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RESEARCH PAPER

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Effects of Different Levels of Salinity on NKA and NKCC Expression in Asian Sea Bass (*Lates calcarifer*)

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Abstract

Asian sea bass, *Lates calcarifer* is one of the most valuable marine fish species that is known to be tolerant to wide range changes of salinity. The species is able to live in freshwater, brackish and marine water, being an ideal model species for studying the effects of salinity on physiological responses. The present study is aimed to evaluate the changes in expression levels of Na^+-K^+ ATPase (*NKA*) and $Na^+/K^+/2Cl^-$ (*NKCC*) genes in gill tissue of *L. calcarifer* kept in a range of salinities including freshwater (0 ppt), brackish water (15 ppt) and saline marine water (35 and 50 ppt). Totals of 180 individuals were randomly distributed into 12 fiberglass tanks (volume 300 L) that contained different water salinity variants. After 30 days of experimental captivity, fish were euthanized and gill tissues were collected for *NKA* and *NKCC* mRNAs expression analysis. The U shape expression pattern for both *NKA* and *NKCC* was recorded. The highest brachial expression was seen in 50 ppt that was statistically different from all other salinity variants. The lowest expression was recorded in 35 ppt. In turns, observed *NKA* and *NKCC* expression levels in freshwater were significantly higher than 15 and 35ppt. The current findings showed that the expression levels of major gill transporters, as are *NKA* and *NKCC* has the highest expression in unusual conditions, having the lowest expression levels under the most habitual salinity conditions present in the wild environment.

Introduction

Nowadays, the increasing world's human census and changing dietary preferences cause the enlarged demand for aquatic products. As the classic fishery based on the wild-catches has reached its limit the aquaculture is expected to satisfy this demand in the future. Aquaculture is also considered as one of the most economically important branch of agriculture, continuously growing since 1990 and presently exceeding wild-catches (FAO, 2020). On the other hand, survival of many freshwater and marine species in endangered due to the pollution by the inflow of urban wastewater into rivers and seas, the loss of spawning grounds and habitats, the overexploitation by fisheries and inappropriate fishing methods, e.g. trawling (Sarvi *et al.,* 2006). In aquatic organisms, the electrolyte-balancing mechanism is of vital importance that enables them to live in changing conditions of the hyper and hypoosmotic environments. The observed variety of osmotic adjustment mechanisms in fish is one of the crucial factors playing role in adaptation to diverse environments (Farshadian *et al.,* 2018; Salati *et al.*, 2011). Adaptation to changes in environmental salinity requires synthesis of specific transport proteins (McCormick, 2001). In fishes many different transporters are involved in osmoregulation, i.e. Na^+-K^+ ATPase (NKA), Aquaporins, H^+-ATPase, Cystic Fibrosis

Transmembrane Conductance Regulator (CFTR), Na^+/H^+ exchangers (e.g. NHE2 and NHE3), Na^+/Cl^- cotransporter (NCC) and Na-K-Cl cotransporter (NKCC) (Lin *et al.,* 2006; Yan *et al,* 2007; Bodinier *et al.,* 2008; Ivanis *et al.,* 2008; Hiroi *et al.,* 2008; Salati *et al.,* 2014). The crucial importance of NKA and NKCC in the osmoregulation mechanism has been previously evidenced by numerous studies (e.g. Patterson *et al*., 2012).

Asian sea bass, *Lates calcarifer* is a valuable marine fish known in Australia as Barramundi. The fish is commercially important species for aquaculture in Southeast Asia, being widely farmed in Australia, Thailand and Indonesia (Longbaf Dezfouli et al., 2019). The fish's distribution range extends from the North Indian Ocean to the Western Pacific, covering the areas from Iran to the northern Australia with optimum temperatures range of 28 to 30ºC (Tian and Qin, 2003).

L. calcarifer is considered as very attractive species for aquaculture production due to easy use of commercial food, rapid growth rate (the species' growth rate is low in the early stages of life, but rapidly raises when reach 30 g) and the high price on the market (Venkatachalam *et al*., 2018). The species is tolerant to wide span of salinity (0-50 ppt) and water temperature (15-40º C) that makes it able to live and growth in various environments, such as: the sea, estuaries, coastal wetlands and rivers (Katersky and Carter*,* 2005; Sorphea *et al.,* 2019). This feature also makes it as a good model species for studying the effects of salinity on physiological process. If the saltwater or brackish water could be used for rearing economically valuable fish, animal protein deficiency can be largely offset (Harpaz *et al*., 2005; Nafisi Bahabadi, 2016).

