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Transient Response of Brain Heat Shock Proteins 70 and 90 to Acute Osmotic Stress in Tilapia (*Oreochromis mossambicus*)

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Meng-Wen Yang, Wei-Tung Huang, May-Jywan Tsai, Ing-Fong Jiang, and Ching-Feng Weng (2009) Transient response of brain heat shock proteins 70 and 90 to acute osmotic stress in tilapia (*Oreochromis mossambicus*). *Zoological Studies* **48**(6): 723-736. Previous studies demonstrated that gills play a major role in osmoregulation during salinity changes. However, the regulatory mechanism in the tilapia brain under osmolarity challenge remains unexplored. Tilapia *Oreochromis mossambicus* were transferred directly from fresh water (FW) to 25 ppt seawater (SW) for 1, 2, 4, 8, and 24 h; or to 35 ppt SW for 1, 2, and 4 h, to determine whether heat shock protein (HSP)70 and HSP90 in the brain respond to acute hyperosmotic stress. After transfer to 25 ppt SW, HSP70 and HSP90 messenger (m) RNA remained at higher levels compared to the FW group. HSP70 and HSP90 protein levels in the anterior and middle brain regions at 1 h after SW transfer were higher than those of the FW group, and they had increased in the whole brain at 1 h and were sustained to 4 h following transfer. After transfer to 35 ppt SW, HSP70 mRNA had decreased at 1 h and increased at 4 h compared to values in FW; however, a lower HSP90 mRNA level was observed at 4 h. For the whole brain, protein levels of HSP70 and HSP90 after transfer to 35 ppt SW increased compared to those in FW except at 2 h after transfer. The results suggest that HSP70 and HSP90 may be involved in regulating the tilapia brain when faced with acute osmotic stress and may subsequently facilitate adaptation to a SW environment.
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Key words: HSP70, HSP90, Hyperosmotic, Brain, Tilapia, *Oreochromis mossambicus*.

Tilapia, an euryhaline teleost, can adapt to different salinity levels of seawater (SW). Our previous study demonstrated that the physiological responses involve 2 isoforms of creatine kinase expressed at slightly higher levels in gills after SW transfer (Gong et al. 2004), and creatine kinase provides an energy source in gills (Weng et al. 2002a, Lin et al. 2003) and the brain (Weng et al. 2002b) when tilapia are transferred from freshwater (FW) to 25 ppt SW. However, the regulatory mechanism in the tilapia brain under osmolarity challenge remains unexplored. Astrocytes, the principal component of glial cells in the brain, support normal a central nervous system by optimizing ion and pH homeostasis. Other studies demonstrated that cultured astrocytes under varying osmotic conditions have numerous biochemical and physiological responses, such as augmentation of water channel (Arima et al. 2003) messenger (m) RNA and protein expression, osmolyte synthesis of enzymes and transporter expression (Bitoun and Tappaz 2000), and activation of p38 kinase activity (Xu et al. 2001) during hyperosmotic stress, whereas an alteration in volume regulation through the chloride channel (Parkerson and Sontheimer 2003) and induction of a calcium response (Fischer et al. 1997) were found under a hypo-osmotic challenge. Previously,

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our *in vivo* study demonstrated that the mRNA level of the pituitary adenylate cyclase-activating polypeptide ($tPACAP_{38}$) in the tilapia brain substantially increased within 8 h after transfer from FW to SW (Lo et al. 2007). Additionally, that study first demonstrated that PACAP expression of tilapia astrocytes significantly increased after NaCl treatment but not with mannitol treatment, suggesting that the expression of $tPACAP_{38}$ is induced by ions (Na^+ and Cl^-) in a hyperosmolar condition, and that PACAP can play a regulatory role in tilapia's adaptation to SW.

Heat shock proteins (HSPs), a family of molecular chaperones, are critical proteins in cellular responses to stress. The 70- (HSP70) and 90-kDa (HSP90) proteins are 2 of the most widely investigated HSPs. Furthermore, HSPs are also involved in molecular chaperoning of steroid receptors, and glucocorticoid receptor (GR) regulation is strongly associated with the level of HSPs. Abundant in unstressed cells, HSP90 is crucial to GR functions, such as ligand binding and GR signaling (Pratt 1997). Additionally, an *in vivo* study showed that reduced HSP90 levels lead to a decrease in steroid receptor action. Recently, cortisol was shown to attenuate heat-induced HSP90 mRNA abundance in trout hepatocytes (Sathiyaa et al. 2001). Constitutive HSP70 occurs in unstressed cells, whereas inducible HSP70 is synthesized in response to acute stress, such as exposure of fish to copper (Boone et al. 2002). HSP70 plays an important role in performing protein renaturation (polypeptide folding) in the cytoplasm. It acts as a non-specific molecular chaperone which protects ion transporters from degradation and helps maintain normal ion concentrations in the cytoplasm (Smith et al. 1999). Fish HSP70s have been cloned from many species; most studies examined HSP70 at the RNA level (Basu et al. 2002 and references therein). Some HSP70s in fish or cultured cells are heat inducible, e.g., Japanese flounder embryonic HSP70 (Yokoyama et al. 1998), HSP70 in platyfish fibroblast cells (Yamashita et al. 2004), and HSP70 in rainbow trout RTG-2 cells (Ojima et al. 2005). Black sea bream (*Spondyliosoma cantharus*) exposed to saline stress (hyperosmotic-hypersaline) or heat shock rapidly upregulate stress hepatic HSP70 proteins (Deane et al. 2002). Fasting increased plasma cortisol levels and brain GR content hindered HSP90 and interrenal cortisol production in anadromous Arctic char (*Salvelinus alpinus*) (Aluru et al. 2004). Overexpressions of HSP70, HSP90, metallothioneins, and cytochrome P^{450} 1A mRNAs were observed in liver and brain tissues of sea bass (*Dicentrarchus labrax*, L.) reared under high population densities (Iwama et al. 1999, Gornati et al. 2004). Chinook salmon (*Oncorhynchus tshawytscha*) exposed to elevated temperatures (10.6-21.6°C) for 5 h showed markedly increased HSP90 mRNA accumulation in heart, brain, gill, muscle, liver, kidney, and tail-fin tissues (Palmisano et al. 2000). However, the extent of osmotic stress in euryhaline fish particularly in portions of the brain remains unclear. This study attempted to investigate whether HSP70 and HSP90 in tilapia brain respond to hyperosmotic stress.

