

Comparative transcriptome analysis on the alteration of gene expression in ayu (*Plecoglossus altivelis*) larvae associated with salinity change

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ABSTRACT

Ayu (*Plecoglossus altivelis*) fish, which are an amphidromous species distributed in East Asia, live in brackish water (BW) during their larval stage and in fresh water (FW) during their adult stage. In this study, we found that FW-acclimated ayu larvae exhibited a slower growth ratio compared with that of BW-acclimated larvae. However, the mechanism underlying FW acclimation on growth suppression is poorly known. We employed transcriptome analysis to investigate the differential gene expression of FW acclimation by RNA sequencing. We identified 158 upregulated and 139 downregulated transcripts in FW-acclimated ayu larvae compared with that in BW-acclimated larvae. As determined by Gene Ontology annotation and Kyoto Encyclopedia of Genes and Genomes pathway mapping, functional annotation of the genes covered diverse biological functions and processes, and included neuroendocrinology, osmotic regulation, energy metabolism, and the cytoskeleton. Transcriptional expression of several differentially expressed genes in response to FW acclimation was further confirmed by real-time quantitative PCR. In accordance with transcriptome analysis, *iodothyronine deiodinase (ID)*, *pro-opiomelanocortin (POMC)*, *betaine-homocysteine S-methyltransferase 1 (BHMT)*, *fructose-bisphosphate aldolase B (aldolase B)*, *tyrosine aminotransferase (TAT)*, and *Na⁺-K⁺ ATPase (NKA)* were upregulated after FW acclimation. Furthermore, the mRNA expressions of *b-type natriuretic peptide (BNP)* and *transgelin* were downregulated after FW acclimation. Our data indicate that FW acclimation reduced the growth rate of ayu larvae, which might result from the expression alteration of genes related to endocrine hormones, energy metabolism, and direct osmoregulation.

Keywords: *Plecoglossus altivelis*; Salinity change;

Transcriptome analysis; Growth rate; Real-time quantitative PCR

INTRODUCTION

A variety of fish undergo migration to cope with environmental fluctuations, such as temperature variation and food availability (Jorgensen & Johnsen, 2014). Some fish species migrate seaward after hatching to grow in marine habitats, and then migrate riverward to further develop in freshwater. This type of diadromy is categorized as amphidromy (McDowall, 1992). Ayu (*Plecoglossus altivelis*), the only member in the genus *Plecoglossus* of the family *Plecoglossidae*, is an amphidromous fish distributed in East Asia. After hatching in estuaries, ayu larvae migrate to the sea where they spend the winter. In spring, they move to the middle reaches of rivers, before returning to estuaries in autumn where they spawn and die. Hence, osmoregulation in ayu seems to be related to their growth and development; however, it is still unclear which genes mediate osmoregulation and growth of ayu larvae.

The growth rates of teleosts can be affected by salinity (Gillanders et al., 2015; Schwarz & Allen, 2014). Salinity induced-change in growth rates mainly results from standard metabolic rates and hormonal stimulation (Boeuf & Payan, 2001). Fish can acclimate to different salinity conditions during migration by several osmoregulatory mechanisms. Osmoregulation occurs in two consecutive phases: (1) an initial period with changing osmotic variables; and (2) a chronic regulatory period during which the variables reach new homeostasis (Holmes & Donaldson, 1969). During these two phases, altered gene expressions play an important role (Weng et al., 2002).

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Changes in the expression of $Na^+K^+ ATPase$ (NKA) in teleosts, for example, provide the primary driving force for the operation of different ion transporters according to environmental salinity (Hwang & Lee, 2007; Kang et al., 2015). In addition to affecting the ion pump, fish respond to changes in salinity with compensatory acclimations that also include regulating hormones (Peter, 2011; Sakamoto & McCormick, 2006; Yada et al., 2010), re-establishing osmotic homeostasis (Hwang & Lee, 2007), altering cell structure and function (Avella et al., 2009), and changing energy metabolism (Tseng & Hwang, 2008). The regulating genes of the above physiological processes are directly or indirectly implicated in the change in growth rates. However, gene expression alteration is dependent on species after acclimation to different osmotic stresses (Tseng & Hwang, 2008).

Transcriptome sequencing technologies are a powerful and cost-effective approach in the application of teleost investigation. Several economically or scientifically important fish species have been analyzed at the transcriptome level, including grass carp (*Ctenopharyngodon idellus*), Atlantic salmon (*Salmo salar* L.), and rainbow trout (*Oncorhynchus mykiss*) (Salem et al., 2010; Tian et al., 2015; Wang et al., 2014). Development of transcriptome sequencing in these species provides access to functional and evolutionary analyses previously restricted to genetic model organisms.

