in inland alkaline brackish water that affect the growth and survival of aquatic animals (Dinh et al., 2015; Partridege, 2008). Previous studies have shown that the main factor affecting the survival and growth of mussels and marine crustaceans is imbalance of the Na^+ and K^+ ratio (Perez-Velazquez et al., 2012). Ca^{2+} changes greatly in inland alkaline brackish water, participates in neurotransmission and osmotic pressure regulation, and plays a very important role in the growth of marine invertebrates, especially crustaceans and bivalves (Yang, 2011). Mg^{2+} is present in the active sites of many enzymes, acts as a co-factor in metabolism, and is

Abbreviations: C_A, Carbonate alkalinity; JSC, Juvenile *Sinonovacula constricta*; TBW, Candidate target inland area brackish water.

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important for the active transport of substances across the cell membrane. Additionally, other ions present in inland alkaline brackish water also affect the survival of aquatic animals in various ways. For example, SO_4^{2-} and Cl- ions are involved in the regulation of osmotic pressure in shrimp (Boyd, 2006), and toxic effects of some inland alkaline brackish water factors have been reported for *Cyclaina sinensis* (Lin et al., 2012).

Razor clam (*Sinnovacula constricta*) is an economically important bivalve that belongs to *Mollusca*, *Lamellibranchia*, *Heterodonta*, *Veneroida*, *Pharellidae*, *Sinonovacula*. It is widely distributed in the intertidal zone and estuary areas of the western Pacific. Annual output of this species is the 4th largest among marine bivalves in China. *S. constricta* is characterised by a short breeding cycle (Xie et al., 2015), the species can adapt to changing environmental conditions, and it can tolerate wide temperature and salt ranges (Liu et al., 2009).

Previous work explored the tolerance and physiological responses of Juvenile *S. constricta* (JSC) to major components in inland alkaline brackish water, including salinity (Peng et al., 2019a), carbonate alkalinity and pH (Peng et al., 2018), Na^+/K^+ (Peng et al., 2019b) and Ca^{2+} and Mg^{2+} (Peng et al., 2019c). However, whether JSC can survive or adapt to ion levels present in inland alkaline brackish water remains unknown. In other words, it is unknown whether the major ions present in inland alkaline brackish water act synergistically or antagonistically in JSC to mediate stress. This is important for aquaculture of *S. constricta* in inland alkaline brackish water. In the present work, an ion composition of inland alkaline brackish water from the Gansu area of northwest China (a candidate target area for *S. constricta* aquaculture) was simulated to formulate the artificial brackish water (TBW). The TBW was used in long-term stress experiments on JSC, and evaluated survival, growth, enzyme activities and haemocyte immune responses. Research in this work provide a theoretical basis for aquaculture of *S. constricta* in TBW, and breeding salt-tolerant *S. constricta* varieties.

2. Materials and methods

2.1. Animals and water parameters

Animals were treated according to the Guidelines for the Care and Use of Experimental Animals of the Institutional Animal Care and Use Committee of Shanghai Ocean University (IACUC-SHOU), Shanghai, China. Animals were not endangered, and only artificially propagated juveniles were employed in experiments.

JSC individuals (45-day-old) were obtained from Zhejiang Sanmen Donghang farm. In total, 1800 JSC of uniform shell length were selected with an average shell length of 1.379 ± 0.160 cm and body weight of 0.170 ± 0.05 mg. All JSC were acclimated for 3 days in 8 ppt seawater then in 6 ppt artificial seawater for 4 days before the test. During the test, the water temperature was maintained at 20–22 °C.

Artificial seawater was prepared by addition of AR-grade salts to distilled water using and artificial seawater B recipe (Shupin Zhu method; Table 1) (Yao et al., 2010; Zhu, 1980). The final test inland alkaline brackish water parameters were set based on the water quality measurement results (unpublished data) for TBW (C_A = 5.040 ± 0.681 mmol·L^{-1}, pH = 9.033 ± 0.681, Na^+/K^+ = 15.704 ± 1.934, Ca^{2+} = 4.170 ± 0.882 mmol·L^{-1}, Mg^{2+} = 19.670 ± 3.320 mmol·L^{-1}, and SO_4^{2-} = 21.040 ± 4.747 mmol·L^{-1}). The salinity of test water was 6 ppt, equal to that of most carbonate-type saline water in northwest China (Me et al., 2010; Yao et al., 2010). Total salinity was adjusted by altering NaCl and KCl. C_A was adjusted by altering Na_2CO_3 and $NaHCO_3$, and pH was adjusted with 0.1 mol L^{-1} HCl and 0.1 mol L^{-1} NaOH. C_A was measured by acid-base titration with phenolphthalein and Methyl Orange and Aniline Blue as indicators (Xiao et al., 2004).

