

Effects of Salinity on Growth, Feeding and the mRNA Expression of Na⁺/K⁺-ATPase and HSP 90 in *Liza haematocheila*

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Abstract The effects of various salinities on growth, feeding and the mRNA expression of related genes in *Liza haematocheilus* were investigated. Fish were maintained at four salinities for 30 days to analyse the specific growth rate (SGR), the feeding rates (FR) of *L. haematocheilus*, and the mRNA expression of Na⁺/K⁺-ATPase (NKA) and Hsp 90 were measured at 0, 5, 10, 20 and 30 d, respectively. The results showed that the SGR of *L. haematocheilus* at 14 psu group was higher than the 2 and 42 psu group significantly; the FR of *L. haematocheilus* at 14 psu group was the highest and the 2 psu group was the lowest at 20 or 30 day; the NKA mRNA abundances in *L. haematocheilus* were increased significantly in the 2, 14 and 42 psu groups at 5 day ($P < 0.05$), and the lowest level emerged in the control group (28 psu), the same relationship was observed for Hsp 90 but the change scopes were smaller. The results indicate that the increased expression of NKA and Hsp 90 mRNA in *L. haematocheilus* is a part of the molecular responses to the osmotic stress of changes in salinity.

Keywords *Liza haematocheilus*, Salinity, Specific Growth Rate, Feeding Rates, Na⁺/K⁺-ATPase, HSP 90

1. Introduction

Euryhaline teleosts present the capacity to tolerate the different environmental salinities by using efficient osmoregulatory mechanisms to maintain homeostasis [1]. Fish in an isotonic medium had the lowest standard metabolic rates and fish must impose energetic regulatory costs for active ion transport if environmental salinities differ from the internal osmotic concentration [2-3]. Many species (early developmental stages) optimal for intermediary salinity conditions of brackish water (BW) and grow optimally in estuaries and coastal systems [2-5].

Na⁺/K⁺-ATPase (NKA) mediates the active secretory functions of chloride cells in fish by generating a chemical

gradient for ion transport [6-7], which is responsible for the active transport of Na⁺ out of and K⁺ into relative cells [8-10]. The functional pump is composed of a catalytic α (α 1-4 isoforms) subunit and an accompanying glycosylated β (β 1-3 isoforms) subunit and among these isoforms, α 1 subunit that is predominantly expressed in the transporting epithelia in higher vertebrates functions primarily in a housekeeping capacity to maintain osmotic balance and cell volume regulation [11]. There are two NKA α 1 isoforms in the fill of some fishes such as *Oncorhynchus mykiss* [12], *Salmo salar* [13-17] and *Oreochromis mossambicus* [18], the mRNA level of NKA α 1a isoform increases after transferred from freshwater (FW) to seawater (SW), and that of the α 1b isoform increases after transferred from SW to FW [17-18]. NKA mRNA expression increased when the European sea bass *Dicentrarchus labrax* were transferred from 15 psu to 50 psu or FW [19]. The euryhaline killfish *Fundulus heteroclitus* often move between SW and FW environments and NKA expression also increased whether fish were transferred from BW to SW or FW [20]. Thus, there are many kinds of NKA change way in fish. Hsp 90 is active in supporting various components of the cytoskeleton and steroid hormone receptors [21]. It is now widely accepted that Hsp amounts in the cell, are not only altered upon exposure to temperature stress but also to a range of abiotic, biotic and chemical stressors [15]. Previous studies have reported that the relationship between Hsp 90 mRNA expression and environmental salinities in some species [22].

Liza haematocheilus (Temminck and Schlegel, 1845) (Mugiliformes: Mugilidae) is a common species in the northwestern Pacific inshore waters and was the dominant species in salt marsh intertidal creek in Yangtze River estuary [23-24]. The water salinity would be fluctuated greatly in Yangtze River estuary every year due to flooding and salt tide. In addition, the normal salinity fluctuation was changed because of the building of large-scale water projects such as Three Gorges Dam, which may be lead to change the habitat selection, growth performance and other indices of redlip mullet. However, few studies have focused on the

process of salinity changes in Mugilidae [25-26]. Therefore, the aim of the present study was to assess the affection of salinity on the growth, feeding, NKA α 1b subunit and Hsp 90 β mRNA expression patterns of *L. haematocheilus*.