Ayu is a popular and highly valued edible fish in East Asia. In industrial aquaculture, ayu larvae are incubated in brackish water (BW) for eight weeks after hatching and then transferred to fresh water (FW) for further growth. In this study, we found that BW-acclimated ayu larvae grew faster than FW-acclimated larvae. Furthermore, we sequenced the transcriptome of a variety of tissues in ayu larvae acclimated to BW or FW. We found that FW acclimation altered the expression of genes mainly implicated in neuroendocrinology, osmotic regulation, energy metabolism, and the cytoskeleton.

MATERIALS AND METHODS

Fish and experimental conditions

Ayu larvae were kept in BW or FW tanks at 20-22 °C in a recirculating system with filtered water after hatching, and were fed with commercial pellets once a day. Groups containing 20 fish each were subjected to BW (containing 10 mg/mL) in four tanks (BW groups) or to FW in four tanks (FW groups).

Tissue sampling and sequencing

Heart, liver, spleen, gill, kidney, skin, muscle, intestine, and brain tissues from ayu after BW or FW acclimation for four weeks were mixed. Total RNA was extracted using TRIzol (Invitrogen, Shanghai, China) according to the manufacturer's instructions. The extracted RNA was treated with DNase I for 30 min at 37 °C (New England BioLabs, Beverly, MA, USA) to remove residual DNA. Oligo (dT) beads were used to isolate mRNA. After the mRNA was fragmented as templates, the cDNA first-strands were synthesized using random hexamer-primer and reverse transcriptase (Invitrogen). Second-strands were synthesized using RNaseH (Invitrogen) and DNA

polymerase I (New England BioLabs). Paired-end libraries with average insert lengths of 200 base pairs (bp) were synthesized according to Illumina (CA, USA) protocols. Transcriptome sequencing of ayu macrophages was performed at the Beijing Genomics Institute (BGI) in Shenzhen, China, using the Illumina HiSeq™ 2000 platform. Transcriptome data were deposited in the Gene Expression Omnibus (GEO) database under accession no. GSE73321.

Assembly and analysis of differential expression

Data quality control and sequence filtering of repetitive, low-complexity, and low-quality reads prior to assembly of sequenced reads for non-redundant consensus were performed using the FASTX_Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) and PRINSEQ software (Schmieder & Edwards, 2011). The default parameters were applied when using Trinity (Grabherr et al., 2011) for first assembly, and parameters “-new_ace -minmatch 12 -minscore 20 -repeat_stringency 0.9” were set for the Phrap software (Ewing & Green, 1998). The Markov Cluster Algorithm (MCL, <http://micans.org/mcl>, Stijn van Dongen) was performed as follows. First, similarity was detected using Blastn (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences with more than 90% identity and more than 60% coverage were filtered out. Subsequently, the similar sequences were used by the TRIBE_MCL algorithm. The longest sequences were selected as the representative transcripts. Gene expression levels were estimated by mapping clean reads to a reference set of assembled transcripts using RSEM (Li & Dewey, 2011). FPKM (fragments per kilobase of exon model per million mapped reads) was used as the value of gene expression levels (Trapnell et al., 2010). Differential expression was assessed using DESeq (Anders & Huber, 2010). Genes with fold change >2 or <0.05 and $P < 0.001$ indicated significant expression abundance.

BLAST against sequence databases and functional annotation

The homology of transcriptome sequences was searched using the BLASTX program against sequences in the NCBI non-redundant (NR) protein database and in the SwissProt database ($E\text{-value} < 1e-5$). Genes were tentatively identified according to the best hits against known sequences. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were performed using DAVID software (Huang et al., 2009).

Real-time quantitative PCR (RT-qPCR)

The ayu samples were collected and preserved at -80 °C after BW or FW acclimation. Total RNA was extracted and purified from ayu mixed tissue samples. After deoxyribonuclease I treatment, the isolated RNA was reverse transcribed with M-MLV Reverse Transcriptase (TaKaRa, Dalian, China). Specific primer pairs of selected genes were designed (Table 1). The reaction mixture was incubated for 300 s at 95 °C, followed by 39 amplification cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C, in a StepOne-Real Time PCR platform (Applied Biosystems, Foster City, CA, USA). The threshold cycle data

(Ct) and baselines were determined using auto settings. Ct values of genes for ayu macrophage samples were normalized

to β -actin using the $2^{-\Delta\Delta Ct}$ method, as previously described (Huang et al., 2011; Livak & Schmittgen, 2001).

