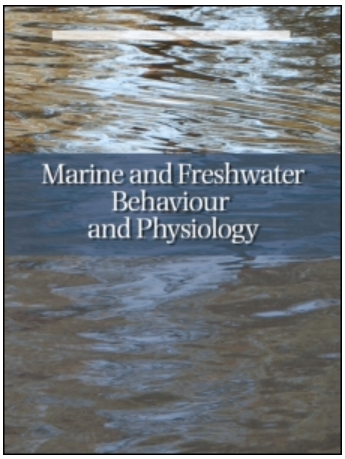

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Physiological responses and expression of arginine vasotocin receptor, prolactin and prolactin receptor mRNA in olive flounder Paralichthys olivaceus during osmotic stress

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Physiological responses and expression of arginine vasotocin receptor, prolactin and prolactin receptor mRNA in olive flounder *Paralichthys olivaceus* during osmotic stress

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We cloned complementary DNA (cDNA) encoding the arginine vasotocin receptor (AVT-R) from the kidney of olive flounder (*Paralichthys olivaceus*). Olive flounder AVT-R cDNA consists of 1155 bp, and encodes a protein of 384 amino acids. To investigate the stress responses and osmoregulatory abilities of olive flounder in changing salinities (35, 17.5, 8.75, 4, and 0 psu), we examined the expression of AVT-R, prolactin (PRL), and PRL receptor (PRL-R) messenger RNA (mRNA) in osmoregulatory organs using quantitative real time PCR (QPCR). AVT-R and PRL-R were expressed in the gill, kidney, and intestine, whereas PRL mRNA was expressed only in the pituitary gland. In addition, we measured the levels of plasma glucose, cortisol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT). The mRNA expression and plasma parameters increased in hypoosmotic environments. Furthermore, osmolality decreased significantly according to salinity concentration decreased. These results suggest that AVT-R, PRL, and PRL-R mRNA play important roles in the hormonal regulation of osmoregulatory organs, thereby improving the osmoregulatory ability of olive flounder in hypoosmotic environments.

Keywords: arginine vasotocin receptor; osmoregulation; osmotic stress; *Paralichthys olivaceus*; prolactin; prolactin receptor

Introduction

In aquaculture, salinity changes in the water cause a variety of physiological stress responses, such as changes in plasma hormones, energy metabolism, and electrolyte equilibrium. They also modify the osmotic pressure of the bodies of fish (Iwama et al. 1994), inducing osmoregulation to maintain homeostasis. Studies on the osmoregulation of fish due to salinity changes have been performed on several marine fish species, such as pufferfish and black porgy (Lee et al. 2006; Chang et al. 2007).

Arginine vasotocin (AVT) is a nonapeptide hormone released by the neurohypophysis of teleost fish and other non mammals (Acher and Chauvet 1995). It provides various physiological activation functions, such as blood pressure maintenance, antidiuretic activation and osmoregulation (Warne 2001). In mammals, this hormone is similar in function to arginine vasopressin (AVP) (Acher 1996). Changes in the osmotic pressure of a fish's body lead to changes in the concentration of AVT in the plasma

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(Kulczykowska 2001; Warne et al. 2005) and pituitary gland (Perrot et al. 1991), suggesting that AVT serves an osmoregulatory function. In addition, while mammals have three types of AVP receptors (V_1, V_{1b}, V_2), teleost fish have only one type (V_1). AVT receptor (AVT-R) has been cloned in white suckers (*Catostomus commersonii*) and flounder (*Platichthys flesus*) (Mahlmann et al. 1994; Warne 2001). Although AVT-R performs an important role in AVT activation, its other functions in teleost fish are unclear (Warne 2001).

Prolactin (PRL) is a peptide hormone released by the pituitary gland that promotes the maintenance and absorption of Na^+ in freshwater and plays an important role in ion regulation (Hirano 1986). Prolactin is a freshwater-adapting hormone (Pickford and Phillips 1959) whose synthesis and release increases as teleost fish adapt to fresh or brackish water (Olivereau et al. 1981). Moreover, during freshwater adaptation, PRL provides propulsive force to ion transportation in the chloride cells of gills by reducing Na^+ loss and moisture permeation and suppressing Na^+/K^+-ATPase activation (McCormick 1995). Therefore, PRL activation is higher in freshwater than in seawater.

Prolactin is activated when it combines with the PRL receptor (PRL-R) located on the cell surface. Prolactin receptor has been reported in several teleost fish and its expression mainly occurs in the gills, kidneys, and intestines (Higashimoto et al. 2001; Cho et al. 2006; Lee et al. 2006), where it interferes with osmoregulation. Therefore, it can be deduced that PRL and PRL-R help regulate the hydromineral balance of these organs (Lee et al. 2006).