2. Material and Methods

2.1. Animals

About 500 *L. haematocheilus*, with a mean body length of 2.08 ± 0.14 cm and wet weight of 0.126 ± 0.024 g (means \pm standard deviation), were collected from the estuary in the Yangtze River, China. The young *L. haematocheilus* were acclimatised in a large aquarium (200 L) for one week before the beginning of the experiment. The salinity of the seawater in the aquarium was 28 psu, and the temperature was maintained at 15 ± 1 °C throughout the acclimatisation. *Liza haematocheilus* were fed on *artemia* nauplius daily at 7:00 and 16:00.

2.2. Experimental Design

Liza haematocheilus were subjected to four treatments in 50 L acrylic aquaria containing 40 L of water under various salinity of 2, 14, 28 (the control) and 42 psu at the room temperature. During all the experiments, *L. haematocheilus* were fed on live enriched *Artemia* nauplius daily at 7:00 and 16:00 and about 50% treatment seawater was replaced every 3 d. In the experiment, the SGR of *L. haematocheilus* was determined at 0 d and 30 d, the method of SGR was previously described by Sampaio and Bianchini (2002). In short, 10 fishes were randomly sampled from each replicate tank when the experiment at beginning and end. SGR was calculated using the formula: $SGR(\%/d) = [(W_t/W_0)^{1/t} - 1] \times 100$, where W_0 was initial wet weight (g) and W_t was the wet weight at the end (g), t was experimental days (d). The FR of *L. haematocheilus* was determined at 10, 20 and 30d, respectively; the method of FR was previously described by Zhu et al. (2006) with minor revision. In short, 400 ml of seawater with *artemia* nauplius (about 70 ind./ml) in 500 ml beaker and 5 fishes in each beaker, the feeding time for 40 mins, 10 ml seawater with *Artemia* nauplius was fixed with formaldehyde and counting when the experiment at beginning and end. Each group with different salinity was replicated. FR was calculated using the formula: $FR = V \times (D_0 - D_t) / nt$, where V was the volume of the seawater (ml), D_0 was the density of *Artemia* nauplius before the experiment (ind./ml), D_t was the density of *Artemia* nauplius after the experiment (ind/ml), n was the number of fishes in each group, t was time during the feeding (min). The mRNA expressions of NKA and Hsp 90 of *L. haematocheilus* were determined at 0, 5, 10, 20 and 30 d, respectively; three individuals were randomly sampled from each aquarium, and the fish was stored in RNAsore reagent (Tiangen) until use.

2.3. RNA Purification and cDNA Synthesis

Total RNA was purified from the samples using the TRIzol reagent (Invitrogen), genomic DNA was digested with Dnase I (Invitrogen), and the purified RNA was stored at -80°C after isolation. The first-strand cDNA synthesis was performed using M-MLV reverse transcriptase (Invitrogen) to transcribe the poly (A) mRNA with Oligo-dT and random 6-mer primers. The reaction conditions were those recommended by the manufacturer's instructions. The cDNA was maintained at -20 °C until use as the template for the quantitative real-time PCR (qRT-PCR).

2.4. Quantification of mRNA Expression

The mRNA expression of the target genes was measured by qRT-PCR. Specific primers were designed based on *L. haematocheilus* mRNA sequences (GenBank accession no. JQ844546 and JX272925) using Primer Premier 5 (PREMIER Biosoft International, Palo Alto, CA). The primer sequences are shown in Table 1. The qRT-PCR amplifications were carried out with an ABI StepOnePlus Detection system (Applied Biosystems, Foster City, CA, USA). The amplifications were performed in a 96-well plate with a 20- μ L reaction volume containing 10.0 μ L of 25 \times SYBR Green I, 0.8 μ L of PCR Forward Primer (10 μ M), 0.8 μ L of PCR Reverse Primer (10 μ M), 0.4 μ L of ROX Reference Dye II (50 \times), 2.0 μ L of cDNA template and 6.0 μ L of diethylpyrocarbonate water (DEPC-water). The thermal profile for the SYBR Green qRT-PCR consisted of an initial step at 95 °C for 90 s, followed by 40 cycles of 95 °C for 5 s and 58 °C for 30 s. All samples were run in triplicate. In this experiment, a preliminary trial showed that the β -actin gene had a steady expression in this experimental species. Therefore, the β -actin gene was used as the housekeeping gene in all of the qRT-PCR assays, and the primers (Table 1) were designed based on the β -actin gene sequence (GenBank accession no. FL589653). The standard curve and the gene expression levels were analysed automatically, as was the setting of the base line. A melting curve analysis of the amplification products was performed at the end of each PCR reaction to confirm that only one product was amplified and detected. For the experimental results of the qRT-PCR, the observations at the beginning in the experiment of 28 psu group were initiated as "1.0", and all the relative ratios of gene expression levels were then calculated to derive the means and standard deviation (SD).

