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Gene expressions levels of 14-3-3a, NKCCla, A_{PO}-14, and Na^+-K^+-ATPaseβ in gill tissue of *Mugil cephalus* acclimated to low salinity

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ABSTRACT. Fishes adapt to salinity changes primarily through osmotic pressure regulation, a process often associated with several genes, including 14-3-3a, NKCCla, A_{PO}-14, and Na^+-K^+-ATPaseβ. The present study investigated the differential expression of genes 14-3-3a, NKCCla, A_{PO}-14, and Na^+-K^+-ATPaseβ in the gill tissue of *Mugil cephalus* acclimated to low salinity. Susceptibility relationships between the four gene expressions levels and salinity were detected and analyzed using polymerase chain reaction-restriction fragment length polymorphism. Homology analysis results indicated significant differences in the correlation between gene expression and salinity. Under low-salt conditions, expression levels for genes Na^+-K^+-ATPaseβ and NKCC1a were significantly elevated ($P < 0.05$), whereas those of genes 14-3-3a and A_{PO}-14 were significantly reduced ($P < 0.05$). Thus, when compared to 14-3-3a and A_{PO}-14, Na^+-K^+-ATPaseβ, and NKCC1a may be better suited to promoting the development of osmotic-regulation mechanisms and increased resistance to environmental stress under low-salt conditions. Furthermore, Na^+-K^+-ATPaseβ and

NKCC1a were identified as suitable potential molecular biomarkers for regulating and controlling genes in low-salinity aquatic environments.

Key words: 14-3-3a; NKCC1a; A_{PO}-14; Na^+-K^+-ATPaseβ; Salinity; Gene expression

INTRODUCTION

Marine fishes exhibit certain ranges of adaptation and tolerance to salinity (Shi et al., 2008), and changes in salinity can elicit a variety of physiological stress responses (Choi et al., 2008). Fishes adapt to salinity fluctuations primarily through osmotic pressure regulation, with the gills functioning as the principle organ for balancing osmotic pressure and ion levels. Osmotic adjustment mechanisms in euryhaline fishes have previously been investigated (Saoud et al., 2007): prior studies have revealed that morphological structure (Martínez-Alvarez et al., 2005) and ion transport regulation (Xing et al., 2015) can affect osmotic regulation. In recent years, molecular mechanisms pertaining to osmotic regulation in fishes (particularly Na^+-K^+-ATPaseβ gene expression and regulation) have gradually become the research focus of both domestic and foreign scholars alike (Hirose et al., 2003). In addition to Na^+-K^+-ATPaseβ, expression levels for genes 14-3-3a, NKCCla, and A_{PO}-14 with regard to osmotic regulation are also being investigated. More specifically, 14-3-3a is a candidate gene that regulates osmotic pressure; its corresponding protein controls the rearrangement of gill epithelia during salt regulation in euryhaline teleostean fishes (Fu et al., 2000). NKCCla ($Na^+/K^+/2Cl^-$ cotransporter) is possibly closely related to salt secretion in the chloride cells of gill filaments as well as to osmotic regulation, and is highly expressed in the gill tissues of *Oreochromis mossambicus* (Hiroi et al., 2005). A_{PO}-14 is a special apolipoprotein that functions in salinity adaptation responses, and is correlated with lipid metabolism in fishes (Jarvis and Ballantyne, 2003).

Recent developments in molecular biotechnology have led to the extensive application of these techniques in studies on biological markers, especially in conjunction with environmental pollution monitoring and ecological risk assessment. Biological integrated marker response values refer to the comprehensive responses of multiple biological markers based on a certain exposure. This method is used in assessing and evaluating the effects of biological toxins on rivers, seas, and other aquatic environments through simulated exposure to pollutants (Cutler and Cramb, 2008).

Mugil cephalus is a euryhaline fish species that can breed in both saltwater and freshwater, and is an economically important fish found in the Yangtze River estuary in China. Expression levels of four functional genes (14-3-3, NKCC1a, A_{PO}-14, and Na^+-K^+-ATPaseβ) in the gill tissue of *M. cephalus* were assessed under low-salt conditions using real-time quantitative RT-PCR. Relationships between gene expression and changes in salinity were compared and analyzed. Potential molecular biological markers involved in osmotic control in gill tissues were screened using the integrated biomarker response method with the intent of providing a basis of future studies on physiological regulation mechanisms in estuarine fishes. This study identifies potential biomarkers for assessing salinity growth responses in fishes, which can be of great use in artificial fish rearing operations and for obtaining higher quality fish stock.