In this study, we investigated the expression patterns of AVT-R, PRL, and PRL-R messenger RNA (mRNA) in the osmoregulatory organs of olive flounder (*Paralichthys olivaceus*), a euryhaline teleost, and analyzed the changes in plasma glucose, cortisol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels.

Methods

Experimental fish

Olive flounder (*P. olivaceus*: average length 17 ± 1.7 cm, weight 52.9 ± 14.5 g) were collected from a commercial fish farm and reared in five semi-recirculating tanks (50 L), 10 fish per tank, in a laboratory. Water temperature and photoperiod were maintained at 20 ± 1°C and a 12L/12D cycle, respectively.

Salinity changes

The flounder were kept in seawater (35 psu) for 48 h and then transferred sequentially to tanks with salinities of 17.5, 8.75, 4, and 0 psu by adding the underground water. The fish were maintained at each salinity for 48 h and were not fed during the experiments.

Sampling procedure

Five fish from each salinity were randomly selected for blood and tissues sampling and then anesthetized with tricaine methane sulfonate (MS-222, 200 mg L^{-1}) prior to blood and tissue collection. Blood was collected from the caudal vasculature with a 3 mL syringe coated with heparin. Plasma samples were separated by centrifugation (4°C, $10{,}000 \times g$, 5 min) and stored at −80°C until analysis. The tissues (pituitary gland, gill, kidney, and intestine) were sampled from five different fish at each salinity. Immediately after collection, the tissue samples were stored at −80°C until total RNA was extracted.

Identification of AVT-R cDNA

Mixed primers for AVT-R were designed using highly conserved regions of European flounder and white sucker AVT-R (GenBank accession no. AF184966 and X76321, respectively): AVT-R forward primer (5′-GCV TCC ACC TAY ATG ATG GTG-3′) and AVT-R reverse primer (5′-GTT RCA GCA GCT GTT GAG ACT-3′). Total RNA was extracted from the kidney using a TRIzol kit (Gibco/BRL, Grand Island, NY, USA). Reverse transcription (RT) was conducted using M-MLV reverse transcriptase (Bioneer, Seoul, Korea), and polymerase chain reaction (PCR) amplification was performed using BS Taq Master Mix (Biosesang, Sungnam, Korea) according to the manufacturer's instructions. Reverse transcription was conducted at 42°C for 50 min and PCR was subsequently performed for 35 cycles as follows: 1 cycle of denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, followed by 1 cycle of 5 min at 72°C for the final extension. Amplified PCR products were processed by electrophoresis using a 1% agarose gel containing ethidium bromide ($0.5\,\mu g\,\mu L^{-1}$). The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA). The colony formed by transformation was cultivated in DH5α (RBC Life Sciences, Seoul, Korea), and then plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Seoul, Korea) and EcoRI (Fermentas, Hanover, MD, USA). Based on the plasmid DNA, the AVT-R cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Rapid amplification of AVT-R cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the PCR reaction, total RNA was extracted from the kidney using the TRIzol kit (Gibco/BRL, Grand Island, NY, USA). With 3 μg total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing^{TM} full length cDNA Premix Kit (Seegene, Seoul, Korea). First-strand cDNA synthesis was conducted using an oligo (dT) anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA $T(T)_{18}$-3′) and a CapFishing^{TM} adaptor (Seegene).

Arginine vasotocin receptor-specific primers were selected from the PCR product obtained by RT-PCR. For 3′ RACE, the 50 μL PCR reaction mixture contained 5 μL 3′ RACE cDNA, 1 μL per $10\,mmol\,L^{-1}$ 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL per $10\,mmol\,L^{-1}$ 3′ RACE gene specific primer (5′-TCC TTG CCA GTC TCA ACA GCT GCT GCA-3′), and 25 μL SeeAmp Taq Plus master mix (Seegene). PCR was performed for 35 cycles as follows: 1 cycle of denaturation at 94°C for 5 min, denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 60 s, followed by 1 cycle of 5 min at 72°C for the final extension.

For 5′ RACE, the 50 μL PCR reaction mixture contained 5 μL 5′ RACE cDNA, 1 μL per $10\,mmol\,L^{-1}$ 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL per $10\,mmol\,L^{-1}$ 5′ RACE gene specific primer (5′-ACA CCA GGC TGC ACA TCC ACG TGG AGA-3′), and 25 μL SeeAmp Taq Plus master mix (Seegene). PCR was performed for 35 cycles as follows: 1 cycle of denaturation at 94°C for 5 min, denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 60 s, followed by 1 final extension cycle of 5 min at 72°C. Amplified PCR products were processed by electrophoresis using 1% agarose gels containing ethidium bromide ($0.5\,\mu g\,\mu L^{-1}$). The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega). The colony formed by transformation was cultivated in DH5α (RBC Life Sciences), and then

plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo) and EcoRI (Fermentas). Based on the plasmid DNA, the AVT-R cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems).