2.5. Data Analysis

Data were expressed as mean \pm SD. The significance of the differences in the FR, SGR, mRNA expression of NKA and Hsp 90 were determined using one-way ANOVA followed by Duncan's multiple range tests; with a threshold significance level of $P < 0.05$. All of the statistical analyses were performed using SPSS 15.0 (SPSS, Chicago, IL, USA).

Table 1. Na⁺/K⁺-ATPase and Hsp 90 βgenes of *L. haematocheilus* and their specific PCR primers used for RT-PCR

Target gene	GenBank#	Primer sequence (5'→3')	Product (bp)
Na ⁺ /K ⁺ -ATPase Fr	JQ844546	GAACCGTCACCATCCTCTGT	167
Na ⁺ /K ⁺ -ATPase Rr	JQ844546	GCCGATTTGTCCGTATGCTA	167
HSP90Fr	JX272925	CGCAAGAACATCGTCAAGAA	219
HSP90Rr	JX272925	CTTCATGCGGGTCAGGTACT	219
β-actin Fr	FL589653	GCCGTGACCTCACAGACTACCT	185
β-actin Rr	FL589653	TTGATGTCTCGCACGATTCTC	185

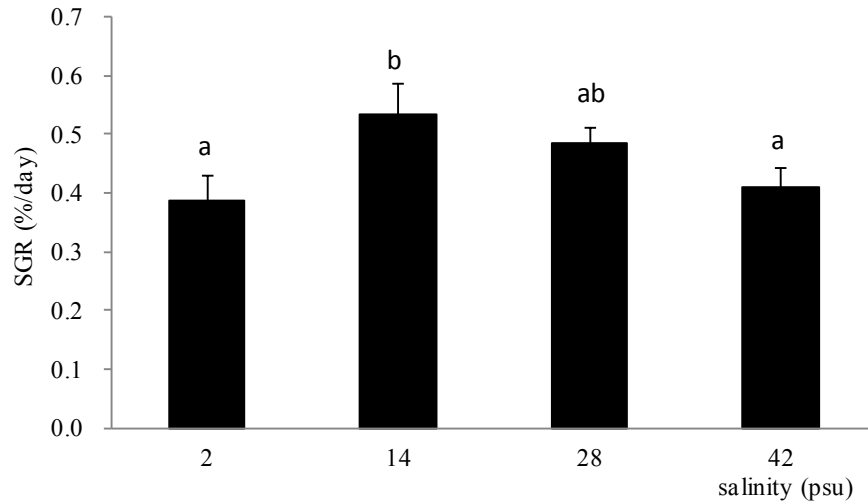


Figure 1. Effects of salinity on the SGR of *L. haematocheilus*, data are expressed as mean ± SD. Different letters indicate significant differences among groups (one way ANOVA, $P < 0.05$).

3. Results

3.1. Effects of Salinity on the SGR

Figure 1 showed the SGR of *L. haematocheilus* stressed by the different salinity (2, 14, 28 and 42 psu) at 30 d. No significant differences were found between 2, 28 and 42 psu ($P > 0.05$), but the SGR of *L. haematocheilus* at 14 psu was higher than 2 and 42 psu groups ($P < 0.05$) and no significant difference with 28 psu group ($P > 0.05$).

3.2. Effects of Salinity on the FR

Figure 2 showed the FR of *L. haematocheilus* stressed by the different salinity at 10, 20 and 30 d, respectively. No significant correlation was found between the FR and salinity at 10 d ($P > 0.05$), but the FR of *L. haematocheilus* at 28 psu group (control group) was the highest and that of the 2 psu group was the lowest; the FR was significantly different between salinity groups at 20 d ($P < 0.05$), the FR of *L. haematocheilus* at 14 psu group was significantly higher than other groups, the FR of *L. haematocheilus* at 2 psu group was

significantly lower than those of other groups; The changes of the FR in different group at 30 d were similar with those changes at 20 d.