Table 1 Oligonucleotide primers used to amplify cDNA of selected ayu genes

Gene	Primer	Nucleotide sequence (5'-3')	Accession number or transcript name
<i>BHMT</i>	BHMT(+)	GCAACAGCCTTAGCTTCACA	FN561754
	BHMT(-)	CTGTCTCACTCTTACAGCTC	
<i>BNP</i>	BNP(+)	CGACGGACTGATGACAAATG	comp127789_c0_seq1
	BNP(-)	TCTGCGGTTGTCCTTTCTCT	
<i>Aldolase B</i>	Aldolase B(+)	CACGAGACGCTGTACCAGAA	comp51413_c0_seq1
	Aldolase B(-)	TTCAGAACGCTCCTCCACTT	
<i>ID</i>	ID(+)	CGCCTACAAGCAGGTAAGC	comp51414_c0_seq1
	ID(-)	GGCGGTCTGAAGACTCAAAG	
<i>POMC</i>	POMC(+)	ACAGCCAGCAGGAGAAGAAG	comp116637_c0_seq1
	POMC(-)	CTGCTGTCCGTTCTTGTTGA	
<i>NKA</i>	NKA(+)	AGAGTGATGTGGGGATCAGG	comp113388_c2_seq1
	NKA(-)	AATGCTCCCCTTTGTGGTAG	
<i>Transgelin</i>	Transgelin(+)	GCCTGGCAGTCACTAAGGAG	comp106110_c0_seq1
	Transgelin(-)	TCCGTAGCTCATACCCTGCT	
<i>TAT</i>	TAT(+)	GCTTCTCAAGTCCAACCTCG	comp117135_c0_seq1
	TAT(-)	CGATGCTGGAAGACAGAACA	
β -actin	β -actin (+)	TACCGGTTGGTACATCAGCA	AB020884
	β -actin (-)	TGACGGTAAAGTTGGTGCAA	

Statistical analysis

All data are shown as means \pm SEM. Data were further analyzed by one-way analysis of variance (ANOVA) using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Growth after BW or FW acclimation

The length and body weight of ayu larvae were recorded to assess growth status after BW or FW acclimation. Three weeks after hatching, there was no significant change in ayu larvae length (Figure 1A). After four weeks, however, ayu larvae acclimated to BW were significantly longer than those acclimated to FW (Figure 1A). After eight weeks, the length of larvae acclimated to FW was only 72.3% that of the larvae acclimated to BW (Figure 1A). Two weeks after hatching, there was no significant change in ayu larvae body weight (Figure 1B). After three weeks, however, ayu larvae acclimated to BW were significantly heavier than those acclimated to FW (Figure 1B). After eight weeks, the body weight of larvae acclimated to FW was only 41.1% that of the larvae acclimated to BW (Figure 1B).

Transcriptome sequencing and assembly

Two cDNA libraries obtained from the poly(A)-selected RNAs of BW- or FW-acclimated ayu tissue samples were sequenced using the Illumina HiSeq™ 2000 platform, and approximately 79.7 (BW) and 79.2 (FW) million 90-bp pair end (PE) reads

were generated. Phrap software was applied successively to improve assembly accuracy in the presence of repeats. Through assembly and elimination of redundancy, 172 623 contigs were generated from both libraries with an average length of 1 319 bp, and an N50 of 2 292 bp. The distribution of contig length is shown in Figure 2, and varied from 201 bp to more than 2 000 bp. According to the P-values, comparison of read occurrences between the two libraries revealed that 297 unique tags were differentially represented between the libraries (absolute value of fold change > 2, composed of 139 contigs more represented in the BW library and 158 contigs more represented in the FW library).

Functional annotation based on GO and KEGG analysis

Starting with a similarity search against the main protein and nucleotide sequence databases, annotated matches were obtained from the Swiss-Prot database (36 203 unigenes) and the NR database (43 128 unigenes). To assess their evolutionary conservation, the 43 128 unigenes mapped to the NR database were searched against the sequences of other fish species in the database using the BLASTx algorithm (Figure 3). Overall, 23.5% of the NR-annotated unigenes were identified in *Maylandia zebra* and 18.6% were identified in *Oreochromis niloticus*.