MATERIAL AND METHODS

Fish

Natural juvenile *M. cephalus* (body length 3.35 ± 0.28 cm, body weight 0.3693 ± 0.1098 g) were collected from the Lvsi Fishing Harbor (Yellow Sea, Jiangsu, China). During a one-week acclimation period, fish were held in aquaria containing 40 L of seawater aerated and sand-filtered to sustain dissolved oxygen values at 7.0 ppm. Temperature was set at 23° ± 2.3°C and pH was 7.5 ± 0.1. Fish were fed twice daily with commercial fish pellets at rates equivalent to 1% total bodyweight for a fixed time; uneaten food was removed. The housing facility was kept in compliance with the Chinese National Standard for Laboratory Animal-Requirements of Environment and Housing Facilities (GB 14925-2001), and the care of all laboratory animals and the conduct of experimental operations conformed to Shanghai Administrative Committee for Laboratory Animals rules.

Experiments

Four different acclimation salinities (0, 5, 10, and 15 ppt) were assessed; fish were exposed to incremental reductions in salinity from 20 to 1 ppt in 5 ppt/day steps. Each experimental group consisted of 30 fish held in 40 L tanks. Daily changes of tank water volume using freshwater were performed to reduce salinity levels by the appropriate measure. Control groups were maintained in 20 ppt for the entire experiment. Fish were sampled at 0, 10, and 20 d following transition to seawater. During sampling, five fishes were netted and anesthetized using a lethal dose of 2-phenoxyethanol (2 mL/L), rapidly decapitated, and 15-20 filaments from the first gill arch were extracted and frozen in liquid nitrogen and stored at -80°C until RNA isolation and analyses of gene expression were conducted (Tipsmark et al., 2011).

Analytical techniques

Total RNA was used as the template for first-strand cDNA synthesis using the SuperScript II First-Strand Synthesis System for reverse transcription (RT)-PCR (Invitrogen, Carlsbad, CA, USA). RNA quality and concentration were verified using an Experion™ Automated Electrophoresis Station, according to RNA StdSens Analysis Kit protocols (Bio-Rad, Hercules, CA, USA). Complementary DNA was synthesized by reverse transcription carried out on 0-6 mg total RNA using qScipt cDNA SuperMix (Quanta, Gaithersburg, MD, USA) according to the manufacturer instructions.

Primers were designed using Primer3 software (Tipsmark et al., 2011) and checked using NetPrimer software (Premier Biosoft International, Palo Alto, CA, USA). All primers were purchased from Applied Biosystems (Foster City, CA, USA). Primer sequences were tested using Oligo6.0 analysis software, and specificity and uniqueness were verified using BLASTX. Additional primers are shown in Table 1.

Quantitative PCR (QPCR) assays were run on a StepOnePlus real-time PCR system (Applied Biosystems). Reactions were conducted using a cDNA amount equivalent to 15 ng total RNA, and 200 nM forward and reverse primers, with Power SYBR Green PCR Master Mix (Applied Biosystems), 15 μL total volume. Real-time PCRs were carried out in an ABI 7500 real-time PCR system (Applied Biosystems) using a program of denaturation at 95°C

for 10 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 60°C for 30 s, with a final extension at 60°C for 10 min (Yeh et al., 2010). The geNorm (version 3.5) software tool was applied to determine a normalization factor from the examined reference genes (β-actin), and was also used to normalize expression for the target genes (Vandesompele et al., 2002).

Table 1. Additional primers used (5'-3').

Primer	Forward	Reverse
β-actin	CGCACTTCCTCACGCCATCAT	GCAGCCGTCTCCATTTCTTGT
14-3-3a	CATCGCAGAGGACAAGGAGA	TCTGCTCTCGCCTGTGATGT
β-actin	TGTGATGGTGGGTATGGGT	TCGTTGTAGAAGGTGTGAT
NaKCC1a	TGTGGAACTTCTGGTTGGTATGGA	GGCTGTGATAAGGACGACGAGTAAG
β-actin	TGTGGAAAAGGCCTCACTTCA	CAGACACGACCACACGCTGT
Na^+-K^+-ATPaseβ	GCAAACCGTGTGTCATTGTGA	GGATGCTGTCGTTGGAGAAG
β-actin	TCGTGCGTGACATCAAGGAG	CGCACTTCATGATGCTGTTG3
A_{PO}-14	AAGCGCCTCCTCAATGCCTACA	GCGGGACAGCTTTTCTACCACT

Data processing

Data are reported as means ± standard deviation. ANOVA was performed using SPSS 16.0. A P value of < 0.05 indicates a significant difference. Origin 8.0 graphing and data analysis software was used for fitting and drawing relevant data.