Numerous studies on fish physiological responses to salinity changes that have been extensively carried out in diverse fish species showed benefits of choosing the optimal salinity for species which can spend more energy on growth (Salati *et al.,* 2011; Salati *et al*., 2014; Farshadian *et al.,* 2018). This especially concerns the species that could be cultured in a wide range of salinities, such as *L. calcarifer*, which can directly lead to increased production efficiency. Therefore, by studying the expression of the *NKA* and *NKCC* genes, a better understanding of the *L. calcarifer* response to the changes in environmental salinity and optimal salinity for its culture can be achieved.

Material and Methods

Experimental Design

One hundred and eighty (n=180) specimens of *L. calcarifer* with a mean initial weight of 34.36 ± 0.41 g were purchased and transferred to the laboratory rearing system. The fish were kept in 12 fiberglass circular tanks of volume 300 L (15 fish per tank) for 2 weeks in 50 ppt salinity for acclimation to experimental conditions. During this time, the fish were hand-fed ad libitum, two times daily at 08:00 and 16:00 (Havoorrash, Iran). Four salinity variants were used, namely: 0, 15, 35 and 50 ppt (g/L) under the current study. After adaptation period, the water salinity was gradually decreased to the desired salinity over a period of 7 days. For this tap water was used to decrease water salinity in experimental groups, excluding already available salinity of 50 ppt. In 0 ppt group Persian Gulf water (50 ppt) was fully replaced by freshwater. Afterwards the fish was kept for 30 days in the obtained salinity. The salinity of the water was monitored daily by the portable meters (WTW Model U10) and 50% of the tank water was replaced daily to maintain its quality. Subsequently, fish were euthanized and gill tissues were collected for further genetic analyses.

RNA Extraction

Primer sequences for *NKA* and *NKCC* genes were designed based on genetic information available in NCBI gene bank database based on the records assigned to the accession numbers: XM_018661006.1 and XM_018701955.1. For this purpose, Primer 3 software (ver. 0.4.0) was used. The resulted primer sequences are shown in Table 1. Total RNAs were extracted from gill tissues using the RNA extraction kit (Roche). The RNA concentration was measured by spectrophotometry and its quality was assessed by 1% agarose electrophoresis. Next, the isolated RNA was used to synthetize of cDNA using M-MLV Reverse Transcriptase (Ferments). The resulted cDNA was used as template for further PCR amplification that was performed under the following conditions: denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 60 s, 58°C for 45 s, and 72°C for 1 min, with a final extension step of 72°C for 10min. The

Table 1. Primer sequences used for cloning and qPCR

Primer name	Primer sequence (from 5 to 3)	Temperature (°C)
NKA	F: CTCAGATGGCCAGGCAAAGA	60
	R: GGGCCAGGATCTCTTTTGCT	60
NKCC	F: TGGTCAGGCTGGGATTGTTC	60
	R: GGGCCCAGACTCCTTGAAAT	60
EF-lα	F: CTTGCCTTTGTCCCCATCTC	60
	R: CTTCCAGCAGTGTGGTTCCA	60

PCR products were separated on a 1% agarose gel and then purged by PCR purification kit (Qiagene). In order to validate the correctness of target genes the obtained fragments DNA was sent for sequencing by the outsource company (Tops gene).

Gene Expression Studies

Real-time PCR analysis was carried out by application of designed primers for *NKA* and *NKCC* genes. The elongation factor 1-alpha (*ef1-α*) was chosen as internal control for qRT-PCR because it was proven to characterize by stable expression across different tissues (Paria *et al*., 2016). The summary of primer sequences used for qRT-PCR is shown in Table 1. Gill tissues were homogenized by liquid nitrogen, and total RNA was isolated using RNA extraction kit (Sinagen) according to the manufacturer' protocol. RNA samples quality was checked using a NanoDrop ND-1000 spectrophotometer. The cDNA was synthesized from 1µg of total RNA using a RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, K1622). The obtained cDNA were used to perform quantitative real-time PCR, where *ef1-α* gene was used as reference gene in all analyses. The real-time PCR was performed with the SYBR Green Real-time PCR Master Mix (Sinagene, Iran) in a Rotor-Gen3000 real-time PCR detection system (Corbett research). Each reaction was carried out in a total volume of 20µL consisting 10µL SYBR Green Real-time PCR Master Mix (Sinagene), 10 pmol of each primer, 1µl of cDNA template and nuclease free water in amount required to fill the reaction volume. The aliquots were run with the following thermal cycling program: an initial activation step at 94°C for 2 min and then 40 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30s. All real-time PCRs were performed in triplicates. Melt curve analysis was performed on the PCR products at the end of each run to ensure that a single product was amplified. Relative targeted gene expression was calculated for each reaction by the ΔΔCt method.

