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Constant Muscle Water Content and Renal HSP90 Expression Reflect Osmotic Homeostasis in Euryhaline Teleosts Acclimated to Different Environmental Salinities

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Cheng-Hao Tang, Ching-San Tzeng, Lie-Yueh Hwang, and Tsung-Han Lee (2009) Constant muscle water content and renal HSP90 expression reflect osmotic homeostasis in euryhaline teleosts acclimatized to different environmental salinities. *Zoological Studies* **48**(4): 435-441. Changes in environmental salinities trigger osmoregulatory mechanisms of euryhaline teleosts in order to maintain the plasma osmolality and water balance. The kidneys are the osmoregulatory organ inside the body which perform ion re-absorption and water regulation. Heat shock proteins (HSPs) are also known as stress proteins, with HSP90 as one of the major classes of HSPs essential for living eukaryotes because it is responsible for the repair and refolding of damaged proteins. In the present study, euryhaline tilapia (*Oreochromis mossambicus*), spotted green pufferfish (*Tetraodon nigroviridis*), and milkfish (*Chanos chanos*), with respective primary natural habitats of freshwater lakes, estuaries, and the sea, were acclimated to fresh water (FW), brackish water (BW; 15‰ salinity), and seawater (SW; 35‰ salinity). The muscle water content (MWC) and relative protein amounts of HSP90 in the kidneys of the 3 studied species acclimated to different salinity environments were analyzed in this study. The MWC of these 3 euryhaline teleosts revealed no significant changes in FW, BW, and SW. Furthermore, relative protein amounts of renal HSP90 were similar among the 3 studied species acclimated to various environments. The physiological (MWC) and stress (HSP90) responses integrated in this study might be indicators of osmoregulatory capacity, illustrating homeostasis of the internal environments of euryhaline teleosts.
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Key words: Euryhaline teleost, Heat shock protein, Osmoregulation.

When organisms including fish experience environmental disturbances, the effects may be dramatic when conditions are outside of a normal range. The physiological systems of fish can be challenged or stressed by a wide range of biological, chemical, and physical factors. Salinity adaptation by euryhaline teleosts is a complex process involving a set of physiological responses by osmoregulatory organs to milieus with differing osmoregulatory requirements (Hwang and Lee 2007, Evans 2008). Teleosts are osmoregulators in both fresh water (FW) and seawater (SW) (Evans 1993, Jobling 1995). Marine species are strongly hypo-osmotic to SW, with plasma osmolalities of temperate, subtropical, and tropical species ranging 370-480 mOsm/kg. FW species, however, are hyperosmotic to these environments, with plasma osmolalities of 230-330 mOsm/kg (Evans 1993, Jobling 1995). Gills and kidneys are the most important organs responsible for osmoregulation in teleosts, with the gills in direct contact with the external environment and the kidneys controlling the internal environment (Marshall and Grosell 2006). In order to maintain plasma osmolality as well as ion and water balance, teleosts take up salts from FW through the gills, produce hypotonic urine to reabsorb salts, and expel excess water by the kidneys. On the other hand, SW teleosts

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excrete salts through the gills and produce a minimal volume of isotonic urine since their kidneys exhibit very low glomerular filtration or even lack glomeruli (Marshall and Grosell 2006). Therefore, water balance is a crucial factor in the survival of euryhaline teleosts in different salinity environments. Muscle water content (MWC) can be used as a physiological index to evaluate the balance of water content in fish.