MATERIALS AND METHODS

Animals

Tilapia (*O. mossambicus*) were obtained from the Mariculture Research Center of the Fisheries Research Institute, Council of Agriculture, Executive Yuan (Tainan, Taiwan), maintained in a FW recirculation tank at 25-28°C, and fed commercial fish feed daily (Tung-Li Feed Industrial, Pingtung, Taiwan). The experiments were performed according to the *Guide for the Care and Use of Laboratory Animals* of National Dong-Hwa University (Hualien, Taiwan). Male and female tilapia (body weight, 80-100 g) were utilized for the experiment. The mortality was 100% at 6 h after tilapia were directly transferred from FW to 35 ppt SW (Weng et al. 2002a). Therefore, exposure for more than 4 h to 35 ppt SW treatment was avoided in this work.

SW transfer treatments

Tilapia were directly subjected to 2 SW treatments: direct transfer from FW to 25 ppt artificial SW for 1, 2, 4, 8, and 24 h; and transfer from FW to 35 ppt SW for 1, 2, and 4 h. For control fish, FW-adapted fish were transferred from FW to FW at each time point of other fish being transferred to SW. We found that no significant differences existed in the expressions of genes examined among various times of FW transfer in our preliminary experiment (data not shown). This result is associated with 1 report which demonstrated that hepatic HSP70 expression in other teleost species (salmonids) was not affected by handling stress (Vijayan et al. 1997a). Thus,

fish retained in FW were considered to be the control group. At the times indicated, fish were anesthetized by immersion in a solution of tricaine methane sulfonate and immediately sacrificed. The brain (sample size shown in the figure legends) was immediately removed for semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and cut into 3 (anterior, middle, and hind) portions for the Western blot analysis. The anterior region contained the telencephalon and olfactory bulb. The middle region comprised the diencephalon, midbrain tegmentum, tectum and valvulae cerebelli, and pituitary. The hind region contained the rhombencephalon, cerebellum, and vagal lobe (Fig. 1). The brain was removed and homogenized for RNA isolation using RNA^{TM}-Bee (Tel-Test, Friendswood, TX, USA) according to the manufacturer's protocol.

Complementary (c) DNA synthesis by RT-PCR

cDNAs were generated using Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA) primed with oligo $(dT)_{25}$ and a random hexamer (Invitrogen). Total RNA (5 μg) from the brain was dissolved in 9 μl of DEPC water with 1 μl oligo $(dT)_{25}$ (0.5 μg/μl), and 1 μl dNTPmix (10 mM) denatured at 65°C for 5 min and then immediately chilled on ice for 1 min. Denatured RNA was mixed with 4 μl of 5 x first-strand buffer, 2 μl of 0.1 M DTT, and 1 μl RNAse Out (40 U), and then denatured at 42°C for 2 min. Subsequently, 1 μl of random hexamer (50 ng/μl) and 1 μl of Superscript II transcriptase (200 U) were added and incubated at 25°C for 2 min. The reaction conditions were set to 1 cycle at 25°C for 10 min, 42°C for 90 min, and 70°C for 15 min. Following reverse transcription (RT), the product was incubated with 1 μl (2 U) *Escherichia coli* RNAse H at 37°C for 20 min. Following reverse transcription, samples were diluted 2 fold in sterilized distilled water and stored at -20°C until the analysis.

PCR and cloning of HSP70 and HSP90 from tilapia brain

The nucleotide sequences of *Oreochromis mossambicus* (GenBank accession no. **AJ001312**) and *Oncorhynchus mykiss* (GenBank accession no. **AB196457**) were used to create primer sequences for HSP70 (sense: 5'-TGAGGTGGA TTTATGTCTATGCTT A-3' and antisense: 5'-CG TTGGCGATGATTTCTACTTTC-3') and HSP90 (sense: 5'-GTTCCGTGCTCTCCTCTTTATC-3' and antisense: 5'-CACCTTCTCTACTTTCTTGTC CAG-3'). And 1 μl of diluted RT products (25 ng cDNA) was amplified by PCR (*Taq* polymerase was purchased from Yeastern Biotech, Taipei, Taiwan). Reactions for HSP70 and HSP90 involved 25 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. All PCR products were purified with a Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taoyuan County, Taiwan), cloned into the pGEM® T-Easy Vector System (Promega, Madison, WI, USA), and sequenced using an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequence was blasted with HSP70 (GenBank accession no. **AJ001312**) or HSP90 (GenBank accession no. **AB196457**), respectively to confirm the amplicon.

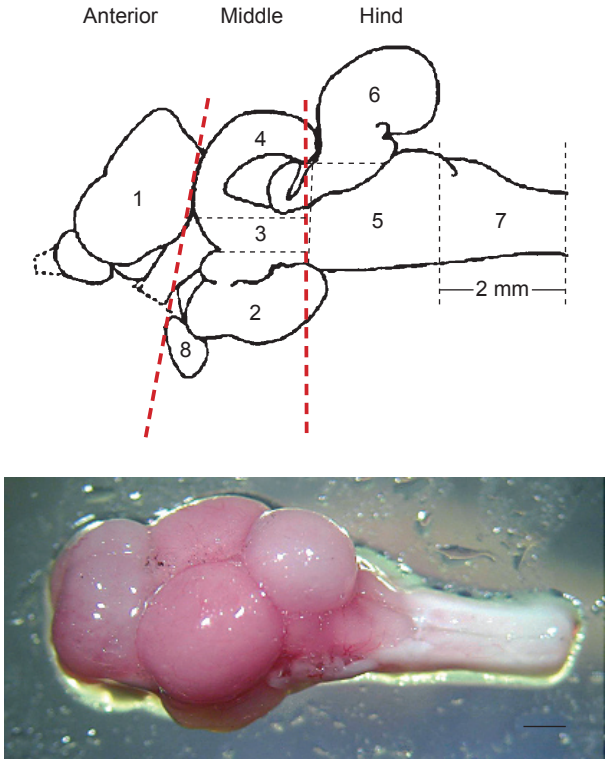

Fig. 1. Dissected regions of tilapia brain. The anterior section contains the telencephalon and olfactory bulb; the middle section contains the diencephalon, midbrain tegmentum, tectum and valvulae cerebelli, and pituitary gland; and the hind section contains the rhombencephalon, cerebellum, and vagal lobe. **1**, Telencephalon and olfactory bulb; **2**, diencephalon; **3**, midbrain tegmentum; **4**, tectum and valvulae cerebelli; **5**, rhombencephalon; **6**, cerebellum; **7**, vagal lobe; **8**, pituitary. Scale bar = 1 mm.