Phylogenetic analysis

Phylogenetic analysis was performed on the amino acid sequences from full-length AVT-R amino acid sequences from mammalian including fish. Amino acid sequences were aligned using the BioEdit software (Hall 1999). Sequences used for comparison and their GenBank accession numbers are as follows: olive flounder (EF451960), European flounder (AF184966), Burton's mouth-breeder (AF517936), Fugu rubripes (AY027887), spotted green pufferfish (CAF96006), white sucker (X76321), zebrafish (XM_678600), giant toad (AB274029), chicken (EU124684), Norway rat (CH473950), and human (CH471054). The phylogenetic tree was constructed using the neighbor-joining method with the Mega 3.1 software package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA).

Quantitative real time PCR

Quantitative real time PCR (QPCR) was conducted to determine the relative expression of AVT-R, PRL, and PRL-R mRNA during salinity changes. Primers for QPCR were designed with reference to the known sequences of olive flounder as follow: AVT-R forward primer (5′-CGC TCC TTG CCA GTC TCA AC-3′), AVT-R reverse primer (5′-TGA AGT CCG TGT TTG CTC TGT G-3′), PRL forward primer (5′-ACT CTC ATT TCC CTC CTA TTG GTC-3′), PRL reverse primer (5′-GTC TTG TCA TTG GGC GTC TGC-3′), PRL-R forward primer (5′-TGAACAATGACAACATGCTGCTAC-3′), PRL-R reverse primer (5′-CTC CAT TCA TCT CTT GGT CCT CAC-3′), β-actin forward primer (5′-GGA CCT GTA TGC CAA CAC TG-3′), and β-actin reverse primer (5′-TGA TCT CCT TCT GCA TCC TG-3′).

QPCR amplification was conducted similar to previous work (Nelson et al. 2007), using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and with the following conditions: 0.5 μL of cDNA, 0.26 μM of each primer, 0.2 mM dNTPs, Sybr green and *Taq* polymerase in buffer (10 mM Tris-HCL [pH 9.0], 50 mM KCl, 1.4 mM $MgCl_2$, 20 nM fluorescein) to a total volume of 25 μL. QPCR was carried out as follows: one cycle of denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s. Each experimental group was run in triplicate to ensure consistency. As an internal control, experiments were duplicated with β-actin, and all data were expressed as the change with respect to the corresponding β-actin calculated threshold cycle (Ct) levels. All analyses were based on the Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR program, QPCR data from three replicate samples were analyzed with analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. The efficiencies of the reactions were determined by performing the QPCR. The efficiencies were found to be as follows: β-actin = 96.3%, AVT-R = 96.2%, PRL = 93.0%, and PRL-R = 97.2%. Also, to ensure that the primers amplified a specific product, we performed a melt curve, melting at only one temperature.

Plasma parameters analysis

Plasma glucose, AST, and ALT levels were analyzed using a biochemistry autoanalyzer (model 7180; Hitachi, Tokyo, Japan). Plasma cortisol was analyzed using a radio-immunoassay kit (Diagnostic Systems Laboratories, Atlanta, GA, USA). Plasma osmolality was measured with a vapor pressure osmometer (Vapro 5520; Wescor Inc., Logan, UT, USA).

Statistical analysis

The data from each experiment were tested for significant differences using the Statistical Package for the Social Sciences software program (version 10.0; SPSS Inc., Chicago, IL, USA). One-way analysis of variance followed by a *post hoc* multiple comparison test (Duncan's multiple range test) was used to compare differences in the data at a significance level of $p < 0.05$.

Results

Identification of AVT-R cDNA

The full-length AVT-R cDNA contained 1155 nucleotides, including an open reading frame (ORF) predicted to encode a protein of 384 amino acids (GenBank accession no. EF451960). The amino acid sequence was compared to that of other teleost fish species to which it showed the following rates of identity: 92% with European flounder, 87% with Burton's mouth-brooder, 75% with white sucker, and 75% with zebrafish. *N*-glycosylation sites are present at position 6–9, 12–15, 42–45, and 169–172 residues and the seven transmembrane spanning domains are well conserved at position 30–50, 61–83, 100–120, 142–162, 189–212, 260–277, and 295–318 residues (Figure 1).

Phylogenetic analysis

The phylogenetic tree obtained by Clustal analysis of the sequences described below was shown in Figure 2. The phylogenetic analysis indicated that there are two groups of AVT-R among mammalian and teleost. In teleost group, olive flounder AVT-R was the most closely related to European flounder AVT-R, which include Pleuronectiforms.

Tissue distribution analysis of AVT-R mRNA

In the gill, AVT-R mRNA expression was the highest at a salinity of 8.75 psu, and decreased at 0 psu (Figure 3(a)). In the kidney, it was the highest at 0 psu (Figure 3(b)) and the expression of intestinal AVT-R mRNA was similar to that of the gill, and was the lowest at 0 psu (Figure 3(c)).

