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Time Course Effect of Low Salinity on the Plasma Osmotic Pressure, Ion Concentrations and Na+/K+-ATPase Activity in the Gill of Juvenile Lined Seahorse, *Hippocampus erectus*

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Abstract

The present study is to evaluate the osmoregulatory ability of the lined seahorse *Hippocampus erectus*, a valuable species for traditional Chinese medicine. The effect of low salinity (10‰, 15‰, 20‰, 25‰, 32‰ as control) on the plasma osmotic pressure, ions concentration including Na^+, K^+, Ca^{2+} and Cl^-, and Na+/K+-ATPase (NKA) activity in the gill of the juvenile seahorses was carried out within 96 h. The results show that plasma osmotic pressure and ions concentration down-regulated significantly with the decreasing salinity at 6 h to 12 h and stabilized at 12 h to 24 h after the salinity stress. The isotonic point of the juvenile seahorses was 317.13 m $Osm \cdot kg^{-1}$ after 96 h transfer, the equivalent of the salinity of 12.05‰, meanwhile, isoionic points of Na^+ and Cl^- were 96.48 $mmol \cdot L^{-1}$ and 113.64 $mmol \cdot L^{-1}$ after 96 h transfer, equivalent to the salinity of 8.82‰ and 10.13‰, respectively. Moreover, the gill NKA activity also down-regulated significantly with the decreasing salinity and reached the lowest value at 12 h to 24 h after transfer to hypotonic water, then stabilized even elevated at 48 h. Juvenile *H. erectus*, is able to stabilize the osmotic pressure and ions concentration at a short time (12 h) after the salinity stressing, suggesting that the species have a strong osmoregulation ability. Moreover, the isotonic point, the equivalent of the salinity of 12.05‰, combined with the gill NKA activity recovered at 24 h after the salinity stress at the salinity of 15‰ and our previous survival and growth data, implying that the juvenile seahorse could be cultured optimally at salinities of 10‰ to 15‰.

Keywords: Seahorse; *Hippocampus erectus;* Salinity; Osmoregulation; Na+/K+-ATPase

Introduction

Salinity influences a series of physiological and biochemistry process in fishes, such as osmotic adjustment [1-7]. Osmoregulation is a major way for aquatic animals to adapt the salinity change [2,4,5,7]. Marine teleost fishes need ability for ion- and osmoregulation, so that they can maintain a stable homeostasis. For aquaculturists, understanding the osmoregulatory ability of the cultured animals is important to develop aquaculture protocols.

Na+/K+-ATPase (NKA) is a trans-membrane protein to exchange K^+ and Na^+ ions [8], which is a primary active pump [9,10]. Gills are the most important organs responsible for osmoregulation in fish [11,12]. In the gill, NKA is located mainly in epithelial mitochondria-rich cells [13]. NKA is responsible for active transport of Na^+ out of and K^+ into fish cells, so that it is important for sustaining intracellular homeostasis and providing a driving force for ion transporting systems, including fish gills and kidneys [14,15].

Significant breakthroughs in the commercial culture of seahorses have only occurred in the past 10 years since all 52 recognized seahorse species were listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora as of March, 2016. This has in part been the result of increasing culture effort to meet growing demand in traditional Chinese medicine (TCM) and ornamental market due to overexploitation of the wild populations [16]. To date, more than 10 seahorse species have been reared successfully in captivity [17]. Although a big progress in technique of rearing seahorses has been made, variation in juvenile survival is still high. The variation in juvenile survival may result from intrinsic juvenile quality [18] and extrinsic factors, such as environment factors, food, and management protocols [19-22].

The lined seahorse (*Hippocampus erectus*) is a highly-valued species in both TCM and aquarium trades [16,20,23]. In natural environment, lowest salinity of the distribution is about 10‰ [24]. Although the lined seahorse has become an important commercial culture species, we still know very little about its osmoregulatory ability. To date, only growth performance of the species in a narrow salinity range of 27‰ to 35‰ was reported [20]. In the previous study, we determined the effect of salinity on the growth and survival of the species over a broad salinity range of 5‰ to 32‰ as reported by Zhang et al. (unpublished data). In the present study, to improve the culture protocols, the osmoregulatory ability of the juvenile seahorse, including the plasma osmotic pressure, plasma ion levels (Na^+, K^+, Ca^{2+} and Cl^-), and NKA activity in the gill of the juvenile seahorses, was investigated. Meanwhile, isotonic point was determined as well, because it is a good implication to aquaculturists. Generally, the energetic cost of osmoregulation is the lowest in iso-osmotic conditions, and these energy savings are allocated to increase growth [25].