3.3. Effects of Salinity on the mRNA Expression of NKA and HSP 90

The mRNA expression of NKA α1b subunit in *L. haematocheilus* were increased significantly at 5 d stressed by 2, 14 and 42 psu groups ($P < 0.05$), especially in 42 psu group, the mRNA expression level of NKA α1b subunit was 8 times of that of 28 psu group. In the later days, the mRNA expression of NKA α1b subunit of 2 and 42 psu groups were decreased and there were high level in 14 psu group (Fig. 3A, B and D). However, in the control group, the mRNA expression of NKA α1b subunit was not significantly increased until 10 d and decreased significantly later (Fig. 3C).

The fluctuations of the mRNA expression of Hsp 90 β stressed by the different salinity were very similar with those of NKA α1b subunit, but the change scopes were different (Fig. 4).

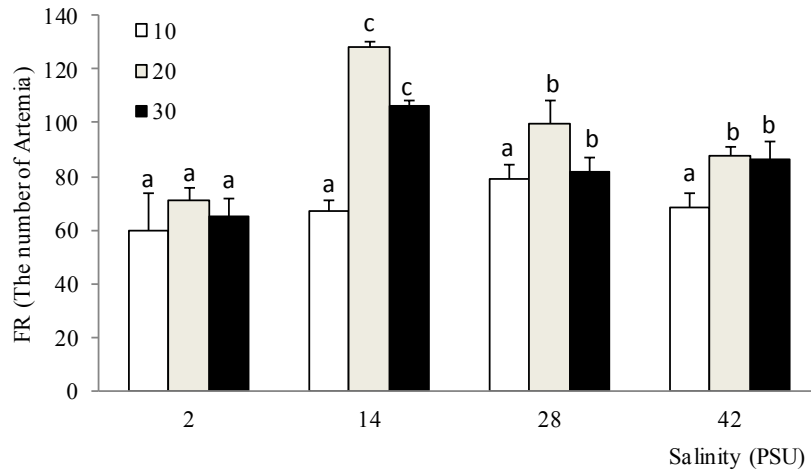
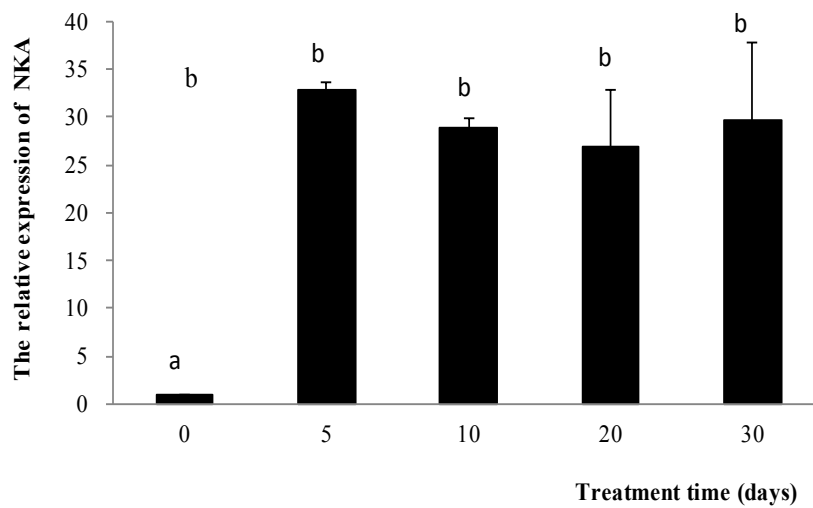
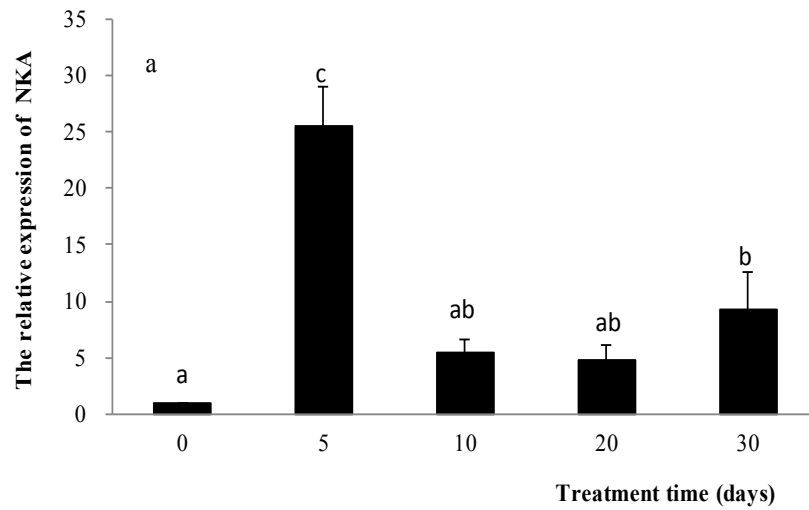


Figure 2 . Effects of salinity on the FR of *L. haematocheilus*, the results are shown as the number of *Artemia* nauplius fed by one fish in one minute. The different superscripts of the same row values are significantly different ($P < 0.05$).