Heat shock proteins (HSPs) are commonly called stress proteins and are constitutively expressed in cells to maintain a number of critical cellular processes relating to protein folding, fidelity, and translocation (Iwama et al. 1998). The protective roles of HSPs have been widely studied (Morimoto and Santoro 1998, Basu et al. 2002, Iwama et al. 2006), and one of the major classes of HSPs induced in cells in response to stress is the HSP90 family (Parsell and Lindquist 1993, Zhao and Houry 2005). HSP90 is abundant at 1%-2% of cellular proteins in most tissues (Lai et al. 1984, Parsell and Lindquist 1993, Csermely et al. 1998), and it contributes to various cellular processes including signal transduction, morphological evolution, folding newly synthesized proteins, and the stabilization and refolding of proteins denatured due to stress (Burrows et al. 2004, Sreedhar et al. 2004, Wegele et al. 2004). In fish, the induction of HSP families was reported not only in cell lines and primary cultures of cells, but also in various tissues from whole animals (Iwama et al. 1998). Previous reviews described the correlation between elevated levels of HSPs and exposure to stressors within an ecologically relevant range (Iwama et al. 1998 1999, Feder and Hofmann 1999, Basu et al. 2002). In order to determine whether homeostasis of the internal environment of euryhaline teleosts was maintained when acclimated to different salinity media, kidneys, the main osmoregulatory organ in the body cavity were selected to investigate stress responses in this study.

Teleostean species are about 95% stenohaline, living entirely in either FW or SW. The remaining 5% are euryhaline, and have the capacity to resist dramatic changes in environmental salinities (Evans 1984). In this study, 3 euryhaline species from different primary natural habitats were used. Mozambique tilapia (*Oreochromis mossambicus*) is a euryhaline FW inhabitant that tolerates salinities of up to 120 ‰ (Stickney 1986). The spotted green pufferfish (*Tetraodon nigroviridis*) is a tetraodontid teleost and the native range of which includes rivers and estuaries of Southeast Asia (Rainboth 1996). The milkfish (*Chanos chanos*), a marine species, is widely distributed throughout the tropical and subtropical Indo-Pacific (Bagrinao 1994). This study showed that the MWC and protein abundance of renal HSP90 were constant among the studied euryhaline teleosts acclimated to environments with various salinities reflecting a stable internal environment in euryhaline teleosts.

MATERIALS AND METHODS

Experimental animals and environments

Tilapia (*O. mossambicus*; with body weights of 5.8 ± 0.4 g) were obtained from laboratory stocks. Spotted green pufferfish (*T. nigroviridis*; with body weights of 4.6 ± 0.3 g) were purchased from a local aquarium. Juvenile milkfish (*C. chanos*; with body weights of 28.5 ± 6.8 g) were obtained from a local fish farm. Seawater (35‰ salinity; SW) and brackish water (15‰ salinity; BW) used in this study were prepared from local tap water with proper amounts of synthetic sea salt (Instant Ocean, Aquarium Systems, Mentor, OH, USA). The fish were reared in SW, BW, or fresh water (FW) at 27 ± 1°C with a daily 12 h photoperiod for at least 2 wk before sampling. The water was continuously circulated through fabric-floss filters. The fish were fed commercial arid shrimp daily.

Muscle water content (MWC)

The procedure for determining the MWC was carried out according to Tipsmark et al. (2002) with little modification. MWC was measured gravimetrically after drying at 100°C for 48 h.

Preparation of kidney homogenates

Fish were removed from the water and immediately killed by spinal pithing. The kidneys were dissected out and blotted dry. Kidney scrapings were suspended in a mixture of homogenization medium (100 mmol/L imidazole-HCl, 5 mmol/L Na_2EDTA, 200 mmol/L sucrose, and 0.1% sodium deoxycholate; pH 7.6) and proteinase inhibitor (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 ml aprotinin; v/v 100: 1). Homogenization was performed in 2 ml tubes with a POLYTRON PT1200E (Kinematica, Lucerne, Switzerland) at maximal speed for 25 strokes. The homogenates were then centrifuged

at 13,000 g and 4°C for 20 min. The supernatants were used for determination of the protein concentration and subsequent immunoblotting. Protein concentrations were identified by reagents from the BCA Protein Assay (Pierce, Hercules, CA, USA), using bovine serum albumin (BSA) as a standard (Pierce). Samples were stored at -80°C before use.

Antibodies

The primary antibody used in the present study was an anti-HSP90 rabbit polyclonal antibody (#4874; Cell Signaling Technology, Beverly, MA, USA) corresponding to human HSP90. The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG; Jackson ImmunoResearch, West Grove, PA, USA).