Materials and Methods

Juvenile seahorses

Twenty pairs (one male and one female) of F_3 generation of *H. erectus* were selected as the broodstock for producing juveniles at the Qionghai Research Center of East China Sea Fisheries Research

Institute, Hainan, China. Upon newborn juveniles being released, they were collected immediately and transferred to the raising tanks (50 × 30 × 30 cm). Juveniles were reared at a stocking density of 3 individuals L^{-1} (80 juveniles per tank). Each flow-through tank contained 30 L seawater with gentle aeration and flow rate of 0.2 L min. Nylon nets were provided for holdfasts for the juveniles. The juveniles were fed twice at 0800 and 1500 h each day with copepods at approximately 15 individual's mL^{-1}. Before each feeding, the bottom of the tanks was siphoned to remove faeces and dead food. Salinity, temperature, light intensity, and photoperiod were 31‰ to 33‰, 28.0 ± 0.5°C, 2000 ± 300 lx, 14L/10D, respectively. Salinity, temperature, and light intensity were measured daily with refractor meter, thermometer, and lux meter, respectively.

Salinity stress

Four salinities, 10‰, 15‰, 20‰ and 25‰, were tested. The waters were prepared by adding distilled water to natural seawater (32‰) which was employed as control. The tests were conducted in 20L white plastic buckets containing 15 L of the experimental salinity seawater, each with 3 replicates. The juvenile seahorses (total length: 5.0 ± 0.20 cm) were transferred into the buckets containing the experimental water from the juvenile rearing system, each bucket with 30 juveniles. Fifty percent of the experimental water was changed daily. If sampling, water change was conducted after sampling. The experiment lasted for 96 h. The experimental seahorses were fed copepods at approximately 15 individuals/ml. The cultured conditions were the same as for the juvenile seahorses. Three individuals from each bucket were randomly sampled at the time point of 0 h, 1 h, 6 h, 12 h, 24 h, 48 h and 96 h. There was no mortality in any of experimental treatments during the experiment.

Sample preparation and analysis

The sampled seahorses were placed into a bucket containing a solution of 0.035% MS-222 (Sigma-Aldrich, Castle Hill, NSW, Australia) in experimental salinity water and anaesthetized for 2 min, then the seahorses were rinsed three times with distilled water, and the body surface was dried with filter paper. Afterwards, they were decapitated and the blood was collected with a capillary. For individual replicate, 3 seahorses were sampled at each time point, except 0 h. The blood was centrifuged at 10,000 rpm for 10 min at 4°C. Then 250 µl of the plasma was collected and stored under -40°C until for the use of osmotic pressure and ion concentration examination. Plasma osmotic pressure (m Osm·kg^{-1}) was determined with VAPRO osmotic pressure dew point meter (VAPRO-5520, Wescor, Inc., Logan, Utah, USA). Plasma cations (Na$^+$, K$^+$, and Ca^{2+}) and anion (Cl$^-$) concentrations were determined with Radiometer ABL80 (Diamond Diagnostics, Hollinston, MA, USA). The isosmotic and isoionic points were estimated as the intersect of the isosmotic or isoionic lines and the regression lines of plasma and water osmolality or ionic concentrations at 96 h after transfer.

After blood sampling, the gills were collected as well. The gills were homogenized with 0.68% NaCl on the ice. The homogenate was centrifuged at 2500 rpm for 10 min at 4°C. The supernatant was obtained for NKA activity measurement. Gill NKA activity was measured using the commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China) following the manufacturer's instructions, and was determined at 636 nm using Epoch microplate spectrophotometer (BioTek, Winooski, Vermont, USA). One unit of NKA activity is defined as ATP enzyme in one milligram tissue protein breaks down ATP to produce 1 µmol inorganic phosphorus in one hour. Total protein concentration was measured using Coomassie brilliant blue method with bovine serum albumin as the standard.

Statistical analysis

All data were expressed as the mean ± SD and analyzed using SPSS17.0 statistical software (IBM SPSS). Prior to the analysis, normality of the data was evaluated using the Shapiro–Wilk's W-test and homogeneity of variances was assessed. Two-way analysis of variance (ANOVA) was used to evaluate the differences among the treatments over time course followed with LSD multiple comparison test if ANOVA results are significant.