Semiquantitation of PCR products

Semiquantitative RT-PCRs were performed by co-amplifying specific genes with β-actin as the internal control. Reactions involved 25 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The PCR products were electrophoresed on 1% agarose gels for signal visualization. Relative band intensities were determined with Phoretix gel analysis software 1D standard vers. 3.0 (Nonlinear Dynamics, Newcastle, UK). Treatment results were compared following β-actin normalization. Semiquantitative RT-PCR assay results were verified, and the amplified cycle numbers of the PCR were determined by assessing the linear intensity of the amplified product under various cycles; the ratio of 2 sets of primers for HSP70 or HSP90 combined with β-actin (5'-ACCACCACAGCCGAGAGGGA and 3'-TTGTTGGGCGTTTGGTTGGG) in 1 reaction was measured using the same approach. The HSP70 and HSP90 cloned plasmids were employed as templates to generate a standard curve for controlling the amplified cycle numbers and preventing oversaturation of the bands. Following this calibration, comparisons of semiquantitative brain HSP70 and HSP90 levels were more reliable.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Test samples were homogenized with lysis buffer (137 mmol/l NaCl, 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 0.1 mmol/l sodium orthovanadate, 20 mmol/l Tris-HCl (pH 7.4), 10 g/l NP-40, and 1 mmol/l PMSF) at a volume of 1:3 μg/μl using a homogenizer on ice. The homogenate was centrifuged at 12,000 g for 10 min at 4°C. The supernatant was transferred to a new microcentrifuge tube and stored at -20°C until the assays. The optical density $(OD)_{595}$ was determined with an enzymelinked immunosorbent assay (ELISA) reader (Thermo Labsystems, Beverly, MA, USA). The standard curve based on the OD_{595} of the standard bovine serum albumin (BSA; Promega, Taipei, Taiwan) solution was generated, and protein concentrations in the test samples were derived from a standard curve. A protein sample (40 μg) was mixed with 3 μl of 6 × SDS-PAGE sample buffer in a 1.5 ml microcentrifuge tube, and then added to sterile water to achieve equal volumes for all samples. Samples were centrifuged, heated in a water bath (95°C) for 10 min, and cooled on ice for 3 min. Samples were examined in a 10% gel. The gel was first run at 50 V for 30 min, and then at 100 V for 100 min. The protein was transferred from the gel with 70 V for 2 h at 4°C to a polyvinylidene difluoride (PVDF) membrane (NEN™ Life Science Products, Boston, MA, USA). Membranes were blocked with 3% BSA in PBS-T buffer (137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, and 0.05% Tween-20) for 1.5 h. The membrane was first incubated with mouse anti-HSP70 (1: 5000 dilution, MA3-007, Affinity BioReagents, Golden, CO, USA) or rat anti-HSP90 (1: 1000 dilution, SPA-835F, Stressgen, Victoria, BC, Canada) monoclonal antibodies, both with species reactivity with fish, and mouse anti-β-actin (1: 4000 dilution, A5441, Sigma, St. Louis, MO, USA) at 4°C overnight (12-16 h) after being stripped of HSP70 or HSP90 blots, respectively. After washing twice with 15 ml PBS-T buffer for 10 min, the blot was incubated with a secondary antibody (goat anti-mouse immunoglobulin G (IgG)-alkaline phosphatase, 1: 4000 dilution, 62-6722, Zymed, South San Francisco, CA, USA) at room temperature for 1.5 h. After washing the membrane twice with 15 ml of PBS-T buffer for 10 min, the pH of the membrane was adjusted with buffer 3 (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2 \times 6H_2O$) for 5 min. Buffer 3 was then poured off. Diluted AP-substrate (BCIP/NBT substrate kit 10 ×) (Zymed) was added, and the membrane was shaken gently until color developed. The membrane was air-dried. The relative band intensity was determined as described previously (Lo et al. 2007).

Statistical analysis

Experimental data are presented as the mean ± standard error (SE). The ratio to the FW control was used to present changes in RNA and protein levels in brain HSP70 and HSP90 following β-actin normalization in the SW group compared to the FW group. Results of the FW controls were set to 1. Numbers of experimental fish were equal (n = 5) in the control (FW) and treatment groups. Results were expressed as the mean ± standard error (SE). In all experiments, significance values ($p < 0.05$) were compared by a Duncan multiple-range test after 1-way analysis of variance (ANOVA).

RESULTS

HSP70 mRNA and protein responses to acute osmotic stress

mRNA and protein levels of β-actin in tilapia brain after transfer to 25 ppt SW did not change (data not shown). Thus β-actin was applied as an internal control to normalize specific gene expressions. After transferring tilapia from FW (control) to 25 ppt SW, HSP70 mRNA levels in the whole brain were higher within 24 h than those of the FW group (1.4-1.9 fold, $p < 0.05$; Fig. 2A). HSP70 protein levels of the whole brain significantly increased at 1 and 4 h after transfer to 25 ppt SW (1.5 fold, $p < 0.05$), whereas levels showed a significant decrease at 24 h after SW transfer (0.6 fold, $p < 0.05$; Fig. 2B).

To further investigate the responses of the different brain portions to salinity changes, tilapia HSP70 protein levels in the 3 brain portions (anterior, middle, and hind) at various time points after fish were transferred to 25 ppt SW were evaluated (Fig. 2C). HSP70 protein levels in the anterior brain region at 1 h after SW transfer were higher than those of the FW, and 8 and 24 h groups (1.7 vs. 1.0, 0.8, and 0.7 fold, $p < 0.05$), but no significant differences were observed compared to those at 2 and 4 h (1.7 vs. 1.2 fold, $p > 0.05$). No significant differences also existed between the levels at 2 and 4 h (1.2 vs. 1.2 fold, $p > 0.05$), and at 8 and 24 h (0.8 vs. 0.7 fold, $p > 0.05$). Nevertheless, HSP70 protein levels in the anterior brain region showed a decreasing tendency 1 h after transfer. HSP70 protein levels in the middle brain region at 1 and 2 h were significant higher than those of other groups after SW transfer (1.7 and 2.0 vs. 0.6-1.0 fold, $p < 0.05$), and they began to decrease 2 h after transfer. Subsequently, HSP70 protein levels in the middle brain region were significantly lower 24 h after SW transfer (0.6 fold, $p < 0.05$). For the hind brain region, HSP70 protein levels remained at similar levels within 8 h after SW transfer (0.7-1.7 fold, $p > 0.05$), and levels had significantly declined at 24 h after SW transfer (0.5 fold, $p < 0.05$), even though no differences existed among those of 2, 8, and 24 h (0.8 vs. 0.7 vs. 0.5 fold, $p > 0.05$). Significant differences in HSP70 protein levels in the hind brain region were only observed 2 h after SW transfer (0.8 vs. 2.0 fold, $p < 0.05$) compared to those in the middle brain region, which were higher than those of other groups. There were no significant differences among HSP70 protein levels in the 3 regions of the brain at other time points (1, 4, 8, and 24 h; $p > 0.05$).