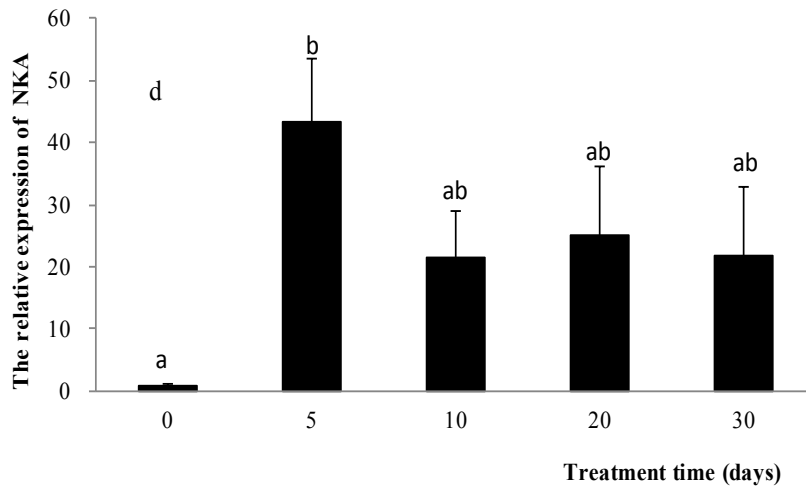
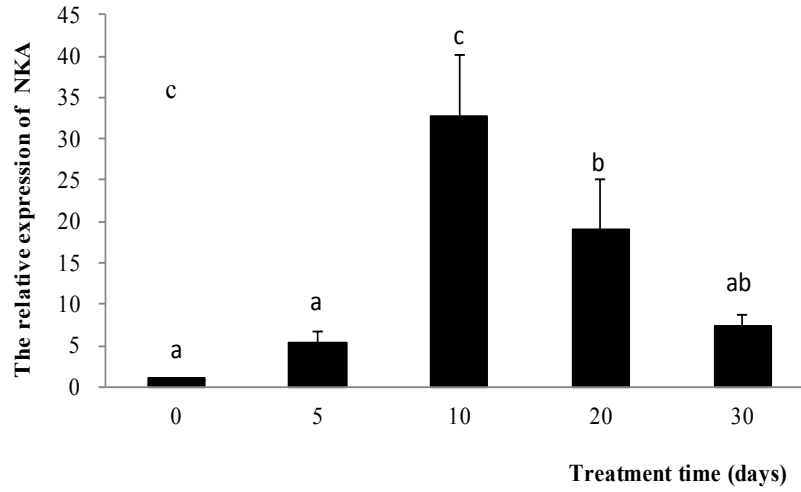
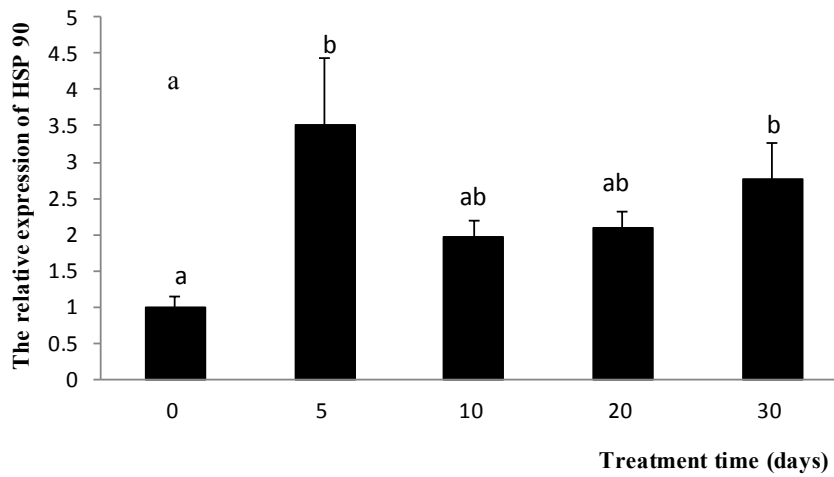


Figure 3. NKA mRNA expression in *L. haematocheilus* stressed by 2 psu (A), 14 psu (B), 28 psu (C) and 42 psu (D) at 0, 5, 10, 20 and 30 d, respectively. Data are expressed as mean \pm SD. Different letters indicate significant differences among groups (one way ANOVA, $P < 0.05$).