Results

Effect of salinity decline on the ions concentration of the lined seahorse juveniles

Generally, the plasma Na$^+$ ion concentration of the seahorse juveniles gradually decreased with salinity decline (Figure 1). The plasma Na$^+$ concentration among the salinity groups was significantly different (Two-way ANOVA, $F_{[4,104]}$ = 948.109, P<0.001), The plasma Na$^+$ concentration changed significantly (Two-way ANOVA, $F_{[6,104]}$ = 388.574, P<0.001) over 96 h after transfer.

Generally, the plasma K$^+$ ion concentration of the seahorse juveniles gradually decreased with salinity decline (Figure 1). The plasma K$^+$ concentration among the salinity groups was significantly different (Two-way ANOVA, $F_{[4,104]}$ = 82.361, P<0.001), The plasma K$^+$ concentration changed significantly (Two-way ANOVA, $F_{[6,104]}$ = 40.720, P<0.001) over 96 h after transfer.

Generally, the plasma Ca^{2+} ion concentration of the seahorse juveniles gradually decreased with salinity decline (Figure 1). The plasma Ca^{2+} concentration among the salinity groups was significantly different (Two-way ANOVA, $F_{[4,104]}$ = 54.151, P<0.001), The plasma Ca^{2+} concentration changed significantly (Two-way ANOVA, $F_{[6,104]}$ = 20.954, P<0.001) over 96 h after transfer.

The plasma Cl$^-$ ion concentration of the seahorse juveniles gradually decreased with salinity decline (Figure 1). The plasma Cl$^-$ concentration among the salinity groups was significantly different (Two-way ANOVA, $F_{[4,104]}$ = 191.499, P<0.001), The plasma Cl$^-$ concentration changed significantly (Two-way ANOVA, $F_{[6,104]}$ = 62.102, P<0.001) over 96 h post transfer.

Effect of salinity decline on osmoregulation and isosmotic of the juveniles

The plasma osmotic pressure of the seahorse juveniles gradually decreased with salinity decline (Figure 2). The plasma osmotic pressure among the salinity groups was significantly different (Two-way ANOVA, $F_{[4,104]}$ = 389.447, P<0.001), The plasma osmotic pressure changed significantly (Two-way ANOVA, $F_{[6,104]}$ = 123.394, P<0.001) over 96 h after transfer.

The plasma isosmotic point of the juveniles was 317.13 m Osm·kg^{-1} and corresponded to 12.05‰ salinity (Figure 3). The plasma isoionic points for Na$^+$ and Cl$^-$ were 96.48 mmol·L^{-1} and 113.64 mmol·L^{-1} (Figure 3), respectively, which corresponded to salinities of 8.82‰ and 10.13‰, respectively.