Further experiments were performed to test the HSP70 mRNA and protein levels in a more restricted salinity condition. After transferring tilapia from FW to 35 ppt SW, HSP70 mRNA levels were significantly reduced at 1 h after transfer and began to increase from 2 h (0.3 vs. 1.2 fold, $p < 0.05$; Fig. 3A). Levels at 4 h were significantly higher than those of the other 3 groups (1.6 vs. 0.3-1.2 fold, $p < 0.05$). Whole-brain HSP70 protein levels were reduced at 2 h after 35 ppt SW transfer compared to those of the FW group (0.7 vs. 1.0 fold, $p < 0.05$; Fig. 3B), while no significant differences at other time points were found. These results showed that changes in HSP70 protein levels after transfer to 25 ppt SW tended to be a short-term response.

HSP70 protein levels in the anterior brain had dramatically decreased at 2 h after 35 ppt SW transfer (0.4 fold, $p < 0.05$), and had increased at 4 h, although they were still significantly lower than those of the FW and 1 h groups (0.9 vs. 1.0 and 1.3 fold, $p < 0.05$; Fig. 3C). However, no significant differences in the levels in the middle part were observed in the FW, and 1 and 2 h groups (1.0 vs. 1.5 vs. 0.9 fold, $p > 0.05$), and levels had significantly increased at 4 h after SW transfer (1.8 fold, $p < 0.05$). There were no significant differences among the levels in the hind regions of the brain at each time point (0.7-1.3 fold, $p > 0.05$). There were no significant differences among HSP70 protein levels in the 3 regions of the brain at 1 h after SW transfer (1.3-1.5 fold, $p > 0.05$). Levels in the anterior brain region at 2 h after SW transfer were lower than those in the middle region (0.4 vs. 0.9 fold, $p < 0.05$), but did not differ from those in the hind region (0.4 vs. 0.7 fold, $p > 0.05$). The same results were observed at 4 h after SW transfer: levels in the anterior brain region were lower than those in the middle region (0.9 vs. 1.8 fold, $p < 0.05$), and no differences existed between the anterior and hind regions (0.9 vs. 1.2 fold, $p > 0.05$).

HSP90 mRNA and protein responses to acute osmotic stress

After transferring tilapia from FW to 25 ppt SW, HSP90 mRNA levels in the whole brain had markedly increased at 24 h compared to those of the FW and 2 h groups (2.1 vs. 1.0 and 0.7 fold, $p < 0.05$; Fig. 4A). Within 8 h after SW transfer, no significant differences in HSP90 mRNA levels