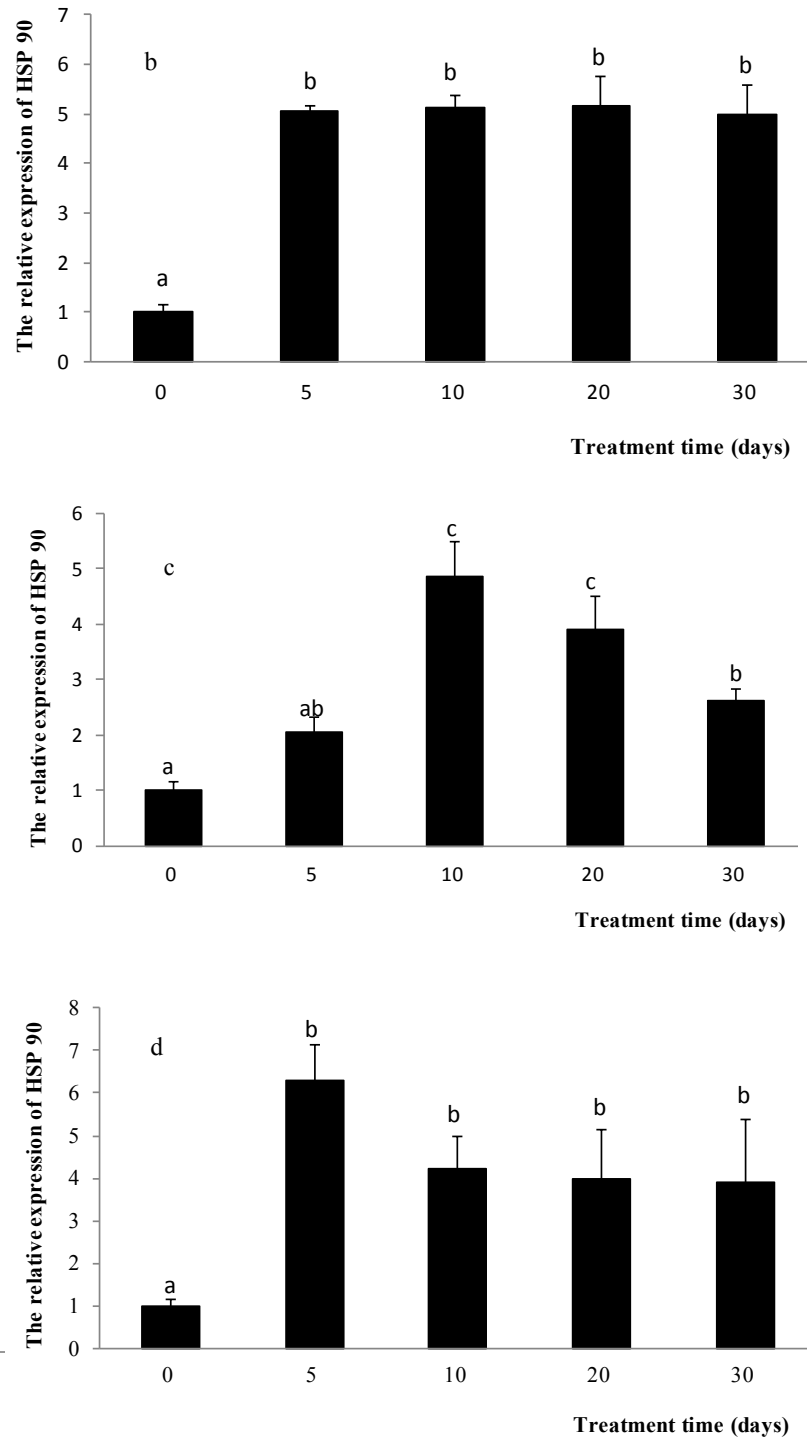


Figure 4. Hsp 90 mRNA expression in *L. haematocheilus* stressed by 2 psu (A), 14 psu (B), 28 psu (C) and 42 psu (D) at 0, 5, 10, 20 and 30 d, respectively. Data are expressed as mean \pm SD. Different letters indicate significant differences among groups (one way ANOVA, $P < 0.05$).