Immunoblotting

Immunoblotting procedures were carried out according to Tang et al. (2008) with little modification. Proteins of those samples were heated together with the sample buffer at 95°C for 5 min. A pre-staining protein molecular weight marker was purchased from Fermentas (SM0671; Hanover, MD, USA). All samples were divided by electrophoresis on sodium dodecylsulfate (SDS)-containing 8% polyacrylamide gels (20 μg of protein/lane) on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). The separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) using a tank transfer system (Bio-Rad, Mini Protean 3) by electroblotting at 100 V for 40 min. After preincubation for 3 h in PBST (phosphate-buffered saline with Tween 20) buffer (137 mmol/L NaCl, 3 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4, and 0.2% (vol/vol) Tween 20) containing 5% (wt/vol) nonfat dried milk to minimize nonspecific binding, the blots were incubated for 2 h at room temperature with the primary antibody diluted in 1% BSA and 0.05% sodium azide in PBST, washed in PBST, then incubated at room temperature for 1.5 h with the secondary antibody. Blots were developed after incubation with a BCIP/NBT kit (Zymed, South San Francisco, CA, USA). Immunoblots were photographed and imported as TIF files. Immunoreactive bands were analyzed using MCID software vers. 7.0, rev. 1.0 (Imaging Research, Ontario, Canada). The integrated density of selected bands was quantified by optical density × area. Results were converted to numerical values in order to compare the relative protein amounts of the immunoreactive bands.

Statistical analysis

The significance of differences among various treatments was compared using a one-way analysis of variance (ANOVA; Tukey's pair-wise method), with $p < 0.05$ set as the significance level. Values are expressed as the mean ± standard error of the mean (SEM).

RESULTS

MWCs and the expression of renal HSP90 in tilapia

There was no significant difference in the MWC of FW-, BW-, or SW-acclimated tilapia (Fig. 1A). Immunoblotting of homogenates of kidneys dissected from tilapia acclimated to different environments was performed. The primary antibody used for immunological detection of HSP90 resulted in a single immunoreactive band with a molecular weight (MW) of about 90 kDa (Fig. 2A). Immunoreactive bands of renal HSP90 in different environmental groups were quantified. The expression levels of renal HSP90 were similar in all groups (Fig. 2B).

MWCs and the expression of renal HSP90 in spotted green pufferfish

No significant difference was found in the MWC of pufferfish acclimated to different salinities (Fig. 1B). Immunoblotting of kidney lysates from *T. nigroviridis* acclimated to FW, BW, and SW revealed a single immunoreactive band with MW at about 90 kDa (Fig. 3A). Quantification of the protein levels of renal HSP90 revealed no significant difference in pufferfish acclimated to various environments (Fig. 3B).

MWCs and the expression of renal HSP90 in milkfish

MWCs were similar among the different salinity groups of milkfish (Fig. 1C). The antibody used for HSP90 protein recognized a single band after immunoprobing of the homogenates of kidneys from milkfish acclimated to FW, BW, and

SW (Fig. 4A). Immunoreactive bands of HSP90 in different environmental groups were quantified. Relative protein amounts of renal HSP90 were similar among milkfish from the 3 different environments (Fig. 4B).