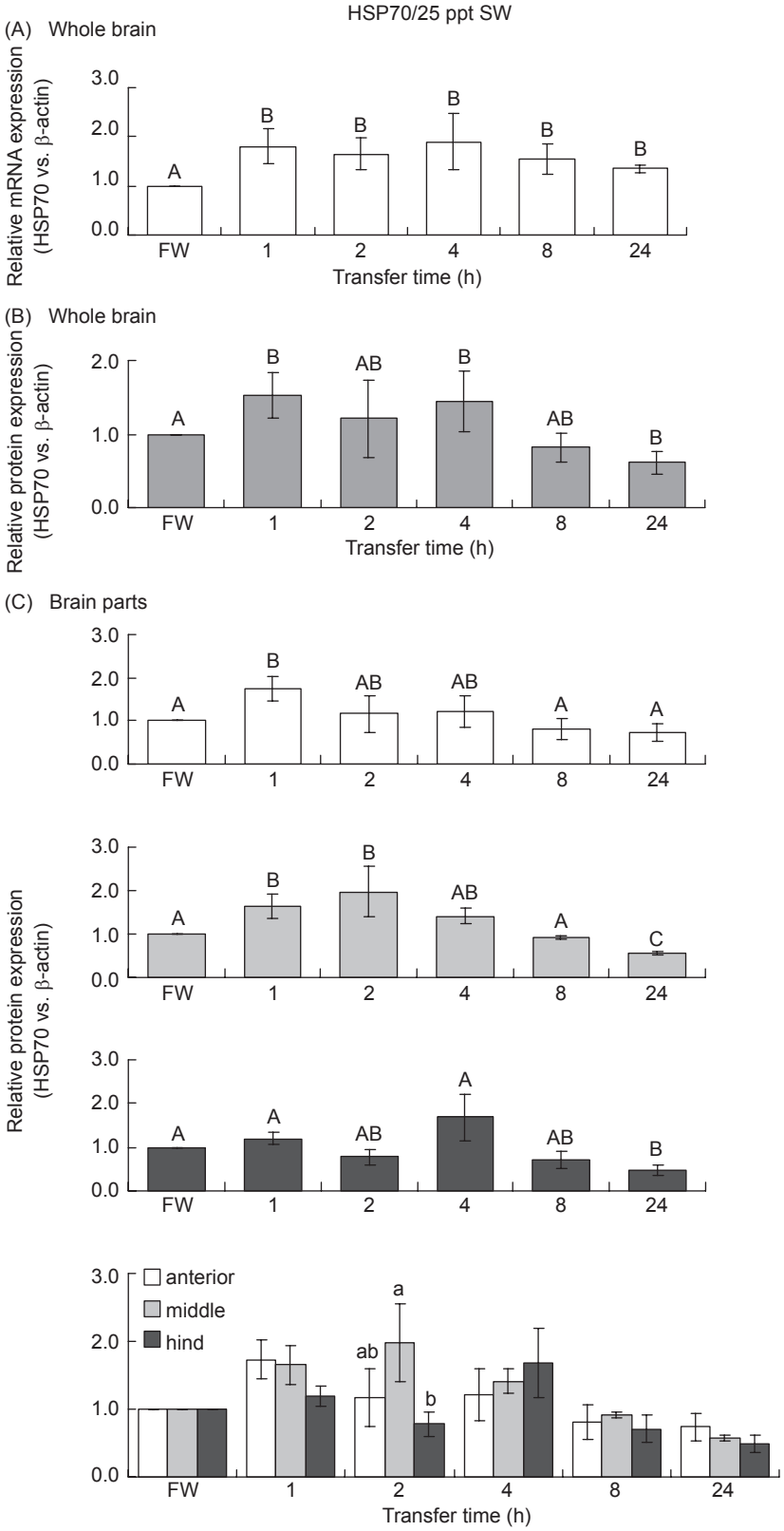

Fig. 2. Expression of heat shock protein (HSP)70 mRNA and protein (ratio to the freshwater (FW) control) in tilapia brain at various times after transfer of tilapia from FW to 25 ppt seawater (SW). HSP70 mRNA (A) and protein (B) in whole brain, and (C) HSP70 protein in brain parts (anterior, middle, and hind). Comparisons of the intensity between the FW control and treatments after β-actin normalization. Values are the mean ± SE (n = 5). $^{A, B, C:}$ Different superscripts among groups indicate statistically significant differences (p < 0.05). $^{a, b:}$ Different superscripts among groups at the same transfer time point indicate statistically significant differences (p < 0.05).

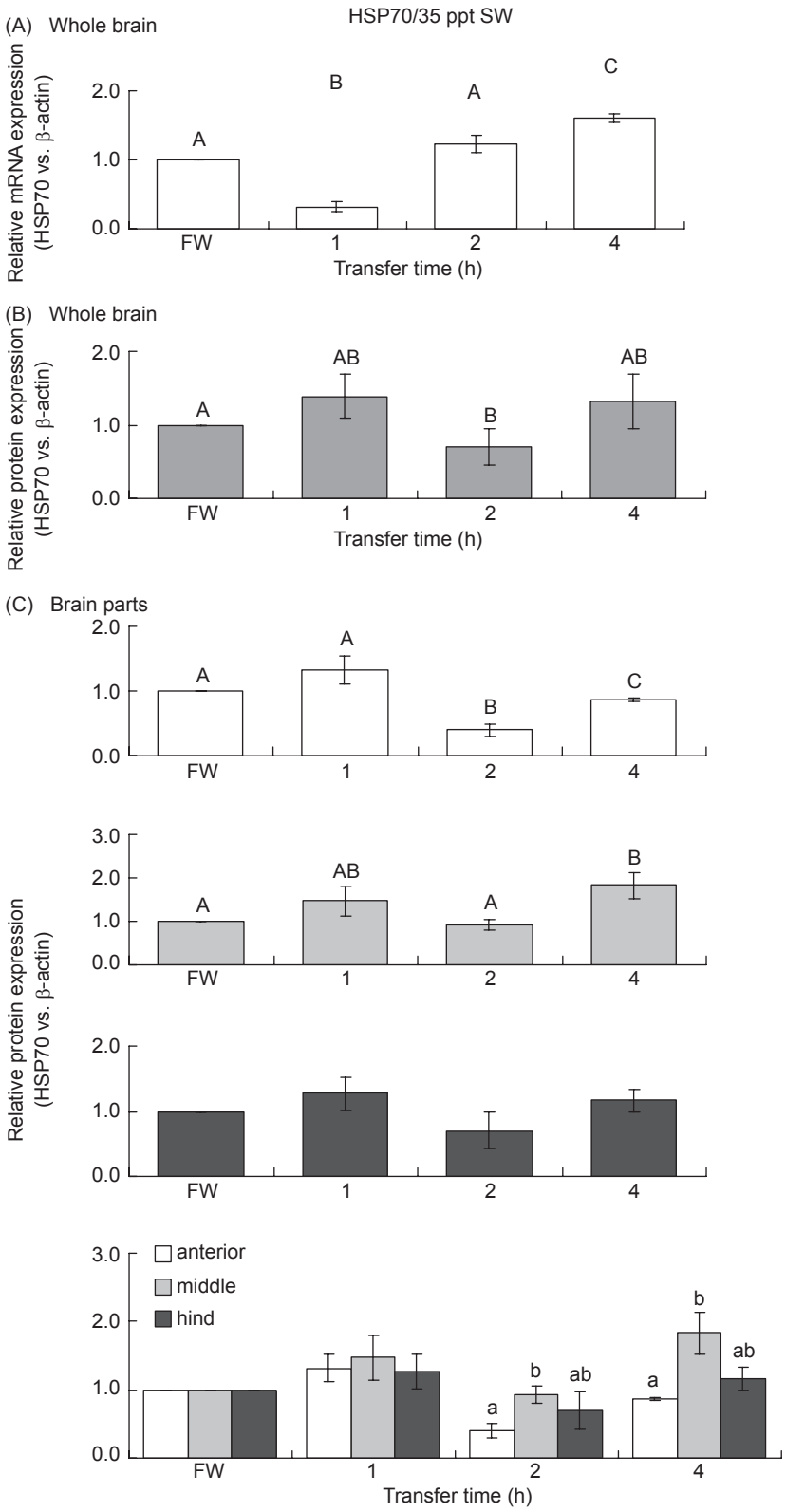

Fig. 3. Expression of heat shock protein (HSP)70 mRNA and protein (ratio to the freshwater (FW) control) in tilapia brain at various times after transfer of tilapia from FW to 35 ppt seawater (SW). HSP70 mRNA (A) and protein (B) in the whole brain, and (C) HSP70 protein in brain parts (anterior, middle, and hind). Comparisons of the intensity between the FW control and treatments after β-actin normalization. Values are the mean ± SE (n = 5). $^{A, B, C:}$ Different superscripts among groups indicate statistically significant differences ($p < 0.05$). $^{a, b:}$ Different superscripts among groups at the same transfer time point indicate statistically significant differences ($p < 0.05$).

in the whole brain were observed (0.7-1.4 fold, $p > 0.05$). HSP90 protein levels in the whole brain had markedly increased at 1 h (1.8 fold, $p < 0.05$; Fig. 4B); however, levels began to decrease after 2 h (1.4 fold), and significantly lower expression was observed at 24 h compared to those of the other groups (0.4 vs. 0.8-1.8 fold; $p < 0.05$).