4. Discussion

Fish growth is affected by many ecological factors, such as salinity, temperature, light, etc. Among them, salinity is a key factor in controlling growth [2, 28]. The effects of salinity on the growing capacities in fishes have been studied widely, and most species grow optimally between 5 and 18 psu [2]. [4] found that the optimum range of salinity for

6-d-old to 20-d-old *Argyrosomus japonicus* larvae was 5-12.5 psu. [3] have reported that BW-acclimated fish (sea bream *Sparus aurata*, 20 g mean body weight) showed a better growth with respect to SW- or LSW-acclimated fish (12 psu > 38 psu > 6 psu). The similar results was found in the present study: SGR of *L. haematocheilus* was better in intermediate salinity (14 psu) than other groups (14 > 28 > 42 > 2 psu) during 30 days (Fig. 1). In contrast, other studies

have shown that salinity did not affect fish growth significantly. [29] found that salinity did not affect the growth of *A. japonicas* juvenile, the size of individuals was 9.4 g weight and the salinity treatments was 5-45 psu. [30] have found that there were no differences in growth of rabbitfish *Siganus rivulatus* in all treatment (10-25 psu, the size of individuals were 7.4 g). [31] have reported that there were no significant differences in somatic growth rate in European flounder (*Platichthys flesus*) when stressed by the different salinities (0-30 psu, post larvae). The different SGR in fishes are related to duration of experiment and/or the developmental stage of the same fish [2]. The earlier developmental stage such as larvae which SGR was higher in brackish water and the larger fish (>6.0 g/fish) showed no significant difference in growth when exposed to the different salinities [4, 29]. Therefore, further experiment about osmotic analysis and a range of temperature regimes at different salinity exposure were needed to test this hypothesis in different developmental stages of *L. haematocheilus*.

In the present study, the effect of salinity on the FR was significantly different in each group at 20 d and 30 d ($p < 0.05$); the FR of 14 psu group was significant higher than those of other groups (Fig. 2). The FR was also significantly affected by salinity in gray snapper *Lutjanus griseus* ($p < 0.05$) [32]. Based on the results of the FR and SGR, the lowest and highest values of the FR and SGR were 2 and 14 psu respectively. Therefore, our results also support the hypothesis that the energy costs of osmoregulation were lowest in isotonic medium and that the energy savings were sufficient to result in increased growth from the index of the FR.

The gene expression of NKA $\alpha 1b$ and Hsp 90 β in response to salinity changes was also studied in the present study. We used the juveniles in the salinity stressed experiments. During the life of *L. haematocheilus* in Yangtze River estuary, the natural high salinity usually occurs at April, and the fish size is small at this time. So we chose juvenile to do salinity experiment in order to make it more close to the actual situation. The different tissues of the fish such as the gill, heart, liver and kidney are difficult to isolate at this stage. The whole fish were used to extract RNA, so the detected expression can reflect the actual situation of the whole fish. In addition, some genes expression (Hsp 90 β mRNA transcript) was found in the different tissues such as gill, heart, muscle, brain, liver and kidney [33].

Euryhaline fish have the capacity to adapt to different environmental salinities by activating their osmoregulatory system and some relative gene expression such as NKA [19-20, 34]. Previous papers described two major salinity-induced gill NKA responses: the “higher-NKA-in-hyperosmotic media” response, i.e., the NKA expression was increased after transfer to hyperosmotic environments, has been observed such as FW-residing euryhaline fishes and diadromous fishes. On the other hand, the “higher-NKA-in-hyposmotic media” response, the NKA expression were increased after transfer