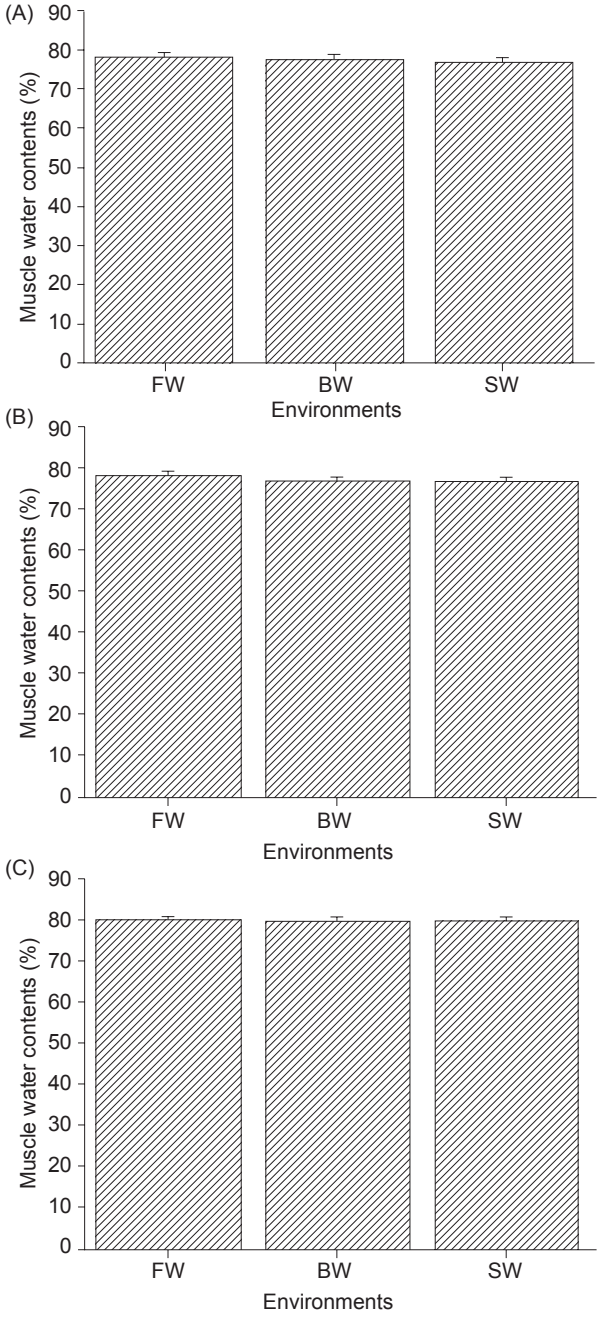

Fig. 1. Muscle water contents (MWCs) of tilapia (*Oreochromis mossambicus*) (A), pufferfish (*Tetraodon nigroviridis*) (B), and milkfish (*Chanos chanos*) (C) acclimated to environments with different salinities; $n = 5$ for all groups. There was no significant difference in the MWCs of tilapia, pufferfish, or milkfish acclimated to various salinities. Tukey's multiple-comparison test after 1-way ANOVA was used for the statistics. Values are the mean ± SEM. M, marker; FW, fresh water; BW, brackish water; SW, seawater.

DISCUSSION

In aquatic animals, all freshwater (FW) animals are osmoregulators, but marine species may be osmoregulator (all teleostean fishes) or osmoconformer (marine invertebrates in general, many crustaceans in particular) (Freire et al. 2008). Euryhaline teleosts have the ability to survive changes in the salinity of the surrounding medium through the function of osmoregulation related to controlling the composition of the extracellular fluid (i.e., plasma, lymph, and interstitial fluid) (Freire