HSP90 protein levels in the anterior brain region at 1 h after transfer to 25 ppt SW were higher than those of the other groups (2.1 vs. 0.5-1.1 fold; $p < 0.05$) and decreased thereafter (Fig. 4C). Significantly lower levels at 24 h after transfer than those of FW and at 1 h were observed (0.5 vs. 1.0 and 2.1 fold; $p < 0.05$). However, no differences existed among the levels at 2-24 h (0.5-1.1 fold; $p > 0.05$). Similar results were observed in the middle brain region, where HSP90 protein levels had significantly increased at 1 h after SW transfer (1.9 fold, $p < 0.05$) and had begun to decrease at 4 h (0.8 fold). Subsequently, significantly lower HSP90 protein levels were observed at 24 h after transfer (0.2 fold, $p < 0.05$). For the hind brain region, although HSP90 protein levels at 4 h after SW transfer were higher than those of the other groups, no significant differences existed among them (2.4 vs. 0.5-1.2 fold; $p > 0.05$). Levels at 24 h after transfer were significant lower than those of the FW and 1 h groups (0.5 vs. 1.0 and 1.2 fold; $p < 0.05$). Significant differences in HSP90 protein levels were only observed at 1 h after SW transfer, when levels in the hind brain region were lower than those in the anterior and middle regions (1.2 vs. 2.1 and 1.9 fold, $p < 0.05$). There were no significant differences among levels in the 3 brain regions at other time points ($p > 0.05$). A short-term response pattern of HSP90 was concurrent with changes in the levels of HSP70 protein after transfer to 25 ppt SW.

HSP90 mRNA levels were lower at both 1 and 4 h after transfer to 35 ppt SW than those of the FW group (0.5 and 0.6 vs. 1.0 fold, $p < 0.05$; Fig. 5A), although they did not differ from those at 2 h (0.5 and 0.6 vs. 0.7 fold, $p > 0.05$). HSP90 protein levels at 2 h after SW transfer were significantly lower compared to those of the FW and 1 h groups (0.4 vs. 1.0 and 1.2 fold, $p < 0.05$; Fig. 5B); however no significant difference existed among the levels of the FW group at 1 and 4 h after transfer to SW (0.5 vs. 1.2 vs. 0.9 fold, $p > 0.05$). The above results were inconsistent with those observed for the expression of HSP70 after transfer to 35 ppt SW.

HSP90 protein levels in the anterior brain region had significantly decreased at 2 h after transfer to 35 ppt SW (0.2 fold, $p < 0.05$; Fig. 5C), and had increased at 4 h (0.4 fold, $p < 0.05$). Similar results were observed for levels in the mid and hind brain regions. In the 3 brain regions, lower HSP90 levels were found in the anterior region than in the hind region at 2 and 4 h after SW transfer (0.2 vs. 0.5 and 0.4 vs. 1.0 fold, respectively, $p < 0.05$), and there were no significant differences between levels in the anterior and middle brain regions (0.2 vs. 0.4 and 0.4 vs. 1.3 fold, respectively, $p > 0.05$), or in the middle and hind brain regions (0.4 vs. 0.5 and 1.3 vs. 1.0 fold, respectively, $p > 0.05$).

DISCUSSION

Experimental results of HSP expressions demonstrated that the 3 parts of the brain reacted differentially to salinity changes. When tilapia were transferred to 25 ppt SW, both HSP70 and HSP90 protein levels had increased at 1 h in the anterior, middle, and whole brain. These tendencies in the whole brain were sustained to 4 h following SW transfer. The data showed a short-term response in HSP protein levels after transfer to 25 ppt SW. The increased mRNA levels of HSP70 occurred in the same pattern as seen for HSP70 protein levels of tilapia brain after transfer to 25 ppt SW. However, the expressions of HSP70 in the 35 ppt SW-transferred group markedly differed from those transferred to 25 ppt SW. The expression of HSP proteins had dramatically fallen at 2 h after transfer to 35 ppt SW and then had suddenly increased at 4 h. Changes in HSP90 mRNA were inconsistent with changes in protein levels after transfer to 35 ppt SW. In this work, HSP70 mRNA expressions were concurrent with protein levels in the brain of tilapia after transfer to 25 ppt SW. Taking all data into consideration, this study implies that HSP70 may serve as a regulatory factor for adaptation when tilapia were directly transferred from FW to 25 ppt SW. In addition, the anterior and middle portions of the brain were more sensitive to osmotic stress, particularly in the short term after SW transfer. Our previous studies demonstrated that tilapia directly transferred to 25 ppt SW can survive and adapt to salinity changes by regulating biochemistry and specific genes in the gills and brain (Weng et al. 2002a b). HSP70 is the primary stress-induced protein in vertebrates and has been maintained throughout evolution. HSP70 transcripts are expressed during various physiological stresses, and the resulting