Subsequent to the above analyses, the differentially expressed sequences were functionally classified using the Blast2Go program. GO terms were assigned to 297 unigenes that belonged to biological processes, cellular components, and molecular function clusters, and were distributed among more

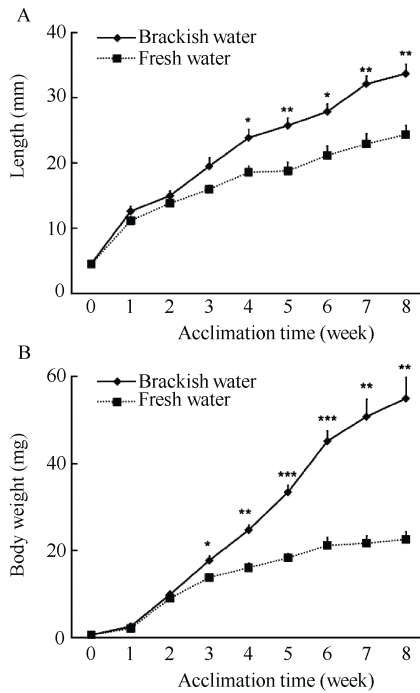


Figure 1 Effect of BW and FW acclimation on length (A) and body weight (B) of ayu larvae

Ayu larvae were acclimated to BW or FW for 8 weeks after hatching. Lengths and body weights were recorded weekly. Bars represent means± SEM of four biological replicates. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

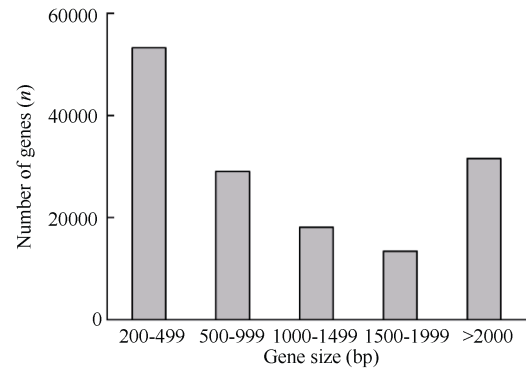


Figure 2 Summary distribution of the lengths of the assembled contigs

The final contig set contained 145 445 contigs with an N50 of 2 292 bp (≥ 200 bp, mean length=1 319 bp, max length=19 352 bp)

than 35 categories (Figure 4), including metabolism, development, apoptosis, response to stimulus, and the immune system. Among them, 62 and 14 unigenes were assigned to GO terms “metabolic process” and “response to stimulus”, respectively. The P -values of GO terms are listed in Table 2.

To investigate the overall biological function of differentially expressed genes, we assigned the unigenes based on the KEGG pathway. A total of 297 unigenes were assigned to 21 known metabolic or signaling pathways (Figure 5). Eight pathways were associated with energy metabolism, as well as the nervous and endocrine systems, including cofactor and

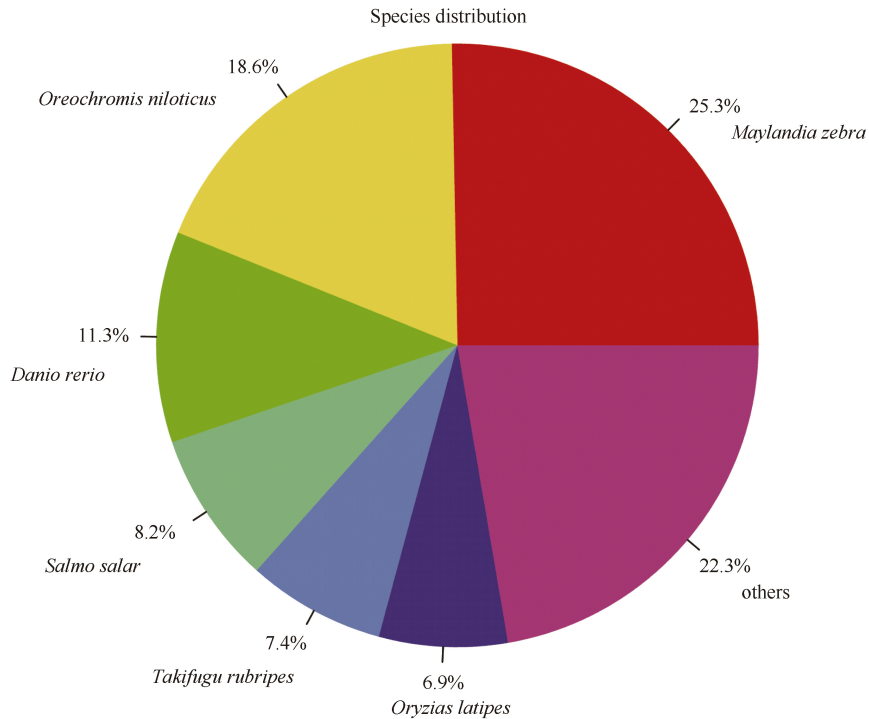


Figure 3 Percentages of the ayu unigenes conserved in other fish species

Unigenes were searched against genomic sequences of other fish species in the NR database using the BLASTx algorithm.