Effect of salinity decline on gill NKA activity of the lined seahorse juveniles

Gill NKA activity of the seahorse juveniles gradually decreased
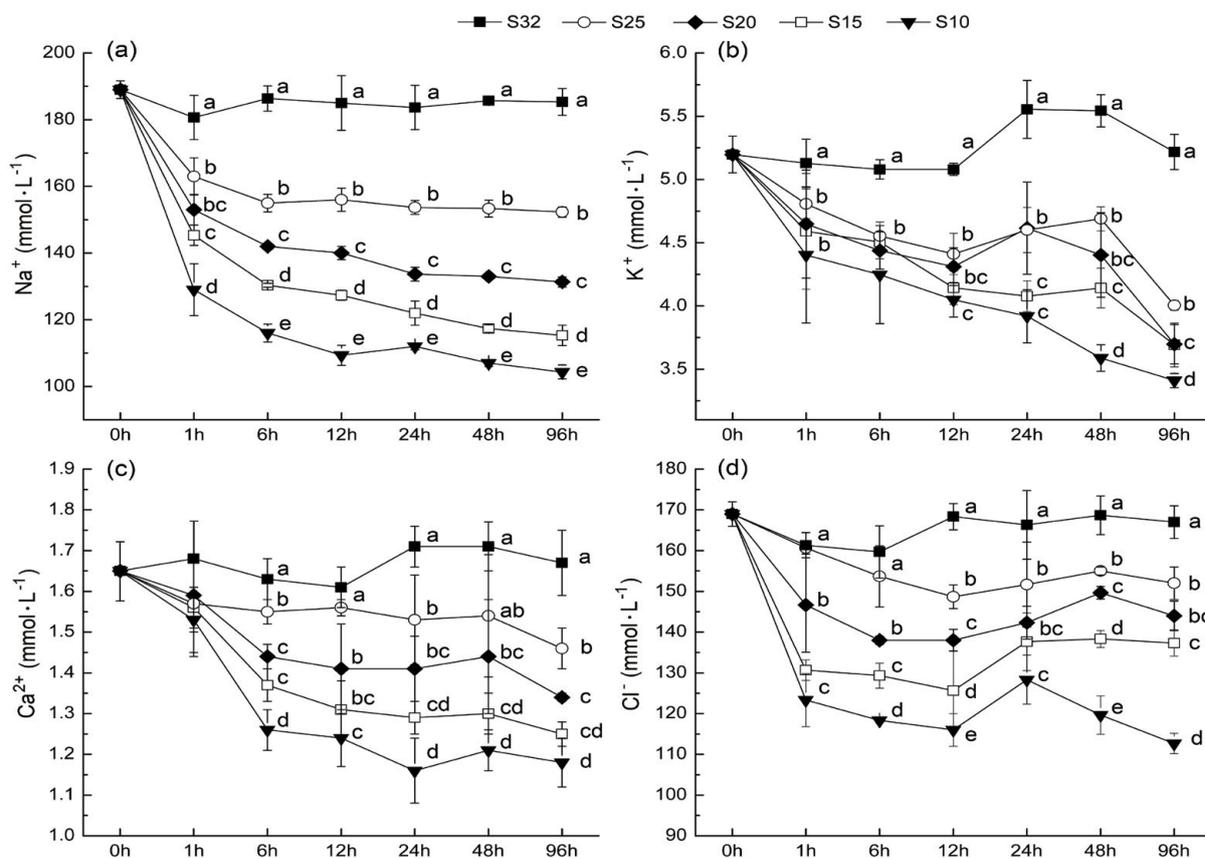
Figure 1: Effect of low salinity stress (10‰, 15‰, 20‰, 25‰, and 32‰ as the control) on the plasma ion (Na⁺, K⁺, Ca²⁺, and Cl⁻) concentrations of juvenile *Hippocampus erectus* over 96 h after transfer. Data presented as mean ± S.D., n=3). Different letters represent significant difference (LSD multiple comparison, $P<0.05$), and the same letters represent no significant difference (LSD multiple comparison, $P>0.05$).
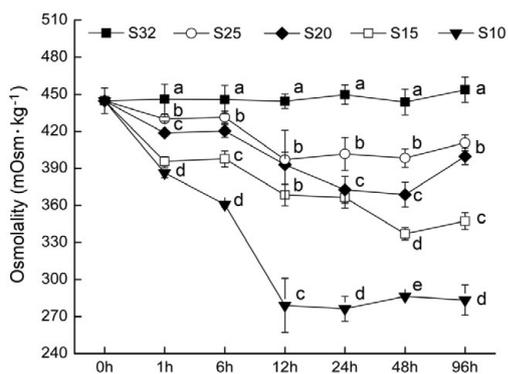
Figure 2: Effect of salinity (10‰, 15‰, 20‰, 25‰ and 32‰ as the control) decrease on osmolality of juvenile *Hippocampus erectus* over 96 h after transfer. Data presented as mean ± S.D., n=3). Different letters represent significant difference (LSD multiple comparison, $P<0.05$), and the same letters represent no significant difference (LSD multiple comparison, $P>0.05$).