Tissue distribution analysis of PRL and PRL-R mRNA

In the pituitary gland, PRL expression was highest at a salinity of 8.75 psu and decreased thereafter (Figure 4(a)). In the gill and intestine, PRL-R mRNA expression was high at a salinity of 8.75 psu (Figure 4(b) and (c)) and the expression of kidney AVT-R mRNA was the highest at 0 psu (Figure 4(d)).

```
ofAVTR    1:MEKPGNITLHPNGSDPFARNEEVAQIEIMVLCITLVVAVIGNVSVLLAMYNTKKKMSRMH   60
efAVTR    1:MEKPGNITLHPNGSDPFGRNEEVAQIEIMVLSITFVVAVIGNVSVLLAMYNTKKKMSRMH   60
bmAVTR    1:MGTSGNGTDQHNGSDPFARNEEVAQIEIMVLSITFVVAVIGNVSVLLAMYNTKKKMSRMH   60
wsAVTR    1:MGRIANQTTASNDTDPFGRNEEVAKMEITVLSVTFFVAVIGNLSVLLAMHNTKKKSSRMH   60
zfAVTR    1:MGNTSNQITANNTTDPFGRNEDVAKMEITVLSVTFLVAVVGNLCVLLAMHNTKKKSSRMH   60

ofAVTR   61:LFIKHLSLADLVVAFFQVLPQLCWKVTFRFYGPDFLCRIVKHLQVTGMFASTYVMVMMTL  120
efAVTR   61:LFIKHLSLADLVVAFFQVLPQLCWEITYRFFGPDFLCRIVKHLQVTGMFASTYMMVMMTL  120
bmAVTR   61:LFIKHLSLADLVVAFFQVLPQLCWEITFRFYGPDFLCRIVKHLQVMGMFASTYMMVMMTL  120
wsAVTR   61:LFIKHLSLADMVVAFFQVLPQLCWEITFRFYGPDFLCRIVKHLQVLGMFASTYMMVMMTL  120
zfAVTR   61:LFIKHLSLADLVVAFFQVLPQLCWEITYRFYGPDFLCRIVKHLQVLGMFASTYMMVMMTL  120

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efAVTR  121:DRYIAICHPLKTLQQPTQRSYIMIVSTWMCSLVFSTPQYFIFSLSEVKNGSTVKDCWAHF  180
bmAVTR  121:DRYIAICHPLKTLQQSTRRSYIMIISTWICSLVLSCSQYFIFSLSEIQNGSGVYDCWARF  180
wsAVTR  121:DRYIAICHPLKTLQQPTQRAYIMIGSTWLCSLLLSTPQYFIFSLSEIQNGSYVYDCWGHF  180
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efAVTR  181:IEPWGARAYITWITGGIFLVPVVILVMCYGFICHTIWKNIKYKKRKTIPGAASKNGLIGK  240
bmAVTR  181:IEPWGARAYITWITVGIFLVPVVLLMMCYGFICHSIWKNIKYKKRKTTAGAASRNGLIGK  240
wsAVTR  181:IEPWGIRAYITWITVGIFLIPVIILMICYGFICHSIWKNIKCKTMR-G-TRNTKDGMIGK  238
zfAVTR  181:IEPWGIRAYITWITVGIFLIPVVILMICYGFICHSIWKNFKCKTKR-GAAHNPKSGMIGK  239

ofAVTR  241:NSVSSVTTISRAKLRTVKMTFVIVLAYIICWAPFFTVQMWSVWDENFMYADSENTAVTLS  300
efAVTR  241:NSVSSVTTISRAKLRTVKMTFVIVLAYIICWAPFFTVQMWSVWDENFQYADSENTAVTIS  300
bmAVTR  241:NSVSSVTTISRAKLRTVKMTFVIVLAYIVCWAPFFIVQMWSVWDENFQWDDSENTAVSLS  300
wsAVTR  239:VSVSSVTIISRAKLRTVKMTLVIVLAYIVCWAPFFIVQMWSVWDENFSWDDSENAAVTLS  298
zfAVTR  240:ASVSSVTIISRAKLRTVKMTFVIVLAYIVCWAPFFTVQMWSVWDENFSWDDSENAAVTLS  299

ofAVTR  301:ALLASLNSCCNPWIYMIFSGHLLQDFVHCFSCCHRANTDFKKEDSDSSIRRTTLLTKM-T  359
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bmAVTR 301:ALLASLNSCCNPWIYMIFSGHLLQDFVQCFSCCRKINTDFKKEDSDSSLRRTTLLTKM-T  359
wsAVTR 299:ALLASLNSCCNPWIYMLFSGHLLYDFLRCFPCCKKPRNMLQKEDSDSSIRRNTLLTKLAA  358
zfAVTR  300:ALLASLNSCCNPWIYMLFSGHLLHDFLGCFPCWNKPQNTLHKEDSDSSIRRNTLLSKLTS  359

ofAVTR  360:NR-SP-TGSTGNWRELD-NSPKTSIQME--------------------------------  384
efAVTR  360:NR-SP-TGSTGNWRDLD-NSPKTSIQME--------------------------------  384
bmAVTR 360:NR-SP-TARTFFSRNPD-AFKGCCASPQCCRTP--FPVKVFTKIKPI-------------  401
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zfAVTR  360:VR-AKDGFDSWKDPCNSRKSSQSIGLDYSRKSSQCLHFDCSRKSSQSVPMES--------  410

ofAVTR  385:----------------  384
efAVTR  385:----------------  384
bmAVTR 402:----------------  401
wsAVTR 419:PLDCSRKSSQCMSKES  434
zfAVTR  411:----------------  410
```