RESULTS

NKCC1a, 14-3-3a, Na+-K+-ATPaseβ and A_{PO}-14 gene expression

Figure 1 shows the effects of low salinity on NKCC1a, 14-3-3a, Na^+-K^+-ATPaseβ, and A_{PO}-14 gene expression in the gill tissue of *M. cephalus*. NKCC1a gene expression was significantly higher in the 0, 5, and 10 ppt salinity groups than in the control group ($P < 0.05$). A_{PO}-14 gene expression in the gill tissues of *M. cephalus* exposed to low-salinity (0 ppt) stress was significantly reduced after 5 d compared to levels seen in the control group ($P < 0.05$). In contrast, gene expression was not significantly reduced in the other salinity groups. Na^+-K^+-ATPaseβ expression gradually increased as salinity declined, and was significantly elevated in the low salinity groups (0, 5, and 10 ppt) ($P < 0.05$), whereas 14-3-3a expression in the gill tissue of *M. cephalus* initially increased and then decreased under low-salinity stress, especially in the 0 and 5 ppt salinity groups.

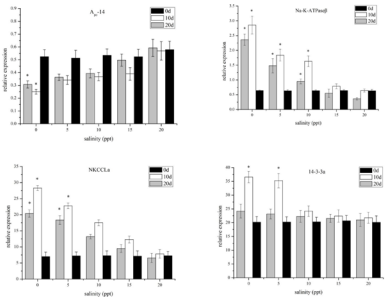

Figure 1. Relative expression of NKCC1a, 14-3-3a, Na^+-K^+-ATPaseβ, and A_{PO}-14 genes in gill tissue of *Mugil cephalus* under exposure to salinity stress. *No significant difference between salinity stress groups and the control group (20) at 0.05 level.

Comparison of 14-3-3a, NKCC1a, A_{PO}-14, and Na-K-ATPaseβ gene expression with salinity sensitivity

Variation amplitudes which represent a relative, variable relationship between the different salinity groups and the control group (20 ppt) for genes 14-3-3a, NKCC1a, A_{PO}-14, and Na^+-K^+-ATPaseβ are shown in Figure 2. Results of the present study suggest that the magnitude of low-salinity stress in *M. cephalus* under low-saline conditions was reflected by the alteration of biochemical parameters (14-3-3a, NKCC1a, A_{PO}-14, and Na^+-K^+-ATPaseβ). A significant difference is observed in both the average and amplitude values among the different gene types. The highest average and amplitude values occurred in Na^+-K^+-ATPaseβ, whereas A_{PO}-14 exhibited the lowest values. Results indicate that the sensitivities of NKCC1a and Na^+-K^+-ATPaseβ were significantly higher than those of 14-3-3a and A_{PO}-14 under low-salinity stress conditions.

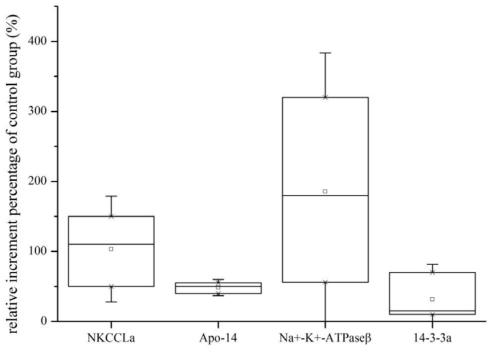

Figure 2. Relationships between gene expression levels and control group relative variety.

Table 2 shows regression relationships between expression levels of genes 14-3-3a, NKCC1a, A_{PO}-14, and Na^+-K^+-ATPaseβ and salinity among the various experimental periods. These results indicate that gene expression was not significantly correlated with salinity at inception (0 d).

Table 2. Correlation analysis of salinity and gene expression in *Mugil cephalus*.