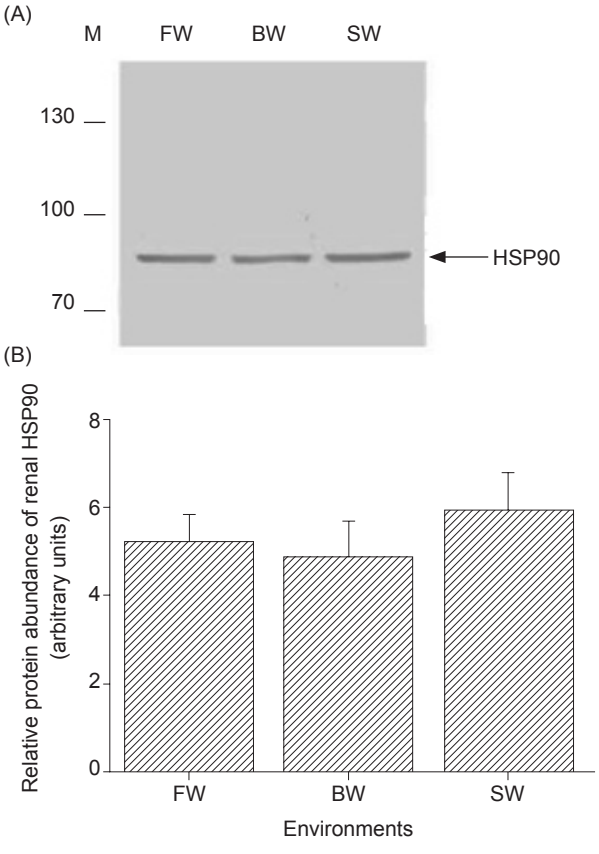

Fig. 2. Expression of HSP90 in the kidneys of tilapia (*Oreochromis mossambicus*) acclimated to environments with different salinities. (A) The arrow indicates immunoreactive bands at 90 kDa in kidneys of tilapia in fresh water (FW), brackish water (BW), and seawater (SW). (B) Relative intensities of HSP90 immunoreactive bands in different salinity groups ($n = 5$ for all groups). No significant difference was found among fish of different salinity groups using Tukey's multiple-comparison test after 1-way ANOVA. Values are the mean ± SEM. M, marker.

et al. 2008). In FW environments where there are osmotic water gain, teleosts produce large volumes of exceedingly dilute urine in the kidneys. On the other hand, seawater (SW) teleosts osmotically lose water (Marshall and Grosell 2006). Therefore, the water contents of euryhaline species must be efficiently regulated to achieve homeostasis when they were acclimated to environments with different osmolalities. The studied species Mozambique tilapia (*O. mossambicus*), spotted green pufferfish (*T. nigroviridis*), and milkfish (*C. chanos*) are all euryhaline teleosts, and their respective primary natural habitats are lakes and rivers, estuaries, and the ocean. A recent study (Freire et al. 2008) indicated that muscle water control in crustaceans and fish can be a function of the habitat acclimation, osmoregulatory capacity, and degree of euryhalinity. Similar physiological features of the MWC that did not dramatically change when acclimated to different salinity environments were found in the 3 species examined in this study as well as in many other euryhaline species, including medaka (*Oryzias latipes* and *O. dancena*; Kang et al. 2008), European sea bass (*Dicentrarchus labrax*; Giffard-Mena et al. 2008), brown trout (*Salmo trutta*; Tipsmark et al. 2002), flounder (*Paralichthys lethostigma*; Tipsmark et al. 2008), perciforms species (*Geophagus brasiliensis* and *Diapterus auratus*; Freire et al. 2008), "California" Mozambique tilapia (*O. mossambicus* × *O. urolepis hornorum*; Sardella et al. 2004), gilthead sea bream (*Sparus auratus*; Laiz-Carrion et al. 2005), sturgeon (*Acipenser naccarii*; Martinez-Alvarez et al. 2002), and