Figure 1. Comparison of the amino acid sequence of the AVT-R of olive flounder (*P. olivaceus*), European flounder (*Platichthys flesus*), Burton's mouth-brooder (*Haplochromis* [*Astatotilapia*] *burtoni*), white sucker (*Catostomus commersonii*), and zebrafish (*Danio rerio*), optimally aligned to match identical residues, as indicated by the shaded box. The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. The AVT-R sequences used for alignment were olive flounder AVT-R (ofAVT-R, EF451960), European flounder AVT-R (efAVT-R, AF184966), Burton's mouth-brooder AVT-R (bmAVT-R, AF517936), white sucker AVT-R (wsAVT-R, X76321), and zebrafish AVT-R (zfAVT-R, XM_678600). The seven putative transmembrane-spanning domains are lined and *N*-glycosylation site are boxed.

Plasma osmolality

Plasma osmolality was $337.50 \pm 2.42\,\mathrm{mOsm\,kg^{-1}}$ at the start of the experiment, and then it reached its lowest levels at 0 psu ($294.5 \pm 4\,\mathrm{mOsm\,kg^{-1}}$; Figure 5).

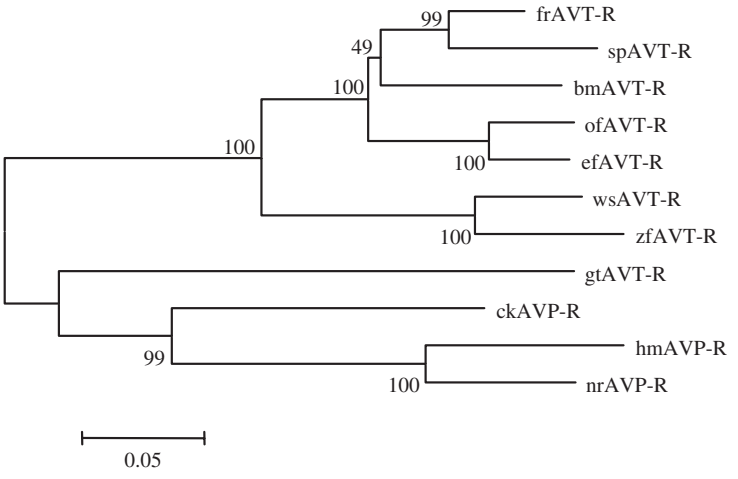

Figure 2. Phylogenetic tree based on an amino acid alignment for AVT-R in teleost fish. Bootstrap values (%) are indicated (1000 replicates). The score between two protein sequences, which is a measure of the relative phylogenetic relationship between the two proteins, is represented by the horizontal distance in this tree, i.e. the shorter the distance, the more related they are. Genbank accession number of the sequences are: olive flounder AVT-R (ofVAT-R, EF451960), European flounder AVT-R (efAVT-R, AF184966), Burton's mouth-brooder AVT-R (bmAVT-R, AF517936), Fugu rubripes AVT-R (frAVT-R, AY027887), spotted green pufferfish AVT-R (spAVT-R, CAF96006), white sucker AVT-R (wsAVT-R, X76321), zebrafish AVT-R (zfAVT-R, XM_678600), giant toad AVT-R (gtAVT-R, AB274029), chicken AVP-R (ckAVP-R, EU124684), Norway rat AVP-R (nrAVP-R, CH473950) and human AVP-R (hmAVP-R, CH471054).

Plasma parameters analysis

Plasma glucose, cortisol, AST, and ALT levels during salinity concentration changes are shown in Figure 6. During the experimental period, no significant differences were observed in plasma glucose levels among the groups. The highest level was measured at 0 psu salinity (Figure 6(a)). Plasma cortisol levels were $0.3 \pm 0.02\,\mu g\,dL^{-1}$ at the start of the experiment, reached their highest level of $0.88 \pm 0.01\,\mu g\,dL^{-1}$ at 0 psu salinity (Figure 6(b)). Aspartate aminotransferase and alanine aminotransferase levels were similar to the cortisol levels, reached their highest levels at 0 psu (Figure 6(c) and (d)).