2.2. Long-term stress experiments

Beach mud was used in the long-term stress test, and was prepared as described in previous work (Peng et al., 2018a). After preliminary acclimation in TBW for 7 days, JSC were transferred to a tank (40 × 40 × 65 cm). Each tank contained 10.4 L of test mud, 12 L of test water, and 300 JSC. Three tanks (three replicates) were prepared for the control group (artificial seawater) and the TBW stress groups, respectively. In each group, JSC were acclimated gradually over 7 days (Table 2). Water was changed completely every day to avoid CO_2 in the air affecting pH and C_A. Before feeding, mixture microalgae (*Chaetoceros calcitrans* and *Platymonas subcordigoramis*, 1:1 rate mix) was separated by centrifugation (1000 × *g* for 10 min) and resuspended twice in test water (Heasman et al., 2000). The concentration of mixture microalgae was adjusted to 400–480 cells μL^{-1} in feed-liquid. A 1.2 L volume of test water was replaced with a same volume of feed-liquid for single feeding, and fed thrice per day (Zhu et al., 2010). Sampling was performed on day 0, 15, 30 and 45 to calculate cumulative mortality, shell length, body weight, oxygen consumption, metabolic activity of haemocytes, Na^+/K^+-ATPase, aspartate aminotransferase, superoxide dismutase enzyme activities, and phagocytic ability.

Table 1
Artificial seawater recipe (Zhu, 1980).

Reagents	Dosage (mg·L^{-1})	Dosage (mmolL^{-1})
NaCl	4,143.01	70.89
$MgCl_2$	879.03	9.23
Na_2SO_4	691.23	4.87
$CaCl_2$	190.03	1.71
KCl	178.99	2.40
$NaHCO_3$	33.88	0.40
KBr	16.94	0.14
H_2BO_3	4.59	0.075
$Al_2(SO_4)_3$	0.53	0.0015
$BaCl_2.2H_2O$	0.016	6.50E-05
$LiNO_3$	0.18	0.0026
$SrCl_2$	4.24	0.027
NaF	0.53	0.017
$NaNO_3$	8.82	0.10
Na_2HPO_4	0.88	0.0062
$Na_2SiO_3.9H_2O$	1.76	0.0062
$MnCl_2$	0.035	0.00028
$FeC_6H_5O_7.3H_2O$	0.095	0.00032
$CuSO_4.5H_2O$	0.0071	2.83E-05
$ZuSO_4.7H_2O$	0.0018	6.14E-06
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0044	3.57E-06

2.3. Oxygen consumption rate and metabolic activity

The method for measuring oxygen consumption rate was performed as previously reported (Peng et al., 2018). Oxygen consumption rate was measured in a sealed conical flask (Fan et al., 2002). A WTW Multi 3420 Set G dissolved oxygen analyser (Xylem Inc., Germany) was used to determine dissolved oxygen in test water. The fifteen JSC of each replicate was used to measure the oxygen consumption rate for 20 times. Each time test ended with a minimum oxygen concentration that tended to be stable (In all measurements, the end point of oxygen level is greater than that 70.5 % of initial point), and then the values were used for calculation oxygen consumption rate.