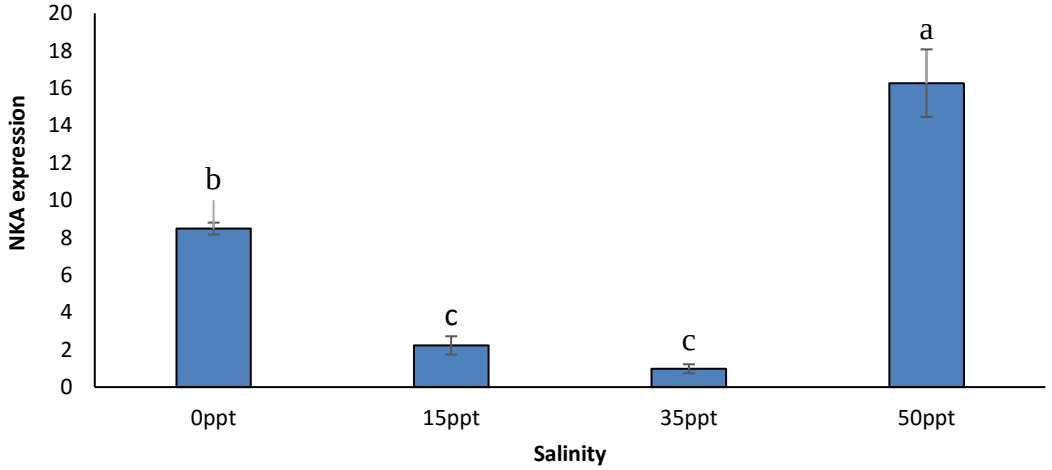

Figure 1. The expression pattern of *NKA* gene in gill tissues of Asian sea bass (*Lates calcarifer*) under the different levels of salinity. The letters a, b and c indicate a statistically significant different results groups (P<0.05).

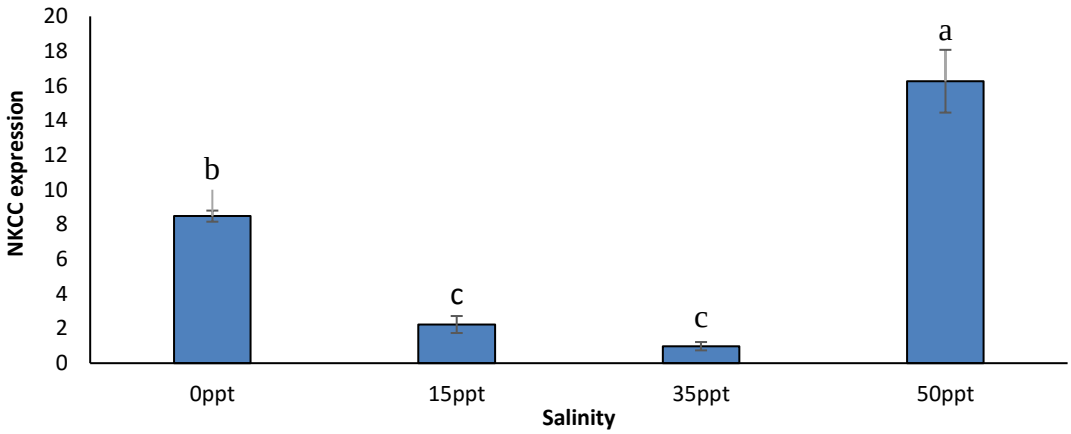

Figure 2. The expression pattern of *NKCC* gene in gill tissues of Asian sea bass (*Lates calcarifer*) under the different levels of salinity. The letters a, b and c indicate a statistically significant different results groups (P<0.05).