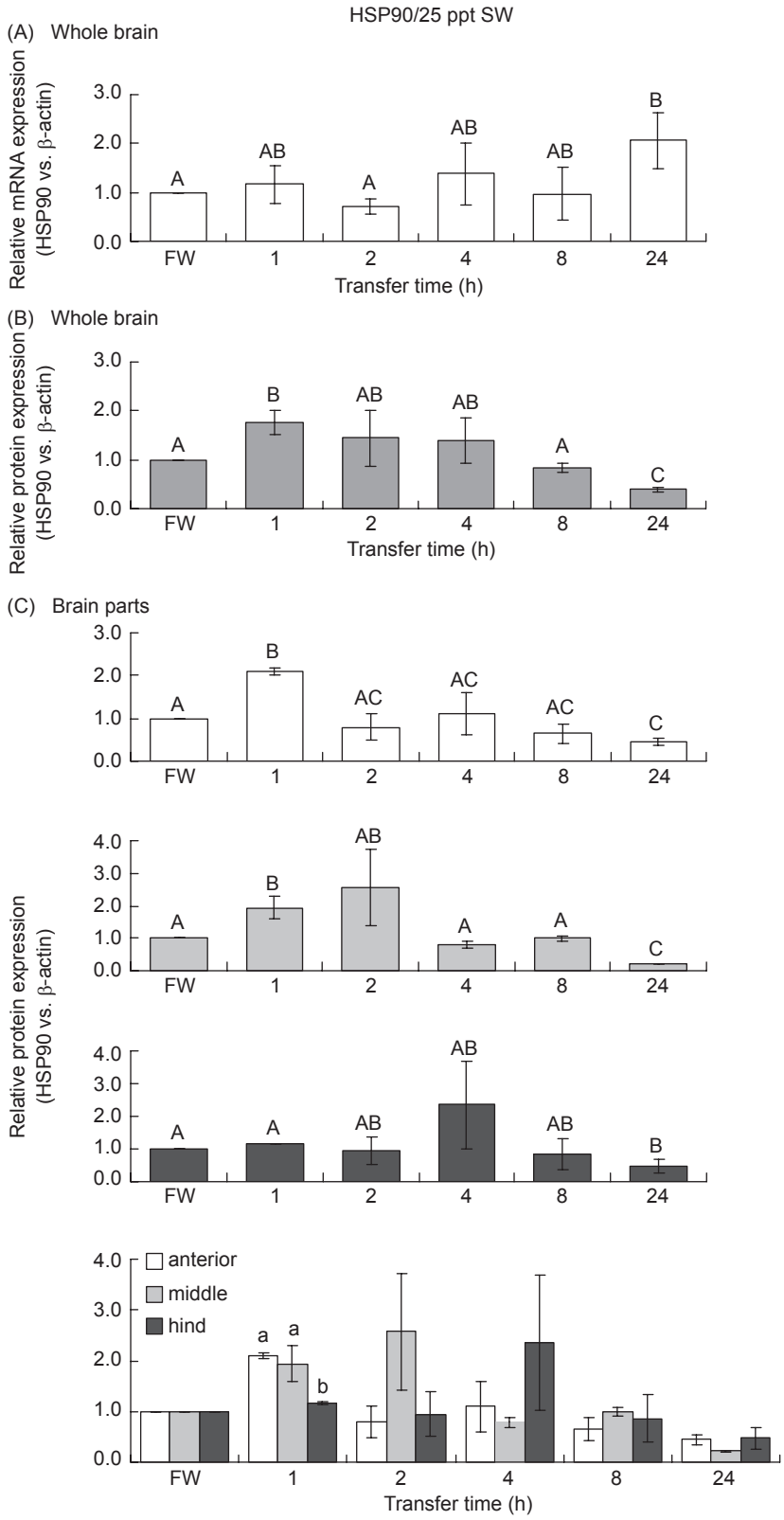

Fig. 4. Expressions of heat shock protein (HSP)90 mRNA and protein (ratio to the freshwater (FW) control) in tilapia brain at various times after transfer of tilapia from FW to 25 ppt seawater (SW). HSP90 mRNA (A) and protein (B) in whole brain, and (C) HSP90 protein in brain parts (anterior, middle, and hind). Comparisons of the intensity between the FW control and treatments after β-actin normalization. Values are the means ± SE (n = 5). $^{A, B, C:}$ Different superscripts among groups indicate statistically significant differences ($p < 0.05$). $^{a, b:}$ Different superscripts among groups at the same transfer time point indicate statistically significant differences ($p < 0.05$).

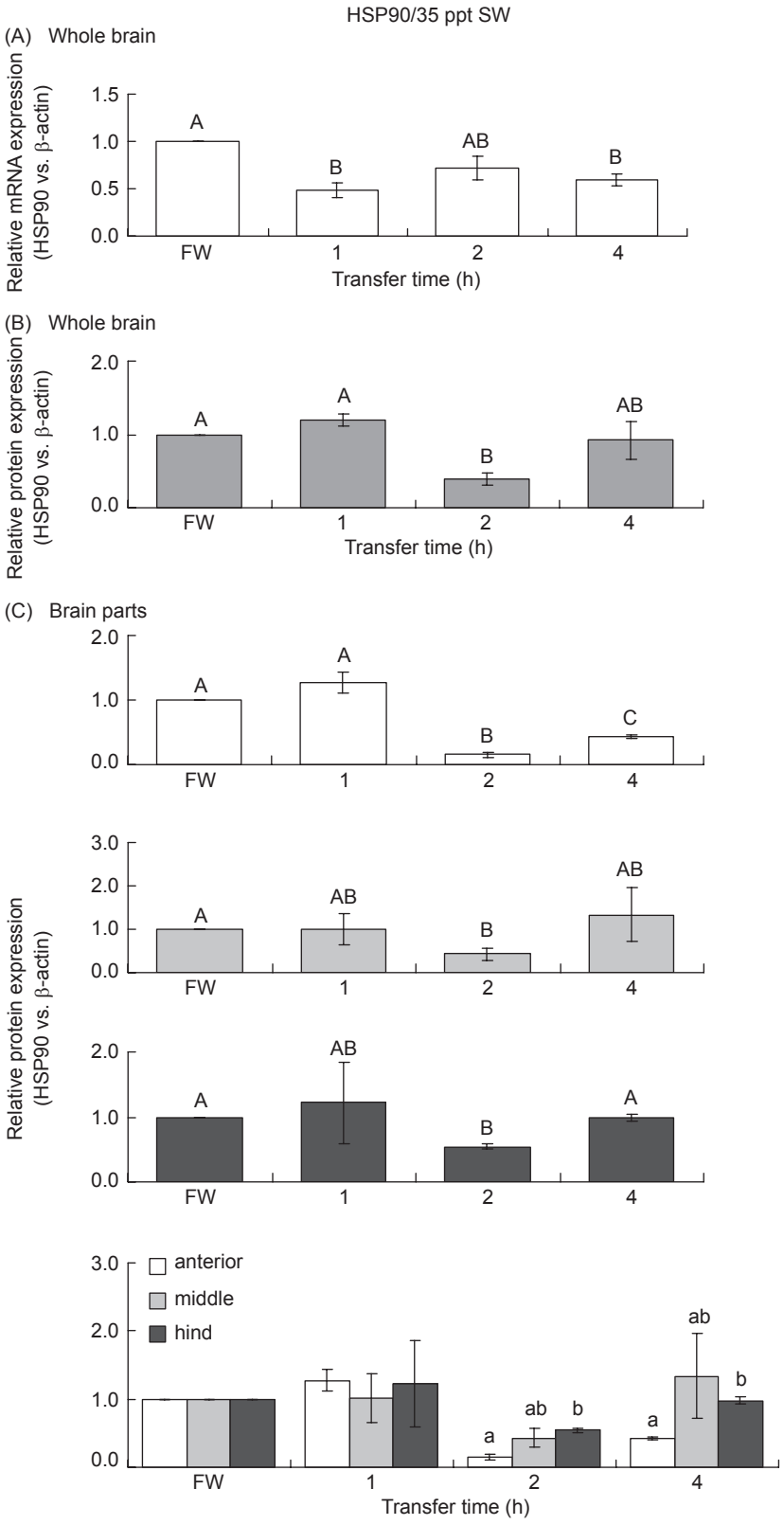

Fig. 5. Expressions of heat shock protein (HSP)90 mRNA and protein (ratio to the freshwater (FW) control) in tilapia brain at various times after transfer of tilapia from FW to 35 ppt seawater (SW). HSP90 mRNA (A) and protein (B) in whole brain, and (C) HSP90 protein in brain parts (anterior, middle, and hind). Comparisons of the intensity between the FW control and treatments after β-actin normalization. Values are the mean ± SE (n = 5). $^{A, B, C:}$ Different superscripts among groups indicate statistically significant differences (p < 0.05). $^{a, b:}$ Different superscripts among groups at the same transfer time point indicate statistically significant differences (p < 0.05).

translation products act as molecular chaperones which govern the correct assembly and localization of intracellular proteins. Numerous studies have demonstrated that fish HSPs are induced by thermal stress (Airaksinen et al. 1998), anoxia (Ossum et al. 2004), and heavy-metal exposure (Zhou et al. 2003); however, the literature on the correlated changes of HSPs with salinity stress is still scant.