with salinity decline (Figure 4). The gill NKA activity among the salinity groups was significantly different (Two-way ANOVA, $F_{[4,104]} = 114.738$, $P<0.001$), The plasma osmotic pressure changed significantly (Two-way ANOVA, $F_{[6,104]} = 140.839$, $P<0.001$) over 96 h post transfer.
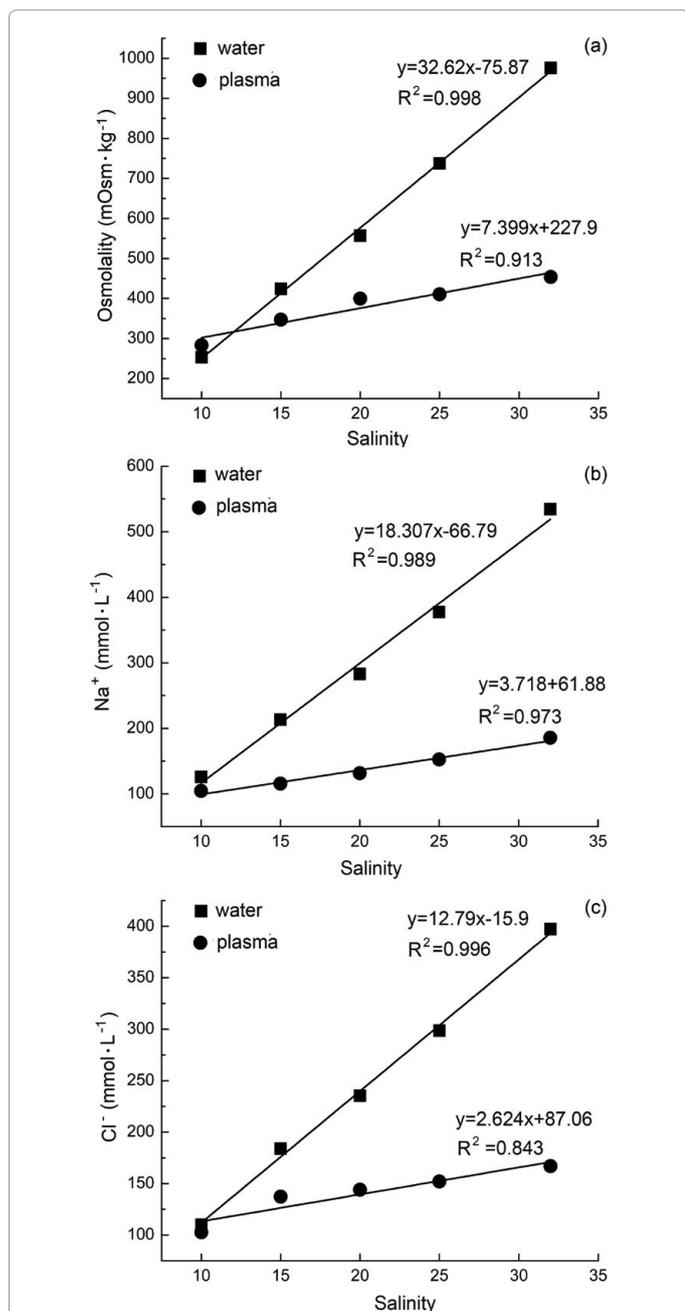
Discussion

The physiological data presented in the current study confirm the capability of juvenile *H. erectus* to survive in salinity of 10‰ as reported by Zhang et al. (unpublished data), [24] and to tolerate a great abrupt salinity changes (>20‰) and maintain the body's homeostasis well over a wide range of salinites, although osmoregulatory perturbations were observed.

Na⁺ and Cl⁻ are the two major blood electrolytes, and they play a crucial role in osmoregulation [26]. When the seahorse juveniles were transferred abruptly into the water of lower salinities from the natural seawater, concentrations of the plasma Na⁺, Cl⁻, K⁺, and Ca²⁺ (Figure 1) decreased as reported in many other marine teleosts [2,27-32]. Similar to reported in many euryhaline teleosts [29-33], there is a distinct crisis phase in plasma ionic level of the seahorse juveniles, i.e. the initial 12 h after abruptly transferred to the different low salinities of water, thereafter a regulatory phase that plasma ions were lost slowly and progressively for 96 h after transfers (Figure 1). The plasma K⁺ (30-55 mmol) in the seahorse juveniles was much higher than Elevation strength of the plasma K⁺ level between 12 h and 48 h weakling with the salinity decrease, especially in the salinites of 32‰, 25‰, and 20‰ (Figure 1), might be affect by food intake. Diet will provide a substantial K⁺ load from copepods intracellular K⁺ [14]. The time-course change pattern of Cl⁻ is usually consistent with Na⁺ in marine teleosts [30-34]. However, difference in change pattern of the plasma Na⁺ and Cl⁻ level between 12 h and 48 h in the lined seahorse juveniles (Figure 1) is interesting and worth to investigate in the future, especially in the effect of food intake [35].

Same as the change in Na^+, Cl^-, K^+, and Ca^{2+} levels, the change of plasma osmolality has a distinct crisis phase, i.e. during the initial 12 h after transfers, and a distinct regulatory phase, i.e. between 12 and 96 h after transfers (Figure 2), suggesting that the seahorse juveniles have the osmoregulatory ability to reach homeostasis within 96 h after transfer to low salinity water. This is the same as in many reported euryhaline teleosts that can stabilize osmolality within 96 h after hypotonic stress [30-33].