Gene	Experiment time/day	Regression equation	Correlation coefficient
A_{PO}-14	0	y = -0.03ln(x) + 0.567	R = 0.217
	10	y = -0.17ln(x) + 0.553	R = 0.818
	20	y = -0.23ln(x) + 0.677	R = 0.787
NKCC1a	0	y = -0.043x + 7.323	R = 0.329
	10	y = 8.794ln(x) + 5.182	R = 0.927
	20	y = 12.33ln(x) + 5.935	R = 0.929
14-3-3a	0	y = -0.026x + 20.39	R = 0.035
	10	y = 2.826ln(x) + 21.27	R = 0.918
	20	y = 1.902ln(x) + 20.61	R = 0.889
Na^+-K^+-ATPaseβ	0	y = 0.000x + 0.633	R = 0.162
	10	y = 1.274ln(x) + 0.330	R = 0.936
	20	y = 1.124ln(x) + 0.063	R = 0.984

However, expression levels of the four genes did show a strong correlation with salinity (R > 0.9) after 10 and 20 d; the correlation between NKCC1a, Na^+-K^+-ATPaseβ, and salinity was especially highest during these periods. Similarly, NKCC1a, Na^+-K^+-ATPaseβ, and 14-3-3a

genes were activated under low-salinity stress (0 and 5 ppt) after 10 and 20 d, whereas A_{PO}-14 showed a negative relative response value, indicating inhibition. Relative response values of NKCC1a and Na^+-K^+-ATPaseβ to low salinity were higher than those of 14-3-3a and A_{PO}-14, indicating a strong susceptibility to low salinity in NKCC1a and Na^+-K^+-ATPaseβ.

DISCUSSION

Osmotic regulation in these fishes reflected a tolerance to salinity changes in the aquatic environment. Gills function as the major organ for regulating osmotic pressure, adjusting the pH value and ion concentration of bodily fluids, excreting ammonia and nitrogen, and regulating other functions that play a vital role in homeostasis (Shi et al., 2008). Tolerance in fishes to changes in salinity depends on the ability to osmoregulate and the metabolic recombination genes related to energy support (Hook et al., 2008).

Effects of NKCC1a gene expression

Salinity adaptation is related to the expression of ion transport protein genes and to the osmotic regulatory mechanisms in the gills. A previous study indicated that expression levels of various transport proteins (e.g., Na^+/K^+-ATPase α-subunit, $Na^+/K^+/2Cl^-$ cotransporter, and V-type ATPase) were significantly elevated in gill tissue under salinity stress (Towle et al., 2001; Luquet et al., 2005). The NKCCla protein is an electrically neutral, transmembrane transport protein; gene expression in high-salinity seawater was 4-7 times greater than that in freshwater (Inokuchi et al., 2008). Furthermore, Li et al. (2008) proved that isosmotic salinity was relatively low in tilapia (20-25 ppt). The present study also revealed low gene expression levels of NKCC1a in the gill tissue of *M. cephalus* at 20 ppt salinity. However, gene expression was significantly elevated at low salinity (0-15 ppt), possibly because the proteins tyrosine kinase or cyclic purine nucleotide-dependent kinase otherwise impaired NKCC1a sensitivity to osmotic pressure (Lemarié et al., 2004).

Effects of Na^+-K^+-ATPaseβ gene expression

Na^+-K^+-ATPase (NKA) regulates osmotic pressure (Hirose et al., 2003) and is a membrane-conjugated enzyme that predominantly exists on cell membranes. Expression of the NKA gene could partially reflect the body's metabolic and energy consumption capabilities (Iwama et al., 1999). Consistent with the characteristic high NKA activity of *M. cephalus* in hypotonic environments (Lin et al., 2006), results from this study also indicated that mRNA expression in Na^+-K^+-ATPaseβ under low-salinity (0-5 ppt) stresses was significantly higher than that under 15- and 20-ppt salinity stress. Under normal environmental conditions, energy loss due to osmotic regulation in *M. cephalus* is possibly minimal, especially given that this fish achieves one of the fastest growth rates (Woo et al., 1997). Salinity adaptation during low-salinity stress requires energy, and thus, animals must mobilize energy resources for metabolism (Iwama, 1998). In doing so, levels of cortisol and growth hormone, which stimulate mRNA expression of Na^+-K^+-ATPaseβ in the chloride cells of the gills, increased, thereby enhancing enzyme activity (McCormick, 2001). A previous study confirmed that low-salinity conditions (0-10ppt) can activate NKA activity in the muscles, enhancing the osmotic regulation ability of juvenile *M. cephalus* (Fiol et al., 2006). Thus, activation of energy metabolism components is a prerequisite for salinity adaptation in fishes (Lingrel and Kuntzweiler, 1994).