Discussion

Olive flounder are euryhaline teleosts, which show relatively little stress to changes in salinity. However, hyposalinity during the summer season caused by local environmental factors, such as torrential downpours, may cause changes in the physiological state of the fish and adversely affect its survival. Therefore, we compared the expression patterns of AVT-R, PRL, and PRL-R mRNA in various osmoregulatory organs of olive flounder under changing salinities, and examined their osmoregulatory capacity through plasma hormone analysis.

The AVT-R of olive flounder contains 1155 bp with 384 amino acids in the ORF, and shows a high level of identity to that of other teleost fish. The olive flounder AVT-R has a

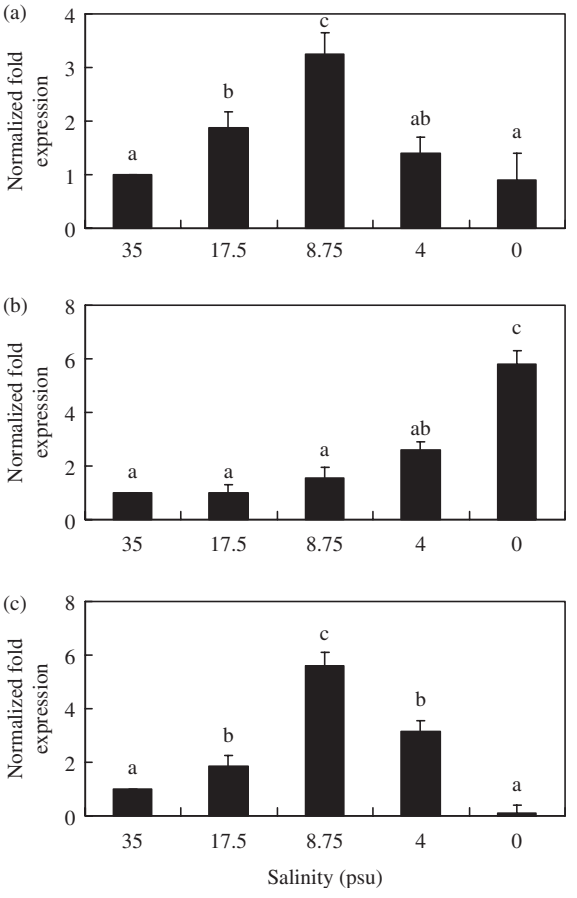

Figure 3. Expression of AVT-R mRNA in tissues of olive flounder. Three micrograms of total RNA prepared from the gill (a), kidney (b), and intestine (c) was reverse-transcribed and amplified using a gene specific primer. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample, and the mean value of the control was set to 1. Values with dissimilar letters are significantly different ($p < 0.05$). Values are mean $\pm$ SD ($n = 5$).

seven-transmembrane spanning structure with the *N* terminus outside the cell. This structure is typical of previously described neurohypophysial peptide receptors and the larger superfamily of G-protein-coupled receptors (Warne 2001; Figure 1). The phylogenetic analysis indicated that olive flounder AVT-R was closely similar to that among other fish, and teleost AVT-R was similar with mammalian AVP-R (V_1 type). In teleost group, olive flounder AVT-R was the most closely related to European flounder AVT-R, which include Pleuronectiforms (Figure 2).

In the gills and intestines, AVT-R mRNA expression was highest at a salinity of 8.75 psu, and decreased thereafter. In the kidneys, expression was highest at 0 psu (Figure 3). Based on this result and the report that AVT interferes with both diuretic and antidiuretic actions in kidneys (Amer and Brown 1995), AVT is also considered to be involved in osmoregulation (Warne and Balment 1995) and the demonstration of AVT-R mRNA expression in the gills and kidney is consistent with the present study. So we deduced that water permeated into the fish and AVT-R mRNA was increased in these