At day 0, eighteen JSC in each group were randomly selected to separate three pools (six JSC each pool) to collect hemolymph. Six JSC hemolymph in each repeat were pooled at day 15, 30 and 45, respectively. To collect hemolymph, the shell was peeled off with a disinfected blade to expose the pericardium, and a sterile needle was directly inserted into the pericardial cavity. Uniform JSC hemolymph suspension was obtained using a 40 μm diameter tissue sieve (Peng et al., 2019d). Metabolic Co-factors $NAD(P)^+$/NAD(P)H (dehydrogenase activity) of hemocytes was measured using a Cell Counting Kit-8 (CCK-8; Dojindo, Japan). For the test tube, hemolymph suspension 20 μL + TBS buffer 180 μL + CCK-8 reagent 20 μL, incubated at 28 °C for 3 h. For the blank tube, serum 20 μL + TBS buffer 180 μL + CCK-8 reagent 20 μL, incubated at 28

Table 2
Design of the gradual acclimation program for the long-term stress test. The conditions of the control group "6 ppt salinity, 0.51 ± 0.09 mmol·L^{-1} C_A, 8.2 pH, 31.91 Na^+/K^+, 1.71 mmol·L^{-1} Ca^{2+}, 9.23 mmol·L^{-1} Mg^{2+} and 4.87 mmol·L^{-1} SO_4^{2-}" remained constant throughout during acclimation. The partial conditions of the TBW group "6 ppt salinity, 9.03 pH, and 15.7 Na^+/K^+" remained constant throughout during acclimation. The inconstant ions in the acclimation process is listed in the table below.

Acclimation time (days)	TBW group C_A (mmol·L^{-1})	Ca^{2+}(mmol·L^{-1})	Mg^{2+}(mmol·L^{-1})	SO_4^{2-}(mmol·L^{-1})
D1	0.72	0.57	2.81	3.00
D2	1.44	1.14	5.62	6.00
D3	2.16	1.71	8.43	9.00
D4	2.88	2.28	11.24	12.00
D5	3.6	2.85	14.05	15.00
D6	4.32	3.42	16.86	18.00
D7	5.04	4.17	19.67	21.04

°C for 3 h. The optical density (OD) value was measured using a microplate reader (Shanghai Dynamax Biotech Company, China) at 450 nm. OD value and total number of hemocytes (obtained from the phagocytosis test) were used to calculate relative activity of energy metabolism, and the unit represented as OD value per million hemocytes.

2.4. Measurement of enzyme activities during long-term stress

Twelve JSC in each group were randomly selected to measure enzyme activity at day 0. Six JSC were selected from each replicate to measure enzyme activity at day 15, 30 and 45. Shells were removed and soft tissue was homogenised with normal saline (tissue weight: normal saline =1 g: 9 mL). Homogenised samples were centrifuged (2000 × *g* for 10 min) and the supernatant was retained. Total protein in the supernatant was measured using a Coomassie Brilliant Blue Total Protein Assay kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Na^+/K^+-ATPase, aspartate aminotransferase and superoxide dismutase enzyme activities were tested according to the instructions of the appropriate kits as previously described (Peng et al., 2018). The units for each enzyme activity were as follows: Na^+/K^+-ATPase = μmolPi·mgprot^{-1}·h^{-1}, aspartate aminotransferase = μmol·mgprot^{-1}·min^{-1}, and superoxide dismutase = μmol·mgprot^{-1}·min^{-1}.

2.5. Phagocytic ability

After hemolymph collection, 20 μL of JSC haemocyte suspension was diluted to 100 μL with TBS, and 10 μL of a 1/10 dilution of carboxylate-modified microspheres (diameter 1 μm, yellow-green fluorescent FluoSpheres; Invitrogen) was added and incubated in the dark at room temperature for 1 h (Wang et al., 2014). Phagocytosis of haemocytes was measured using a BD C6Plus flow cytometer (BD Biosciences, USA). The haemocyte cell density was measured by without fluorescent microspheres for each group using flow cytometry with a Gate A threshold (cells only) based on the relative size of haemocytes (FSC value) and the FITC fluorescence intensity (FITC value).

2.6. Calculation and data analysis

Parameters were calculated as follows:

Cumulative mortality (%) = $100 \times N_f / N_i$

where N_f and N_i are the final number of dead JSC and initial number of live JSC, respectively.

Per-day shell length growth rate (cm day^{-1}) = $[L_f - L_i] / T$

where L_f, L_i and T are the final average length of JSC, initial average length of JSC and time, respectively.