Effects of 14-3-3a gene expression

To overcome the adverse effects of hypoxia or low-salinity stress, fishes and shrimp can specifically activate the expression of some genes, or can increase expression levels of specific proteins (Machera et al., 1996). Because cell volume in the body changes under osmotic stress, such regulations involve intracellular signaling (Kültz et al., 2001). This study indicated that 14-3-3a was abundantly expressed when *M. cephalus* was transferred from a high-salt environment to a low-salt environment (0 and 5 ppt, respectively). As differences in salt concentration increase, more of the 14-3-3a gene is expressed, indicating that *M. cephalus* exhibited effective signaling cascades under salinity stress. Findings indicated that only when the 14-3-3a gene was transferred from high-salinity to low-salinity environments that it was abundantly expressed in those tissues and organs involved in osmotic regulation (Kültz et al., 2001). Elevated expression of these proteins in the gills indicated high metabolism during salinity adaptation. This phenomenon possibly occurred because the protein kinase C at the N-terminal and tyrosine kinase II phosphorylation sites may have reduced 14-3-3a gene response to osmotic pressure (Finnie et al., 1999).

Effects of A_{PO}-14 gene expression

A fish's body maintains a balanced osmotic pressure through osmotic regulation mechanisms as a means of adapting to changes in aquatic salinity levels. Osmotic regulation requires a large amount of energy, usually obtained from lipids, to meet the demands of energy metabolism (Onken and Putzenlechner, 1995). Therefore, lipid metabolism plays a very important role in salinity adaptation in fishes. Apolipoprotein also plays a key role in lipid metabolism (Jarvis and Ballantyne, 2003). In this study, expression of A_{PO}-14 apolipoprotein decreased under low-salt environments (0-5 ppt), indicating that *M. cephalus* might change apolipoprotein distribution in the body. Consequently, lipid metabolism was further altered, thus ensuring that *M. cephalus* maintained its metabolic demand for energy and osmotic balance in hypotonic environments. This is a physiological stress change resulting from adaptation to varying levels of environmental salinity. Cao et al. (2011) showed that the A_{PO}-14 apolipoprotein, as a type of osmotic regulation-associated protein in ayu (*Plecoglossus altivelis*), also played an important role in the salinity adaptation process.

Comparison of salinity sensitivity among the four genes studied

Some scholars have proposed that differential expression of functional genes under stress is indicative of biological damage to a molecular biomarker. When compared with enzyme activity, gene transcription levels could more sensitively and stably reflect the effects of environmental toxins, findings that are widely recognized by the majority of scholars (Hook et al., 2008). The gills are the major organs of ion regulation in fishes subjected to varying salinity levels. ATPase, as a primary ion carrier and channel (Cutler et al., 1995), could not only stabilize intracellular and extracellular osmotic pressure, but could also serve as a driving force within the ion transportation system (Cutler and Cramb, 2008). Increased expression of ATP synthase reflected reinforced NKA activity; thus, the corresponding chlorine cells and ion excretion cells of the gills, increased (Choi et al., 2008). Correlation analysis between gene expression among the four genes studied and salinity levels showed that Na^+-K^+-ATPaseβ

sensitivity at a salinity <20 ppt was significantly stronger than that of the three other genes. Such a significant increase in gene expression, along with enhanced metabolism and energy consumption, indicates that *M. cephalus* is adapted to low-salt environments. In molecular toxicology studies, NKA is widely used to evaluate pollution, and is an index that exhibits both universality and substantivity (Sancho et al., 1997). Whether Na^+-K^+-ATPaseβ, which regulates NKA, could be used as a parameter for evaluating salinity stress is a topic that requires further investigation.

However, low-salinity regulatory mechanisms in fish are complex. In addition to the four genes 14-3-3a, NKCCla, A_{PO}-14, and Na^+-K^+-ATPaseβ, there are many unknown-but-related genes that are similarly associated with osmotic pressure regulation. Because their biological functions remain unknown, further studies are needed.

In conclusion, the results of this study emphasized the different expression levels of 14-3-3a, NKCCla, A_{PO}-14 and Na^+-K^+-ATPaseβ in the gills of *M. cephalus* under low-saline conditions, and indicated that Na^+-K^+-ATPaseβ and NKCCla can act as potential biomarkers for low salinity. The identification of these potential biomarkers for assessing salinity growth conditions in fishes can be of great use in artificial fish rearing operations and for obtaining better quality fish stock. This will also further increase understanding of the physiological and biochemical basis of adaptation in fishes to low-salinity conditions.

Conflicts of interest

The authors declare no conflict of interest.

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