Statistical Analysis

All data were presented as mean ± standard deviation. The observed differences in the analysis results were tested for significance using a one-way analysis of variance (ANOVA) and the Duncan's post hoc ($P<0.05$).

Results

No mortality was recorded during the experimental captive of fish under determined salinity variants. The PCR amplification product length of the *NKA* and *NKCC* genes were 200 and 150, respectively. The results of *NKA* and *NKCC* genes expression analysis in gill tissues of fish exposed to different salinity conditions are shown in the Figures 1 and 2. Both genes displayed specific U-like shape expression pattern. The expression levels of *NKA* and *NKCC* in the 50 ppt condition were the highest, being significantly different from other variants ($P<0.05$). The expression of *NKA* and *NKCC* in freshwater (0 ppt) treatment was significantly higher than in 15 ppt and 35 ppt variants but statistically lower than treatment of 50 ppt. There was no significant difference between 15 ppt and 35 ppt treatments.

Discussion

Most of the teleost species are stenohaline that live exclusively in freshwater or saltwater. The others are euryhaline that have the ability to withstand wide changes in environmental salinity (Evans, 1984). The euryhalinity is considered as one of the most important feature that enables the teleost fishes to colonize all aquatic habitats, taking important part in the development of anadromy and catadromy life styles. Adaptation to different environmental salinity stimulates changes and/or activation of ion specific transport mechanisms that can be observed in euryhaline species. Due to their enzymatic properties, gill mitochondrion-rich (MR) cells are considered to be the major site of osmotic regulation in teleosts (Lin *et al.,* 2004a; Lin *et al.*, 2003; Evans *et al.,* 2005).

Euryhaline species show adaptive changes in gill *NKA* genes activity following salinity changes. The majority of energy spent during osmotic regulation is devoted to the synthesis of ion transporting proteins and the active maintenance of the electrochemical gradient by NKA proteins (Hwang and Lee, 2007). According to the current model of ions secretion in gills of saltwater species, the driving force for chloride secretion is the electrochemical gradient generated by NKA proteins. Sodium secretion occurs through the specific cellular pathway of cations in the direction of their electrochemical gradient (Marshall, 2002).

Despite the *NKA* genes response to environmental changes of salinity is variable in euryhaline fish, two main patterns can be discerned that enable to interpret observed changes: (1) the directly proportional association that characterizes anadromous fish and is based on mechanism where increased salinity induces greater *NKA* genes expression as well as protein activity (McCormick, 1995, 2001; Tipsmark *et al.,* 2002; Tang and Lee, 2007) and (2) the U-shaped relationship described in some euryhaline species (Jensen *et al*. 1998). According to the first expression pattern it can expect higher levels of *NKA* genes expression and greater proteins activity in marine fish group with comparison to the brackish and freshwater ones. Indeed, Tang and Lee (2007) reported that the expression of *NKA* in gills of a euryhaline teleost fish pufferfish (*Tetraodon nigroviridis*) was higher in the marine group than in the freshwater group. The U-shaped *NKA* response pattern mechanism to salinity changes creates potential for improvement of the available energy consumption by its reduction for purposes of osmoregulation and redirecting for other processes.

NKA gene expression in gills of *L. calcarifer* was lower in salinity of 35 ppt in comparison to other experimental variants and increased both under decreased and increased salinity conditions ($P<0.05$). Similar results on *NKA* genes activity in gill tissues was reported by Saoud *et al.* (2007) for marbled spinefoot (*Siganus rivulatus*) kept in 25, 30, 35 and 40 ppt salinity for 6 weeks where the lowest *NKA* expression level was recorded in fish reared in salinity of 35 ppt. Another example of U-like mRNA expression pattern of *NKAα1b* subunit was reported in so-iuy mullet (*Liza haematocheilus*), where the significant increment of gene expression level was observed 5 days after transferring fishes to salinities of 2, 14 and 42 ppt and retaining its lowest expression in control group kept in 28 ppt salinity (Shen *et al.*, 2015). This U-like response pattern in *NKA* expression activity to the changes in environmental salinity was also recorded for such species as: Common carp (*Cyprinus carpio*) (Salati *et al.,* 2011), Dorada (*Sparus aurata*) (Laiz-Carrión *et al.,* 2005) and pufferfish (*Tetraodon nigroviridis*) (Lin *et al*., 2004b). In general, it can conclude that the lowest level of *NKA* gill expression is observed for conditions that are observed in natural habitats of studied fish, which reflects the species' evolutionary-based genetic adaptation to specific environments (Kang *et al.,* 2008).