Per-day weight gain rate (g day^{-1}) = $[W_f - W_i] / T$

where W_f, W_i and T are the final average wet body weight of JSC, initial wet body weight of JSC and time, respectively.

Oxygen consumption rate (mgO_2·g^{-1}·h^{-1}) = $[A_i - A_f] * V / [W * T]$

where A_i, A_f, V, W and T are the initial oxygen concentrations, final oxygen concentrations, volume of test water, wet body weight and test time, respectively.

Phagocytosis rate (%) = $(H_t - H_u)/H_t \times 100$

where H_t and H_u are the total hemocytes and non-phagocytic hemocytes, respectively.

Relative metabolic activity (OD·million hemocytes^{-1}) = $(OD_{tt} - OD_{bt}) / H_t \times 1000{,}000$

where OD_{tt}, OD_{bt} and H_t are the OD value of test tube, the OD value of blank tube, and the total hemocytes, respectively.

2.7. Statistical analysis

SPSS 19.0 software (IBM, USA) was used for statistical analysis. Significant differences were determined using a students *t*-test. The results were plotted using SigmaPlot 12.3 software (Systat Software Inc., USA).

Table 3
Growth and cumulative mortality of JSC in different treatment groups.

Parameters	Groups	Rearing time			
		0-day	15-day	30-day	45-day
Shell length (cm)	CK	1.379 ± 0.160	1.742 ± 0.186	1.963 ± 0.205	2.145 ± 0.199
	TBW		1.380 ± 0.102**	1.508 ± 0.123**	1.728 ± 0.083**
Body weight (g)	CK	0.170 ± 0.050	0.288 ± 0.049	0.442 ± 0.045	0.529 ± 0.043
	TBW		0.202 ± 0.053*	0.287 ± 0.058**	0.437 ± 0.040**
Cumulative mortality (%)	CK	—	0.333 ± 0.577	1.333 ± 1.155	1.667 ± 1.528
	TBW		81.479 ± 7.028**	92.028 ± 2.765**	92.915 ± 2.271**

3. Results

3.1. Effect of TBW stress on the survival and growth of JSC

The results showed that the cumulative mortality under TBW stress was significantly higher than control group at 15, 30 and 45 days ($p < 0.001$; Table 3).

The results showed that per day shell length rate was significantly inhibited by TBW stress at 15 and 30 days ($p < 0.001$) (Fig. 1A), and per day body weight growth was significantly inhibited by TBW stress at 15 ($p < 0.001$) and 30 days ($p < 0.05$) (Fig. 1B). On the contrary, the daily growth rate (shell length and body weight) of the TBW group were not significantly different from that of the control group at 45 days.

3.2. Oxygen consumption rate and metabolic activity

As shown in Fig. 2, oxygen consumption for the TBW group was significantly lower at 15 days ($p < 0.001$), but it was significantly increased at 30 and 45 days ($p < 0.05$).

Fig. 3 shows metabolic activity values for TBW and control groups. The results show that the metabolic activity of TBW group was peaked at 15 days. Value of TBW group was significantly higher than that of the control group at 15, 30 and 45 days ($p < 0.001$).