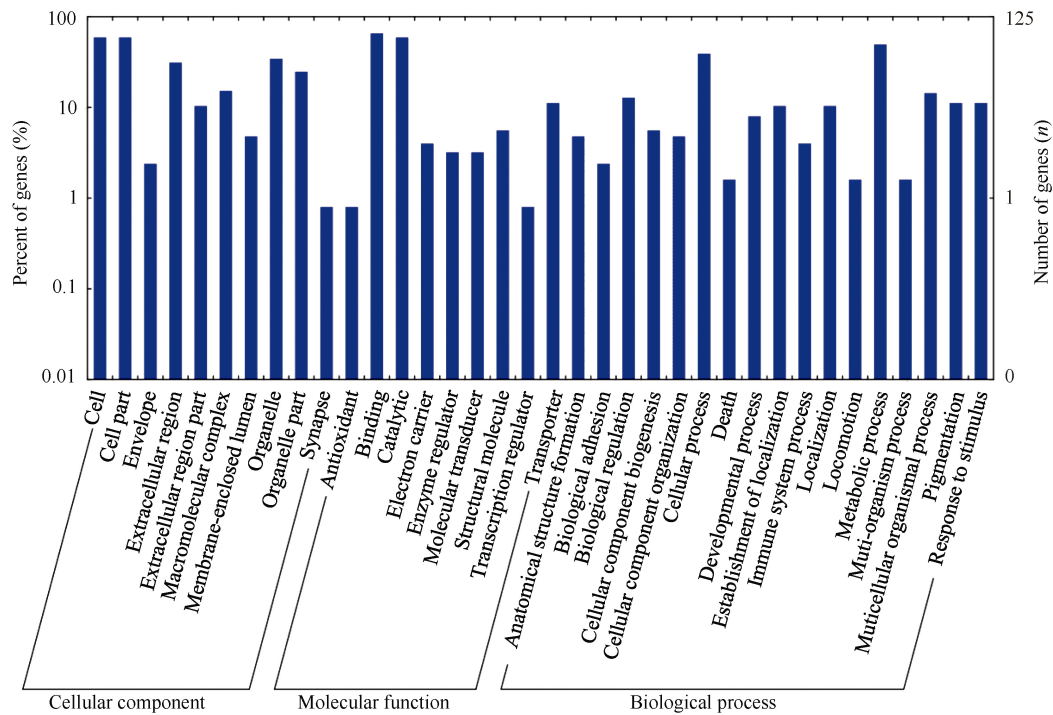


Figure 4 GO classification of differentially expressed genes

Transcripts were annotated by GO terms, and belonged to three main categories: biological processes, cellular components, and molecular functions.

Table 2 P-values of GO terms

Ontologies	GO term	P-value
Cellular Component	Cell	1
	Cell part	7.79E-05
	Envelope	0.876383
	Extracellular region	1.00E-15
	Extracellular region part	0.000665
	Macromolecular complex	0.687542
	Membrane-enclosed lumen	0.983933
	Organelle	1.33E-05
	Organelle part	0.957311
	Synapse	0.992793
Molecular Function	Antioxidant	0.20312618
	Binding	0.97707844
	Catalytic	8.44E-145
	Electron carrier	0.008248341
	Enzyme regulator	0.96959837
	Molecular transducer	2.19E-08
	Structural molecule	0.124043728
	Transcription regulator	0.999432387
	Transporter	0.171271508
	Biological Process	Anatomical structure formation

Ontologies	GO term	P-value
Biological Process	Biological adhesion	0.977677
	Biological regulation	1
	Cellular component biogenesis	0.750927
	Cellular component organization	0.999999
	Cellular process	1
	Death	0.996138
	Developmental process	0.999982
	Establishment of localization	0.000894
	Immune system process	0.52067
	Localization	0.99828
	Locomotion	0.928613
	Metabolic process	0.032464
	Multi-organism process	0.000108
	Multicellular organismal process	0.999769
	Pigmentation	1
	Response to stimulus	0.00449