A link between *NKA* and *NKCC* expression during osmoregulation process by gill's mitochondrion-rich (MR) cells had been reported in some previous studies (Tipsmark *et al.,* 2002; Shirangi *et al.*, 2017). In the current study, the lowest expression of *NKCC* was observed in salinity of 35 ppt and with both positive and negative salinity changes, the expression of this gene increased in gill tissue. Similarly, to *NKA* its expression in 50 ppt treatment was significantly higher compared to other treatments. In euryhaline bony fish, gills usually secrete extra chlorine to maintain a constant plasma chlorine concentration in environments of different salinity (Evans *et al.,* 2005). In the chlorine secretion model of MR cells in gill tissues, *NKCC* is expressed in the basolateral membrane to transfer chlorine from the

blood stream to MR cells that is especially important mechanism in hyperosmotic conditions of marine environment (Marshall, 2002; Evans *et al.,* 2005). Higher expression of *NKCC1a* was also observed in gills of Indian ricefish (*Oryzias dancena*) from sea and brackish waters when compared to freshwater fish (Kang *et al.,* 2010). The increase in the expression level of *NKCC1a* in MR cells of the gill tissues can suggest the higher efficacy of chlorine transfer from the plasma in *O. dancena* that inhabits brackish water, which evidences the adaptation to hypersomatic environment (Kang *et al.,* 2010). It is also reported that freshwater residing *O. latipes* shows better hypoosmoregulatory ability, while *O. dancena* residing of brackish water characterizes by higher hyperosmoregulatory adaptation abilities (Kang *et al.,* 2008).

In the study of Breves *et al*. (2010) on Mozambique (*Oreochromis mossambicus*) and Nile tilapia (*Oreochromis niloticus*) acclimated to brackish water and freshwater, after transfer to seawater, the expression of *NKCC* in gill increased in *O. mossambicus* and decreased in *O. niloticus*. It may be due to the reduced ability of *O. niloticus'* gill to excrete the excess of ions in a hyperosmotic environment and the limited capacity to increase of *NKCC* expression needed for adaptation to saline environment. The pattern of increment in *NKCC* expression levels in relation to increased salinity in *O. mossambicus* is consistent with the results in present study.

Shirangi *et al.* (2017) studied the expression of *NKA* and *NKCC* genes during short-term salinity transfer in Persian sturgeon (*Acipenser persicus*) and found that *NKA* and *NKCC* genes expression in the gills increased after transfer to brackish waters of the Caspian Sea. They suggested that the increased activity of *NKCC* during salt excretion in the Caspian Sea water can lead to sodium penetration into the chloride cells and; therefore, increasing *NKA* activity that tends to maintain intracellular sodium homeostasis.

In striped bass (*Morone saxatilis*), transfer from freshwater to seawater increased *NKA* and *NKCC* expression levels in gills after 3 days. Conversely, the transfer from seawater to freshwater decreased *NKCC* expression level in the species after 24 hours (Tipsmark *et al.,* 2004). In turns, under 15 ppt salinity that is specific for brackish water, the *NKA* activity in the species was stimulated by transfer to either freshwater or seawater conditions, suggesting both a hyper and a hypoosmoregulatory response of the gene expression (Tipsmark *et al.,* 2004).

Conclusion

It could be stated that the effect of different salinities on the expression of *NKA* and *NKCC* genes in gill tissues of *L. calcarifer* showed the lowest expression levels in the salinity conditions (15-35 ppt) that matches to the species' natural habitat. Moreover, increased levels of *NKA* and *NKCC* gene expression were observed in higher and lower salinities. Despite the species' ability to survive in wide salinity levels environments, the salinity variant of 35 ppt requires the least energy consumption needed for osmoregulation and thus some extra dynamism is available for fish metabolic activities, such as growth.

Highlights

NKA and NKCC expression showed U pattern in response to different environmental salinities in gill of *Lates calcarifer*

Lowest expression of studied genes was seen in 35 ppt in gill of *Lates calcarifer*

Highest expression of examined genes was seen in 50 ppt in gill of *Lates calcarifer*

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