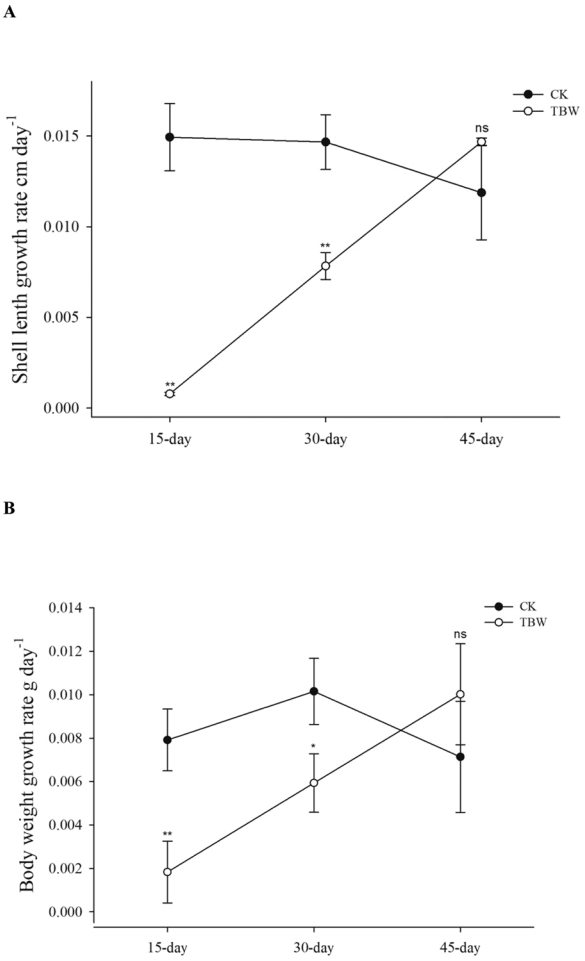

Fig. 1. Shell length growth of juvenile *S. constricta* (JSC) (A) and body weight of JSC (B) under candidate target inland area brackish water (TBW) stress. Lines (mean ± SE, n = 3) with an asterisk denote a significant difference (**$p < 0.001$, *$p < 0.05$, $^{ns}p > 0.05$) between the control group (seawater) and the test group (TBW).

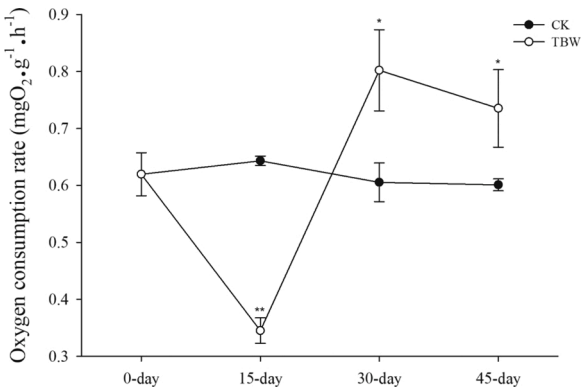

Fig. 2. Oxygen consumption rate of juvenile *S. constricta* (JSC) in the long-term candidate target inland area brackish water (TBW) stress test. Bars (mean ± SE, n = 3) with an asterisk denote a significant difference (*$p < 0.05$, **$p < 0.001$) between the control group (seawater) and the test group (TBW).

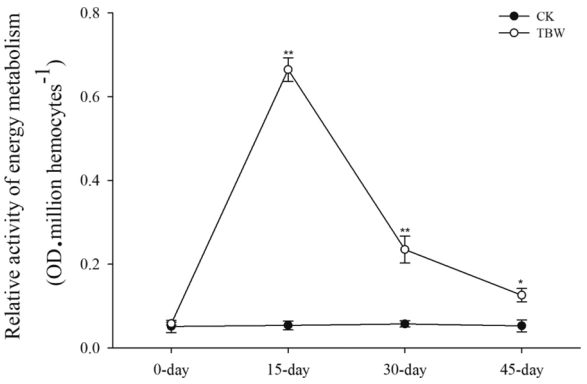

Fig. 3. Metabolic activity of juvenile *S. constricta* (JSC) in the long-term candidate target inland area brackish water (TBW) stress test. Bars (mean ± SE, n = 3) with an asterisk denote a significant difference (*$p < 0.05$, **$p < 0.001$) between the control group (seawater) and the test group (TBW).

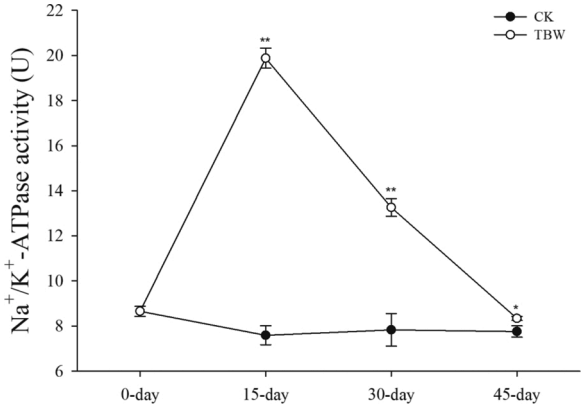

Fig. 4. Na^+/K^+-ATPase activity of juvenile *S. constricta* (JSC) in the long-term candidate target inland area brackish water (TBW) stress test. Bars (mean ± SE, n = 3) with an asterisk denote a significant difference (*$p < 0.05$, **$p < 0.001$) between the control group (seawater) and the test group (TBW).