Changes in osmoregulatory ability of the seahorse juveniles within 96 h after transfer to low salinity water were correlated with changes in
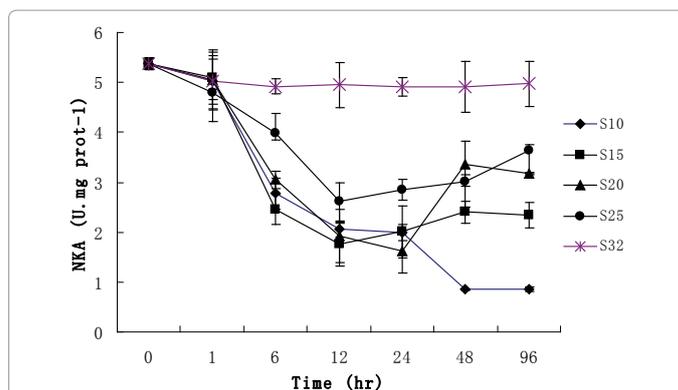
Figure 4: Effect of salinity (10‰, 15‰, 20‰, 25‰ and 32‰ as the control) decrease on the gill NKA activity of juvenile *Hippocampus erectus* over 96 h after transfer. Data presented as mean ± S.D., n=3). Different letters represent significant difference (LSD multiple comparison, $P<0.05$), and the same letters represent no significant difference (LSD multiple comparison, $P>0.05$).

gill NKA activity. Responsiveness of gill NKA activity to environmental salinity is dependent on species, life-history stage, and, in some cases, experimental conditions [36]. Most euryhaline teleosts acclimating to hyperosmotic environments need to experience a rapid increase in gill-ion fluxes accompanied by elevated plasma ions and osmolality, and an increase in gill NKA activity, opposite when exposure to hypotonic conditions [11]. However, gill NKA activity in some euryhaline teleosts performs different pattern. Some euryhaline species show no change or reduction in NKA activity when acclimated to seawater [2,32,37]. In the present study, the gill NKA activity of the seahorse juveniles agreed with most species, i.e. the NKA decreased rapidly within 24 h after transfer the hypotonic environments, thereafter stabilized or elevated except the 10‰ treatment. Same as reported in many other marine teleosts, for example the European sea bass *Dicentrarchus labrax* (Jensen, Madsen and Kristiansen 1998), milkfish *Chanos chanos* [32], changes in osmoregulation and major blood electrolytes (Na^+ and Cl^-) of the seahorse juveniles after transfer to hypotonic water were positively related to changes in the gill NKA activity. On the other hand, some species show a reverse relationship [2,32].

Osmoregulation costs energy [38,39]. Non-optimal salinity waters could result in an increase in NKA activity and concomitant energy expenditure so that causes reduction in fish growth [2,25]. Juvenile fish may maintain internal homeostasis with the lowest NKA activity so as to allow more energy available for growth [40]. Salinity approaching iso-osmotic condition is recognized to improve growth, because aquatic animals cost lower energetic for osmoregulation, and have lower standard metabolic rate, so that allocate a larger proportion of ingested energy to growth [41,42]. In the present study, we found the isotonic point of the juvenile seahorse at 96 h after transfer was 317.13 m Osm·kg^{-1}, the equivalent of salinity 12.05‰. This value is similar to that in many other euryhaline teleost species [2], and falls salinity range of 10‰ to 15‰ in which the gill NKA activity was lower than in other treatments (Figure 4). The results suggest that salinities of 10‰ to 15‰ may be the optimal range for the lined seahorse growth. Actually, the juveniles has been found to perform better growth in the salinity of 10‰ to 15‰ as reported by Zhang et al. (unpublished data), indicating the consistence between the ecological and physiological evidence.

Conclusion

In conclusion, the present study shows that the lined seahorse has a strong osmoregulatory ability to deal with an abrupt salinity change (>20‰), thereafter is able to survive well at the salinities down to

Figure 3: The isotonic and isoionic points of the plasma in juvenile *Hippocampus erectus* at 96 h after transfer. (a) The isotonic point; (b) The isoionic point of Na^+; (c) The isoionic point of Cl^-. Na^+ and Cl^- in the figures represent the levels of sodium and chloride ions in the different salinities.

10‰. In combination of the isotonic point, the gill NKA activity at 24 h after the salinity stress at the salinity of 15‰, and the growth data as reported by Zhang et al. (unpublished data), we recommend the salinities of 10‰ to 15‰ as an optimal range for growth of the lined seahorse, which falls in the range (5‰ to 18‰) reported in most of the marine fishes [25,43]. To further improve the culture protocols for the lined seahorse, more physiological and biochemical studies should be carried out in the future.

Acknowledgements

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