to hyposmotic environments, has been observed such as BW- or SW-residing euryhaline fishes [1, 10]. For example, the NKA mRNA expression was increased in the gill of the euryhaline milkfish *Chanos chanos* from seawater transfer to freshwater after 24 h [10] and the similar result was observed in black porgy *Acanthopagrus schlegeli* [35]. In *Oryzias latipes* and *O. dancena*, whose primary natural habitats are FW and BW environments, respectively, the gill NKA mRNA abundance of the SW-acclimated *O. latipes* was significantly higher than FW- and BW- acclimated individuals. However, the gill NKA mRNA abundance of the FW-acclimated *O. dancena* was significantly higher than BW- and SW- acclimated individuals [1]. The two salinity-induced NKA responses are based on the fishes transfer from SW/BW to FW or transfer from FW to SW/BW. Nevertheless, in the present study, the control group salinity was 28 psu, because the test was in April and this season is the drought period in Yangtze River and the salinity is higher in estuary and adjacent waters, *L. haematocheilus* were transferred to the hyposaline groups (2 and 14 psu) and hypersaline group (42 psu). The mRNA expression of NKA $\alpha 1b$ subunit were increased significantly at 5 d stressed in 2, 14 and 42 psu groups ($P < 0.05$), which “U-shaped” salinity dependence of the NKA expression was different from the above two major salinity-induced NKA responses. Teleosts with “U-shaped” regulation of gill NKA activity or mRNA have been observed in some other euryhaline species, such as, *Dicentrarchus labrax* [19] and *Fundulus heteroclitus* [20]. A “U-shaped” salinity dependence of the NKA expression may be energetically favorable for the fishes, since they ensure that the fishes may maintain a relatively low NKA expression for most of the life stage [19]. In a word, the discrepancy in NKA responses upon salinity challenge between the hypo- or hyperosmotic environment may relate to suggestion that higher NKA mRNA expression may be required to maintain a favorable Na^+ gradient across the apical membrane.

Salinity challenge can result in fishes becoming osmotically stressed and the cellular ion regulation mechanisms can be affected, which in turn can cause alterations in cellular protein damage and subsequent induction of Hsp synthesis [36]. Using a similar experimental approach it was reported that gill Hsp 90 mRNA abundance increased in juvenile Atlantic salmon (*Salmo salar*) that were transferred from FW to SW [37]. Alterations in gill Hsp 90, upon hypoosmotic stress, have also been recently reported as transfer of black porgy (*Acanthopagrus schlegeli*) from SW to 10% SW or FW caused a significant increase in gill Hsp 90 mRNA abundance [35]. The amounts of Hsp 90 mRNA transcript were increased in skin tissue when mudskipper (*Periophthalmus modestus*) was transferred from SW to FW [38]. When juvenile black sea bream (*Mylio macrocephalus*) were acclimated to 6 (hypoosmotic), 12 (isoosmotic), 33 (control) and 50 psu (hypersaline) salinities, the liver Hsp 90 amounts were lowest in black sea bream acclimated to 12 psu [36]. In the present study, the mRNA expression of Hsp 90 β

were increased significantly at 5 d when *L. haematocheilus* were transferred from SW (28 psu) to the BW (2 and 14 psu) or HSSW (42 psu) ($P < 0.05$). Therefore, whatever fishes were transferred from SW to FW (hypoosmotic stress) or FW to SW (hyperosmotic stress), for the majority of euryhaline teleosts, the relative gene expression (NKA $\alpha 1b$ or Hsp 90 β) will be increased. Of course, in view of different experimental time, different fish size, and different species, there are different results, for instance, the abundance of Hsp 90 mRNA transcript remained unchanged in gill, heart, muscle, brain, liver and kidney of chinook salmon (*Oncorhynchus tshawytscha*) that were transferred from FW to SW [33].

Fish in an isotonic medium have the lowest standard metabolic rates and the energetic cost of osmoregulation is lower [2], which may be the result of natural selection. While the isosmotic point was not measured in the present study and the SGR was highest in 14 psu group, which may be isosmotic condition in *L. haematocheilus*. But the lowest gene expression level emerged in 28 psu at 5 d, so we could not make sure the lowest energetic cost of osmoregulation was at the isosmotic point or at control group (28 psu) of *L. haematocheilus* from gene expression level perspective.

5. Conclusions

The results in the present study indicated that *L. haematocheilus* can survive in the different salinity environment. At the individual level, a better growth over a period of 30 d of young *L. haematocheilus* acclimated to 14 psu with respect to those acclimated to 2, 14, 28 and 42 psu (e.g. SGR and FR). At the gene expression level, the mRNA expression of NKA $\alpha 1b$ subunit and Hsp 90 β in *L. haematocheilus* were increased significantly stressed by 2, 14 and 42 psu groups at 5 d ($P < 0.05$), and expression level in the control group (28 psu) was lower. Furthermore, according to the energetic cost theory, the lowest mRNA expressions of NKA $\alpha 1b$ subunit and Hsp 90 β (at 28 psu) were not matched by the best SRG and FR (at 14 psu) in the present study. Therefore, further research is required to determine the effect of salinity on the relationship between growth and gene expression in the different domesticated salinity condition.

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