3.3. Enzyme activities and phagocytic ability under long-term stress

The results in Fig. 4–6 show that the Na^+/K^+-ATPase, aspartate aminotransferase, and superoxide dismutase activities were peaked by TBW stress at 15 days ($p < 0.001$), and then gradually declined at 30 ($p < 0.001$) and 45 days ($p < 0.05$), in TBW group.

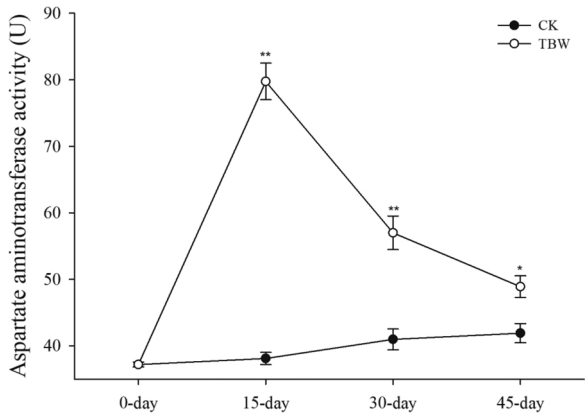

Fig. 5. Aspartate aminotransferase activity of juvenile *S. constricta* (JSC) in the long-term candidate target inland area brackish water (TBW) stress test. Bars (mean ± SE, n = 3) with an asterisk denote a significant difference (*$p < 0.05$, **$p < 0.001$) between the control group (seawater) and the test group (TBW).

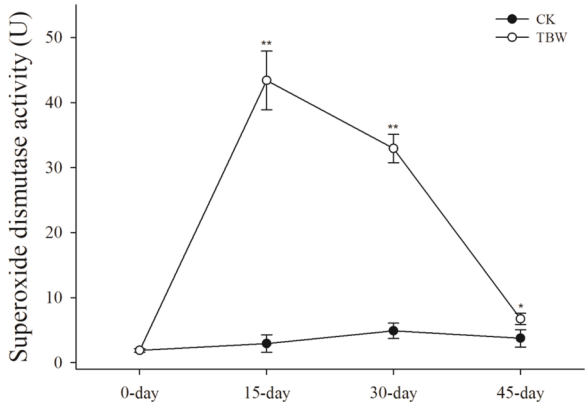

Fig. 6. Superoxide dismutase activity of juvenile *S. constricta* (JSC) in the long-term candidate target inland area brackish water (TBW) stress test. Bars (mean ± SE, n = 3) with an asterisk denote a significant difference (*$p < 0.05$, **$p < 0.001$) between the control group (seawater) and the test group (TBW).

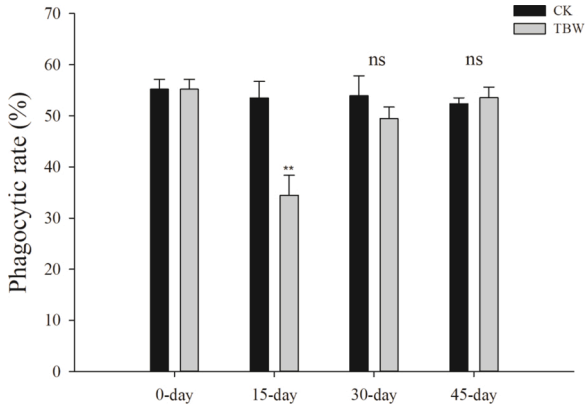

Fig. 7. Phagocytic ability of juvenile *S. constricta* (JSC) in the long-term candidate target inland area brackish water (TBW) stress test. Bars (mean ± SE, n = 3) with an asterisk denote a significant difference (*$p < 0.05$, **$p < 0.001$, $^{ns}p > 0.05$) between the control group (seawater) and the test group (TBW).

Fig. 7 shows that phagocytic ability of the TBW group was significantly decreases at 15 days ($p < 0.001$). However, there were no significant differences between control and TBW groups at other timepoints.