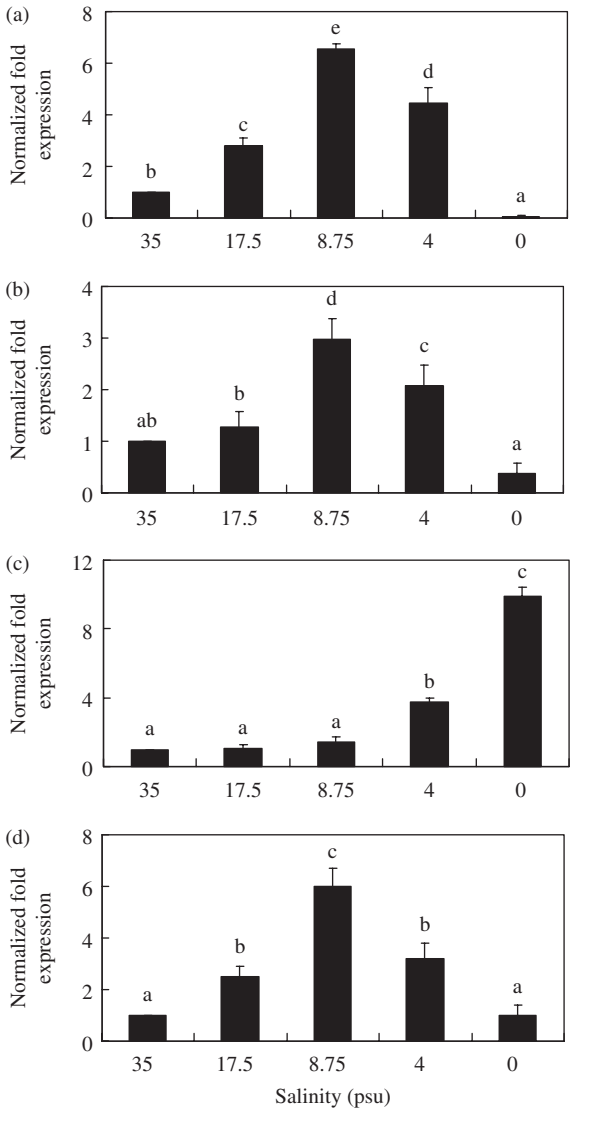

Figure 4. Expression of PRL and PRL-R mRNA in tissues of olive flounder. Three micrograms of total RNA prepared from the pituitary gland (a), gill (b), kidney (c), and intestine (d) was reverse-transcribed and amplified using gene specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample, and the mean value of the control was set to 1. Values with dissimilar letters are significantly different ($p < 0.05$). Values are mean $\pm$ SD ($n = 5$).

tissues to osmoregulate as the external osmotic pressure decreased. This led to a decrease in the plasma osmolality and an imbalance in osmotic pressure. In response, water was discharged from the gills, kidneys, and intestines to regulate internal osmotic pressure.

Prolactin plays an important role in freshwater adaptation of teleost fish. In this study, we found that PRL mRNA expression in the pituitary gland of olive flounder significantly

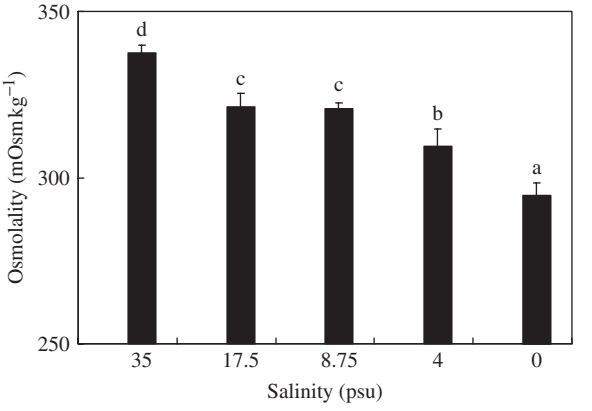

Figure 5. Levels of plasma osmolality in olive flounder after salinity changes. Each value represents the mean ± SD ($n = 5$). Values with dissimilar letters are significantly different ($p < 0.05$).