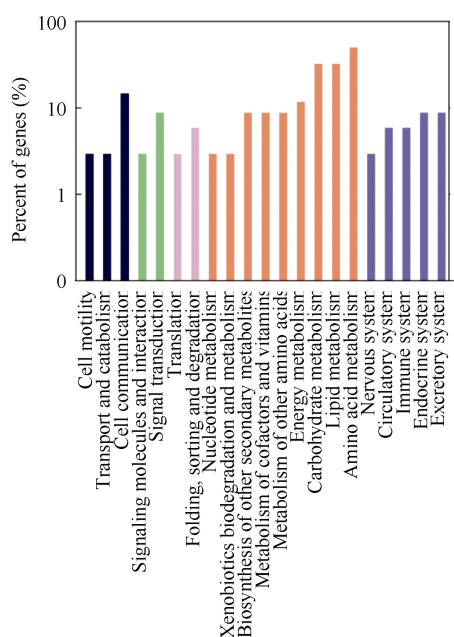


Figure 5 Functional distribution of differentially expressed genes based on KEGG analysis

vitamin metabolism, other amino acid metabolism, energy metabolism, carbohydrate metabolism, lipid metabolism, amino acid metabolism, and nervous and endocrine system processes.

Profiling gene expression patterns

We further analyzed differentially expressed genes related to salinity acclimation. Table 3 shows genes selected due to their responsiveness in the transcriptome, and which were deter-

mined to be involved in neuroendocrinology, osmotic regulation, energy metabolism, and the cytoskeleton. The transcriptional expression of several differentially expressed genes in response to salinity acclimation was further confirmed by RT-qPCR (Figure 6) at different time points. In accordance with the transcriptome analysis, data indicated that mRNA expression of *BHMT*, *aldolase B*, *ID*, *POMC*, *NKA*, and *TAT* were upregulated after FW acclimation (Figure 6); *BHMT*, *aldolase B*, and *TAT* were upregulated 3 and 4 weeks after FW acclimation; *ID* and *POMC* were upregulated at all time points; and, *NKA* was upregulated 2, 3, and 4 weeks after FW acclimation. The mRNA expressions of *BNP* and *transgelin* were downregulated after FW acclimation (Figure 6); *BNP* was downregulated at all time points; and, *transgelin* was downregulated 3 and 4 weeks after FW acclimation.

DISCUSSION

Salinity, an important environmental factor, influences the growth of fish (Boivin et al., 2015). We therefore investigated the mechanism underlying the effect of salinity on the growth of ayu. Ayu larvae live in BW initially, but move to FW habitats after several months. In this study, we found that BW-acclimated ayu larvae had a higher growth rate compared with that of FW-acclimated larvae. Furthermore, transcriptome sequencing was employed to measure changes in ayu larvae gene expression associated with changes in environmental salinity. Genes with significant expression alterations were implicated in neuroendocrinology, osmotic regulation, energy metabolism, and the cytoskeleton. This suggests that growth suppression in FW-acclimated larvae resulted from alteration in the expression of several genes associated with endocrine hormones, osmotic regulation, and energy metabolism.

Table 3 Selected differentially expressed genes as determined by transcriptome sequencing

Transcript name	Fold change (FW/BW)	Identity	P-value
Neuroendocrinology			
comp116637_c0_seq1	Only FW	<i>Pro-opiomelanocortin</i>	1.03E-08
comp51414_c0_seq1	12.846	<i>Iodothyronine deiodinase</i>	3.58E-16
comp127789_c0_seq1	0.242	<i>B-type natriuretic peptide</i>	2.78E-17
comp87338_c0_seq1	5.366	<i>Neurogranin</i>	1.78E-04
Osmotic regulation			
comp127693_c0_seq1	3.122	<i>Betaine--homocysteine S-methyltransferase 1</i>	0
comp108643_c0_seq1	2.174	<i>Inositol oxygenase</i>	1.68E-04
comp87233_c0_seq1	2.115	<i>Allantoinase</i>	5.35E-07
comp113388_c2_seq1	2.096	<i>Sodium potassium ATPase beta</i>	5.51E-08
comp117155_c0_seq1	0.277	<i>Ammonium transporter</i>	5.32E-11
Energy metabolism			
comp107204_c0_seq1	2.105	<i>4-hydroxyphenylpyruvate dioxygenase</i>	1.57E-04
comp117135_c0_seq1	2.315	<i>Tyrosine aminotransferase</i>	5.79E-08
comp51385_c0_seq1	3.125	<i>Homogentisate 1, 2-dioxygenase</i>	5.34E-19
comp117911_c0_seq1	0.048	<i>Phospholipase A2</i>	1.63E-05
comp127763_c0_seq1	2.482	<i>Fatty acid-binding protein</i>	3.60E-09
comp51523_c0_seq1	3.136	<i>Alkylglycerol monooxygenase</i>	1.22E-06
comp51413_c0_seq1	2.635	<i>Fructose-bisphosphate aldolase B</i>	0
comp119933_c0_seq2	2.197	<i>mitochondrial uncoupling protein 2</i>	6.87E-05
comp123015_c0_seq1	Only FW	<i>Vacuolar ATP synthase subunit F</i>	4.19E-06
Cytoskeleton			
comp118771_c0_seq1	0.304	<i>Scinderin</i>	9.17E-08
comp106110_c0_seq1	0.292	<i>Transgelin</i>	6.07E-07
comp112167_c1_seq1	0.325	<i>Myosin heavy chain</i>	5.84E-12
comp120851_c4_seq2	0.454	<i>Myosin-1</i>	6.17E-08
comp123726_c0_seq2	0.358	<i>Titin</i>	1.15E-04