4. Discussion

Inland alkaline brackish water is yet to be fully exploited for aquaculture, and addressing this is a major focus. Among the ions in inland alkaline brackish water, CO_3^{2-} and HCO_3- (C_A), OH- (pH), Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl- and SO_4^{2-} can all affect the growth and development of farmed animals (Partridge et al., 2008; Dinh et al., 2015). In previous study, the tolerance scope of *S. constricta* to salinity was reported to be greater than that in the inland alkaline brackish water in China (Peng et al., 2019a). Thus, in the present study, salinity was maintained constant, while CO_3^{2-} and HCO_3- (C_A), OH- (pH), Na^+, K^+, Ca^{2+}, Mg^{2+} and SO_4^{2-} ions were varied. In addition, TBW acclimation was performed before long-term testing because it may enhance survival under TBW conditions. Previous studies showed that shifting directly from 30 ppt to 5 ppt without adapting to the environment can lead to excessive mortality (Kumlu and Jones, 1995). Jayasankar et al. (2009) found that prolonging the acclimation time can reduce stress caused by osmotic pressure, and the pressure to adapt to the environment is greater, which can boost survival rate. In a preliminary experiment in which JSC were acutely stressed under TBW conditions, we found that the survival rate was only ~2 % at day 7. After a 7-day acclimatisation gradient, the greatest mortality rate increase was observed after 15 days of TBW stress (~18.5 % survival rate), and adaptation to TBW was good after 45 days (~7.1 % survival rate). This implies that *S. constricta* is not suitable for direct migration to TBW aquaculture. But individuals who survived at high elimination intensity can be used as broodstock for selective breeding studies to obtain high survival rate of progeny populations.

Shell length and weight were significantly inhibited ($p < 0.001$) throughout the experiment (Table 3), but the growth rate of TBW treated group continued to rise with time (Fig. 1). This was similar to the growth of *L. vannamei* observed previously in a similar study (Laramore et al., 2001). Some JSC individuals have the ability to adapt to the ionic strength of TBW, as demonstrated in our previous research on single ion analysis (Peng et al., 2018, 2019b; Peng et al., 2019c). This suggests that TBW stress significantly inhibited JSC growth over a short time period, but this can be alleviated slowly by surviving individuals after 15 days. In other words, these surviving individuals who keeping growth can be used for subsequent breeding research (tolerate TBW water).

Oxygen consumption rate is one of the most important indicators of animal metabolism. The stress of ion changes in water can significantly increase metabolism and oxygen consumption rate in aquatic animals (Portz et al., 2006). In the present study, oxygen consumption rate was significantly lower than in control groups in the first 15 days, but was significantly higher than control groups after 30 and 45 days. Although, it was observed that shells were tightly closed at the first 15 days, from the oxygen consumption data, the clams did not completely cut off the exchange with the environment, which may imply that JSC individuals attempted to maintain homeostasis balance for survival in TBW ionic conditions by altering their metabolism.

Na^+/K^+-ATPase is a common transmembrane protein that directly participates in the regulation of intracellular Na^+ concentration and provides energy for ion regulation (Hirose et al., 2004). Fig. 4 shows that Na^+/K^+-ATPase activity was significantly higher in TBW groups than controls groups after 45 days, and similar results have been reported previously for tilapia, pufferfish, milkfish and shrimp when stimulated in seawater (Gao et al., 2016; Jorgensen et al., 2008; Lin et al., 2005; Verbost et al., 1994). As isotonic animal, the euryhaline bivalve change rapidly of body fluids when the environmental ionic strength changes. In our subsequent transcriptome study, it was also proved that the stress of TBW conditions promote Na^+/K^+-ATPase gene to be significantly up-regulated in multiple tissues (unpublished work). It suggest that activity of Na^+/K^+-ATPase be increased accordingly to regulate the ionic balance and recover normal physiology in body (Geng et al., 2011).