Compared to those exposed to 25 ppt SW transfer, mRNA and protein levels of HSP70 in tilapia brain considerably differed with transfer to 35 ppt SW. The change in salinity only affected the mRNA level of HSP70, not the protein level. This discrepancy could have been due to changes at the translational level. A previous report demonstrated that RNA splicing can influence subsequent events in the RNA processing pathway and resulted in a change of translational yields in *Xenopus* oocytes (Matsumoto et al. 1998). However, no such report has demonstrated stress-related changes in cytoplasmic processing bodies in teleosts (tilapia). HSPs, inducible in nearly every other species, were constitutively expressed in *in vivo* experiments on sections of metabolically active brain, spleen, liver, heart, and gill tissues (Hofmann et al. 2000). From the above point view, further study is required to elucidate possible factors affecting why mRNA levels of HSP70 and HSP90 were not associated with their respective protein levels.

Expression of HSP70 in tilapia brain had dramatically decreased at 2 h and was elevated at 4 h after transfer to 35 ppt SW. The death of tilapia at 6 h following transfer from FW to 35 ppt SW may account for this inconsistent expression (Weng et al. 2002a). Upregulation of HSP70 in response to osmotic stress was noted in fish tissues (Smith et al. 1999). The HSP70 family has been widely investigated as a stress biomarker (Ryan and Hightower 1996). Increased accumulation of HSP70 protects cellular protein processes, including renaturing denatured proteins, preventing protein aggregation, targeting damaged proteins for breakdown, and facilitating protein synthesis and translocation (Feder and Hofmann 1999, Fink 1999, Kregel 2002). HSP70 is crucial to protein metabolism under normal and stress conditions, in terms of de novo protein folding, membrane translocation, formation, disassembly of protein complexes, and degradation of misfolded proteins (Hartl 1996, Bukau and Horwich 1998, Kregel 2002). These HSP70s also interact with numerous other proteins, enhancing specific chaperone functions. Expressions of these proteins are regulated by environmental and physiological stresses and non-stressful conditions such as cell growth and development (Santacruz et al. 1997). Recent studies also implicated HSP70 as a general antiapoptotic protein, which protects cells from cytotoxicant-induced cell death (Mosser et al. 1997, Gabai et al. 1998, Jäättelä et al. 1998). Iwama et al. (2004) pointed out that the use of HSPs as indicators for stressed states of fish in most cases is still premature due to variations in the HSP family and types of stressors. Taken together, experimental results in this study imply that HSP70 in the brain may serve as a biomarker for predicting survival during osmotic stress in tilapia and teleostian fish.

mRNA and protein levels of HSP70 changed concurrently when tilapia were transferred to 25 ppt SW; however, no such phenomenon was observed for HSP90. One possible explanation is the specificity of HSP90 isoform antibodies. Various isoforms of HSP90 exist in fish. Previous investigations showed that hyperosmotic conditions disrupt protein synthesis mechanisms and decrease the rate of synthesis of all but a small number of proteins (Kultz 1996, Kurz et al. 1998, Smith et al. 1999). Preferential synthesis of HSP70 during osmotic stress (Rauchman et al. 1997, Santos et al. 1998, Smith et al. 1999) and not of HSP90, may result from the different functions of these 2 chaperones. Translation of HSP90 mRNA lagged behind its transcription due to cellular perturbation caused by elevation of the concentrations of intracellular Na^+ and Cl^-. Synthesis of HSP90 regulated at the transcription and translation levels has not been ruled out. Although osmotic stress substantially stimulated gene expression, message translation may require additional factors for initiation or elongation. Both HSP90 mRNA and protein levels decreased particularly in the short term after transfer to 35 ppt SW. Experimental results in this study are in agreement with a previous report that the accumulation of HSP90 mRNA in *Xenopus laevis* exposed to a thermal shock was significantly less pronounced than the increase in HSP70 mRNA (Ali et al. 1996). In salmon, the response of HSP90 in both branchial lamellae and kidney tissues to thermal shock was less prominent than that of HSP70 *in vitro* and *in vivo* (Pan et al. 2000). Cortisol, the predominant corticosteroid in teleosts, is a primary mediator of stress-induced hyperglycemia and is considered crucial for meeting increased energy demands

associated with stress (Vijayan et al. 1997b, Wendelaar-Bonga 1997, Mommsen et al. 1999, Saplosky et al. 2000). However, HSP90 may be essential to cellular signaling and steroid receptor activation (Pratt 1997, Csermely et al. 1998). By contrast, a standard handling challenge resulted in elevated plasma cortisol levels but did not raise HSP90 mRNA expression in any tissue, clearly differentiating physiological and cellular stress responses (Palmisano et al. 2000). Tissue HSP90 mRNA levels and plasma cortisol concentrations did not increase after exposing Chinook salmon (*Onc. tshawytscha*) to seawater overnight (Palmisano et al. 2000). Our previous experiments showed that plasma cortisol increased 4 fold at 8 h after tilapia were transferred to 25 ppt SW compared to the FW controls (Yang 2004). However, an *in vivo* study assessing the role of cortisol in cellular stress response in fish indicated that liver HSP70 mRNA expression failed to show any change (Deane et al. 1999). Similar reductions in the GR capacity with stress-induced cortisol and/or cortisol treatment were noted in rainbow trout (Shrimpton and McCormick 1999). Taken together, previous experimental results and results from this study suggest that HSP90 is highly predisposed to GR, and increased cortisol levels provide energy against stress. Therefore, energy is not consumed by producing HSP90 under severe and transient changes in salinity (35 ppt SW). In conclusion, experimental results suggest that HSP70 and HSP90 are critical for regulation in tilapia brain through temporal-spatial changes that may be crucial to adapting to SW. HSPs may play an osmoregulatory role in maintaining normal processes of brain functions to prevent osmotic stress when tilapia are directly transferred from FW to 25 ppt SW.

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