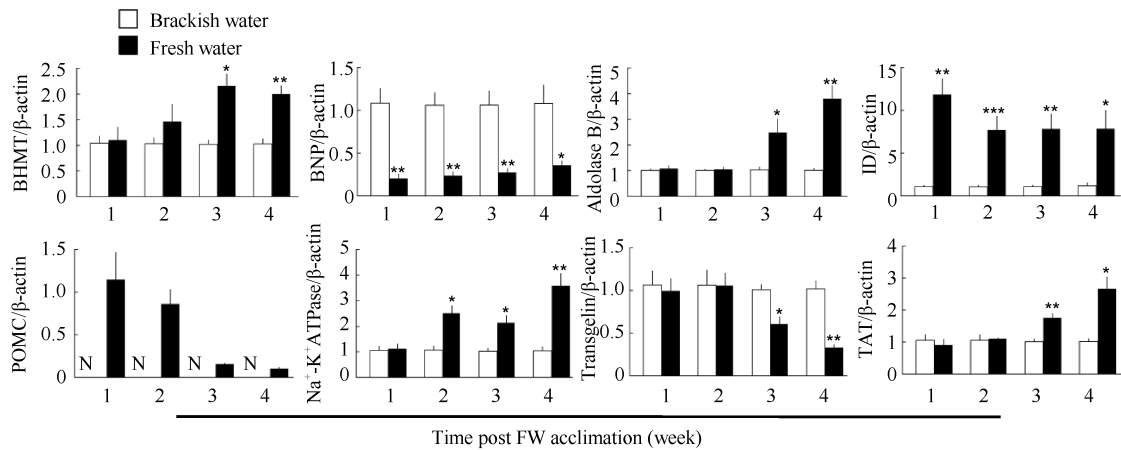


Figure 6 RT-qPCR confirmation of differential gene expression in BW- or FW-acclimated ayu

RNA was isolated from ayu multi-tissue samples and RT-qPCR was performed 1, 2, 3, and 4 weeks after BW or FW acclimation. β -actin was used for normalization. Bars represent means \pm SEM of four biological replicates. N: not detected. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Ayu, an aquacultured fish species, has been previously employed to investigate the immune system in teleosts (Chen et al., 2014; Lu et al., 2013). However, the mechanism underlying salinity acclimation is still poorly known. Hence, we used tissue-mixed samples from FW- and BW-acclimated ayu to detect differentially expressed genes by transcriptome sequencing. Tissue-mixed samples have also been used in other aquaculture fish to investigate gene expression by transcriptome sequencing (Xiao et al., 2015). Although transcriptome comparison of tissue-mixed samples can result in lost information underlying acclimation and certain measurement errors, we found 297 differentially expressed genes between FW- and BW-acclimated ayu. These genes were mainly implicated in neuroendocrinology, osmotic regulation, energy metabolism, and the cytoskeleton, which are all related to salinity acclimation. Hence, transcriptome analysis of tissue-mixed samples in this study successfully found several possible mechanisms underlying ayu salinity acclimation.