When aquatic animals respond to stress, a large amount of energy is

needed to activate various regulatory processes. *S. constricta* typically displays an increase in energy consumption and an increase in energy metabolism-related enzyme activities in response to ionic stress. Due to previous research, we believe that ion-regulated enzymes and the energy required for metabolism rate changes have led to increased enzyme activity such as aspartate aminotransferase (Kirsch, 1984). We also observed a significant increase in aspartate aminotransferase and oxygen consumption was much lower in experimental groups than in control groups at 15 days. Despite shell closure, the metabolic activity of haemocytes (Fig. 3), Na^+/K^+-ATPase (Fig. 4) and superoxide dismutase activities (Fig. 6) in clams under TBW stress for 15 days suggest the anaerobic metabolism or damaged microstructure of gill tissue may were employed in here. Zwaan and Wijsman (1976) also found that shellfish may engage in partial anaerobic metabolism and/or new types of metabolism under certain conditions. For example, *L. vannamei* displays changes in aspartate aminotransferase activity that are consistent with growth rate (Jin et al., 2017). However, aspartate aminotransferase activity and growth were not consistently correlated in our ion stress experiments. Based on recent transcriptomics results (unpublished), we speculate that the increase in aspartate aminotransferase activity under TBW stress may not only be related to the changes of metabolism, but also to the large amount of free amino acids being produced for cell osmotic regulation.

Changes in superoxide dismutase activity can reflect the environmental stress, immune ability and metabolism of mollusc (Guo et al., 2015; Hermes-Lima and Storey, 1995; Wang et al., 2013). When animals are exposed to stress, superoxide dismutase activity can increase to scavenge oxygen free radicals in the surrounding water, thereby maintaining oxidative balance in the body (Li, 2017). For example, when *Ruditapes philippinarum* is exposed to low salinity, superoxide dismutase activity is significantly increased (Nie et al., 2020). In the present work, superoxide dismutase activity was significantly increased at 15 days, then it decreased thereafter. This trend was observed previously in *Apostichopus japonicas* (Dong et al., 2013).

In addition, phagocytosis of haemocytes is an important defence mechanism in invertebrates including bivalves, but phagocytic ability is also easily affected by environmental stress (Pressley et al., 1981). In previous studies, decreases in haemocyte phagocytosis were observed in *Haliotis varia* under environmental stress (vibration stress) (Malham et al., 2003), and clams *Anodonta woodiana* and *S. constricta* exposed to high pH and high C_A also decreased phagocytic ability in haemocytes (Li et al., 2000; Ye et al., 2019). In the present study, phagocytic ability was significantly decreased in TBW groups at 15 days. This, haemocyte phagocytosis may be inhibited in JSC under strong ionic stress, such as when experiencing TBW conditions. We speculate that adhesion and aggregation functions may be limited (Chen and Bayne, 1995). The results for superoxide dismutase and phagocytic ability suggest that the effects of TBW on the immune system of JSC may not be unilateral. Indeed, there may be activation via a respiratory burst and inhibition of the cellular immune system, but this special immune response can be adapted.

In summary, JSC under TBW stress displayed similar responses to those observed in previous studies on single ions. Importantly, not all JSC individuals died under TBW stress (the survival rate was ~8% at 30 days). Compare previously published data (Peng et al., 2018, 2019b; Peng et al., 2019c), this survival rate is close to the effect of Na^+/K^+ on JSC (the survival rate was ~13 % at 30 days), but lower than the effects of other ions (at 30 days the survival rate of C_A-pH, Ca^{2+} and Mg^{2+} were ~92 %, ~75 % and ~50 %, respectively). Therefore, after comparing the results, we speculate that there may be weak or relatively independent interactions between the main ionic components in TBW, and the Na^+/K^+ ratio may be the most important limiting factor. Animals transferred to TBW initially suffered a high elimination rate (~93 %), but surviving clams make future selective breeding experiments possible. "Short-term TBW stress can inhibit the growth of JSC, phagocytic abilities of hemocytes, and response of Na^+/K^+-ATPase, aspartate aminotransferase, superoxide dismutase, and metabolic activities. Surviving animals will slowly adapt to stress conditions. Therefore, *S. constricta* has a strong potential in selective breeding research, that is, to obtain progeny populations with higher survival rate under TBW conditions.

Author statement

The manuscript has been approved by all listed authors, is novel and has not been submitted to any other journals.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2020.100463.

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