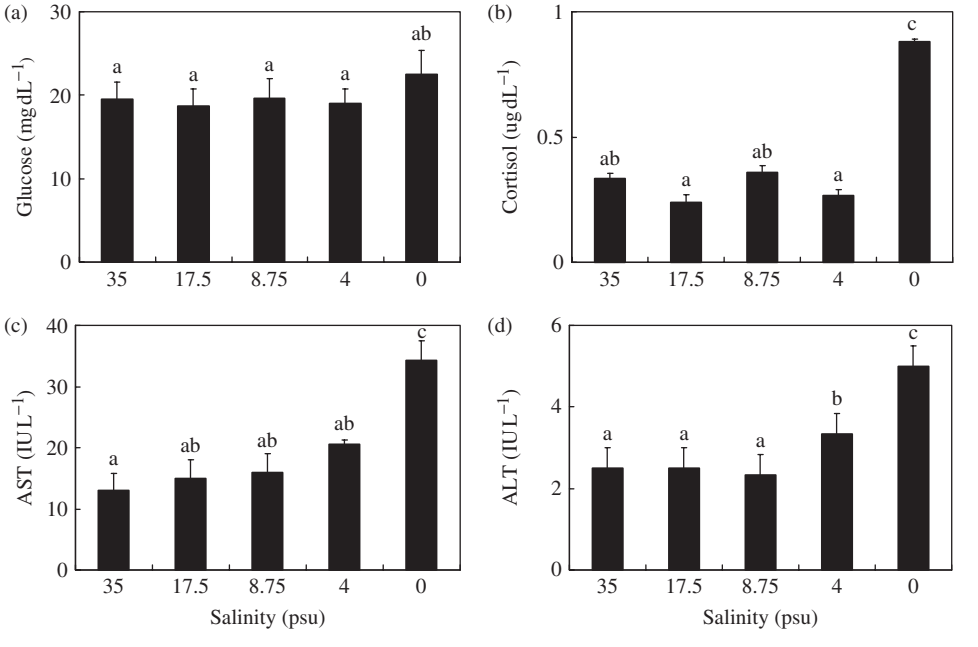

Figure 6. Levels of plasma glucose (a), cortisol (b), AST (c), and ALT (d) in olive flounder after salinity changes. Each value represents the mean ± SD ($n = 5$). Values with dissimilar letters are significantly different ($p < 0.05$).

increased as salinity decreased, suggesting an increase in plasma PRL. Cho et al. (2006) reported the same trend in olive flounder, and other studies have found similar increases in PRL mRNA expression in fugu (*Takifugu rubripes*) moved from 35 to 25 psu seawater (Lee et al. 2006), and freshwater-adapting Mozambique tilapia (*Oreochromis mossambicus*; Yada et al. 1994) and black porgy (*Acanthopagrus schlegeli*; Chang et al. 2007). We also found that PRL mRNA expression peaked at a salinity of 8.75 psu, and then tended to decrease thereafter. It is suggested that there is an imbalance in osmotic pressure at

8.75 psu and increased expression of PRL mRNA to regulate the osmotic pressure during salinity change. We therefore deduced that water permeated into the fish as the external osmotic pressure decreased, and that the increase of PRL mRNA means discharge of water to maintain the osmotic pressure in hyposalinity.

The physiological functions that maintain ion concentration levels in fish during salinity changes are largely influenced by related hormones, but also by the expression of hormone receptors in osmoregulatory organs such as the gills, kidneys, and intestines. The hormone signal becomes functional as it is transported into the cells of these osmoregulatory organs. In this study, the expression of PRL-R mRNA, the receptor of PRL that interferes with osmoregulation, was observed in the gills, kidneys, and intestines. In all of these organs, PRL-R mRNA was increased as salinity decreased, suggesting an imbalance in osmotic pressure. In addition, at this concentration, PRL was released in the pituitary gland, where it combined with PRL-R in the gills, kidneys, and intestines to maintain internal homeostasis by interfering with osmoregulation (Higashimoto et al. 2001; Santos et al. 2001; Lee et al. 2006).

Consequently, increased PRL-R mRNA expression in these tissues activates the PRL–PRL-R system to increase water discharge and Na^+ reabsorption (McCormick 1995) and AVT-R mRNA expression in these tissues means discharge of water by diuretic actions to regulate the osmotic pressure (Amer and Brown 1995). So, we deduce that AVT-R together with PRL–PRL-R system serves an important role in the adaptation of the olive flounder to hyposaline conditions.

We also compared plasma osmolality, glucose, AST, ALT, and cortisol levels. Plasma osmolality decreased with decreasing salinity, possibly because ions were removed from the body as the fish adapted to the hypoosmotic environment, and osmoregulation was disrupted. In addition, we observed that osmolality recovered to the initial (normal) seawater level at salinity levels higher than 0 psu. These results correspond to those of Mancera et al. (1993) and Chang et al. (2007).

Salinity changes cause physiological stress to fish. In this study, we used plasma cortisol levels as a stress index. The plasma cortisol levels tended to increase as salinity decreased, suggesting that changes in salinity caused stress and activated the hypothalamus-pituitary-interrenal axis. This has been reported in many other fish species (Redding and Schreck 1983; Mancera et al. 1993). Plasma cortisol levels were highest at a salinity of 0 psu, which suggests that olive flounder are highly stressed at this salinity. In addition, the increase in plasma glucose at salinities above 0 psu suggests that stress-related increases in cortisol causes gluconeogenesis in the liver and results in high blood sugar to compensate for stress-related increases in energy requirements (Vijayan et al. 1997).

Aspartate aminotransferase and alanine aminotransferase are general indices of liver function in vertebrates. In fish, they are used to evaluate stress reactions due to changes in water temperature, hypoxia, pH, ammonia, and heavy metals (Pan et al. 2003). In this study, AST and ALT levels increased as salinity decrease peaked at a salinity of 0 psu. Their patterns were similar to the changes in cortisol and glucose levels, suggesting that in olive flounder, stress due to changes in salinity damages liver cells and degrade liver function.

In summary, we found that plasma cortisol and glucose levels in olive flounder increased as the fish were stressed by decreased salinity. Increases in plasma AST and ALT levels suggest that this stress damaged liver cells. Furthermore, increases in the expression of AVT-R, PRL, and PRL-R in osmoregulatory organs suggest that olive flounder are able to osmoregulate in a hypoosmotic environment.

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