Endocrine hormones regulate a variety of physiological processes, including stress, growth, energy metabolism, and osmoregulation. In this study, FW acclimation led to the upregulation of three genes in the endocrine hormone system, that is, *POMC*, *ID*, and *BNP*. Moreover, the expression of these genes changed 1 week after FW acclimation, earlier than genes implicated in energy metabolism and osmotic regulation. These results suggest that these gene-related hormones may control osmotic regulation and energy metabolism. *POMC* can be cleaved to give rise to three peptide hormones, α -melanocyte stimulating hormone (α -MSH), adrenocorticotrophic hormone (ACTH), and β -endorphin (Böhm & Grässel, 2012). α -MSH has important roles in the regulation of appetite and sexual behavior. ACTH enhances the secretion of glucocorticoids from the adrenal cortex to regulate stress behavior. β -endorphins are endogenous opioid peptides with widespread action in the brain, including reward and pain. Mammalian α -MSH is a tridecapeptide cleaved from *POMC* that acts to inhibit food intake (Ramos et al., 2005). In the goldfish hypothalamus, α -MSH has also been implicated in constraining food consumption (Shimakura et al., 2008). The upregulated *POMC* in ayu larvae after FW acclimation might induce food intake suppression and thus inhibit growth. ACTH can stimulate gill *NKA* activity in juvenile teleosts (Langdon et al., 1984). Transcriptome sequencing and RT-qPCR data showed that the mRNA expression of *NKA* was upregulated in FW-acclimated ayu larvae. Since *NKA* will cost ATP, FW acclimation could increase energy consumption to induce growth suppression in ayu larvae.

FW acclimation in ayu larvae also upregulated a variety of genes related to energy metabolism, such as *4-hydroxyphenylpyruvate dioxygenase*, *TAT*, *fatty acid-binding protein*, *alkylglycerol monooxygenase*, and *aldolase B*. *TAT* can catalyze the conversion of tyrosine to 4-hydroxyphenylpyruvate, which is converted to homogentisate by 4-hydroxyphenylpyruvate dioxygenase. Hence, the mRNA expressed upregulation of *4-hydroxyphenylpyruvate dioxygenase* and *TAT* may promote tyrosine catabolism. Both fatty acid-binding protein and *alkylglycerol monooxygenase* were upregulated in FW-acclimated

ayu larvae. The fatty-acid-binding protein is in charge of fatty acid transport, while alkylglycerol monooxygenase can catalyze the hydroxylation of alkylglycerol, which is rich in fish liver (Hajimoradi et al., 2009). Hence, FW acclimation in ayu larvae could lead to the enhancement of lipid utilization. Aldolase B plays a key role in carbohydrate metabolism as it catalyzes one of the major steps of the glycolytic-gluconeogenic pathway (Munnich et al., 1985). This suggests that FW acclimation in ayu larvae might lead to the enrichment of carbohydrate metabolism. In addition, ATP synthase was also upregulated in FW-acclimated ayu larvae. Osmoregulation is a physiological process of energy consumption (Tseng & Hwang, 2008). Enhanced expression of ATP synthase may contribute to energy for osmoregulation. Hence, FW acclimation may enhance the utilization of amino acids and lipids to produce more ATP to regulate salinity change according to gene changes between FW- and BW-acclimated ayu larvae.

Direct osmoregulation in FW-acclimated ayu larvae included a variety of genes related to different physiological processes. Firstly, *BNP* was downregulated in FW-acclimated larvae. Natriuretic peptides are in charge of blood volume and osmosis (Loretz & Pollina, 2000; Martinez-Rumayor et al., 2008). In fish, the primary actions of natriuretic peptides are extrusion of excess salt and limitation of drinking-coupled salt uptake (Loretz & Pollina, 2000). *BNP* was downregulated one week after FW acclimation. Hence, the downregulation of *BNP* in FW-acclimated larvae might be the direct mechanism of osmoregulation. *NKA* provides the primary driving force for ion concentration regulation after changes in environmental salinity (Gonzalez, 2012). The mRNA expression of *NKA* was upregulated in FW-acclimated larvae and might directly contribute to osmoregulation via maintaining ion balance. Moreover, we found that *BHMT*, an enzyme involved in a cell volume-regulatory response via regulating betaine (Lu et al., 2010), was upregulated in FW-acclimated larvae, suggesting that FW acclimation may also regulate cell volume in ayu larvae. We also found that *transgelin*, an actin-binding protein involved in protein synthesis and destination (Assinder et al., 2009), was downregulated in FW-acclimated larvae, indicating that many protein synthesis processes might be depressed in FW-acclimated ayu larvae. Furthermore, *ID* was upregulated in FW-acclimated ayu larvae. *ID* is a subfamily of deiodinase enzymes important in the activation and deactivation of thyroid hormones, which are believed to play an osmoregulatory role in freshwater teleosts (Subash Peter et al., 2000). This suggests that the upregulation of *ID* in FW-acclimated larvae might also directly contribute to osmoregulation via regulation of the thyroid hormones.

In this study, we found that ayu larval growth was suppressed in FW-acclimated larvae compared with that in BW-acclimated larvae. This might result from hormones regulating food intake, catabolism of amino acids and lipids in FW-acclimated ayu larvae, and increased consumption of ATP to maintain osmotic pressure.

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