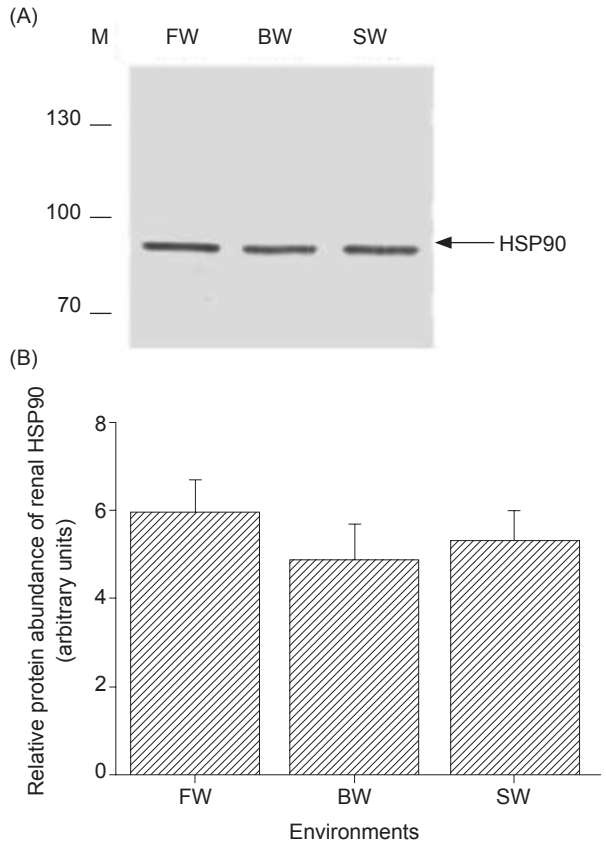

Fig. 3. Expression of renal heat shock protein (HSP)90 in pufferfish (*Tetraodon nigroviridis*) acclimated to fresh water (FW), brackish water (BW), and seawater (SW). (A) The arrow indicates immunoreactive bands at 90 kDa in kidneys of pufferfish acclimated to environments with different salinities. (B) Relative protein abundance of HSP90 immunoreactive bands in different salinity groups (n = 5 for all groups). No significant difference was found among these salinity groups using Tukey's multiple-comparison test after 1-way ANOVA. Values are the mean ± SEM. M, marker.

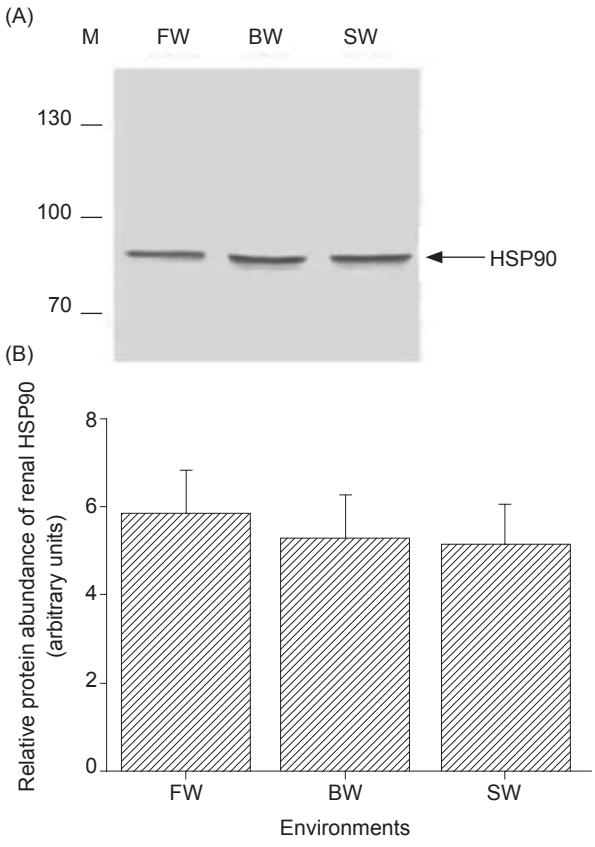

Fig. 4. Expression of renal heat shock protein (HSP)90 in milkfish (*Chanos chanos*) acclimated to environments with various salinities. (A) The arrow indicates immunoreactive bands at 90 kDa in kidneys of milkfish acclimated to fresh water (FW), brackish water (BW), and seawater (SW). (B) Relative protein amounts of HSP90 immunoreactive bands in different salinity groups (n = 5 for all groups). No significant difference was found among the 3 groups using Tukey's multiple-comparison test after 1-way ANOVA. Values are the mean ± SEM. M, marker.

black sea bream (*Mylio macrocephalus*; Kelly et al.1999). Hence, a stable MWC reflects efficient mechanisms of euryhaline teleosts to maintain a water balance in their internal environments.

There is a cellular stress response that involves the induction of a family of proteins, the HSPs (Feder and Hofmann 1999). HSPs are constitutively expressed in cells, are also known as stress proteins, and are involved in protein folding, assembly, stability, and intracellular localization, as well as acting as molecular chaperones (Hightower 1991, Basu et al. 2002, Iwama et al. 2006). Evidence is available that acceleration of HSP accumulation is essential for cell survival in normal conditions or after stress exposure. In general, elevated expression of HSP represents a ubiquitous molecular mechanism to cope with stress, although animals exhibit individual responses with different thresholds of sensitivity (Hofmann 1999). Therefore, this study compared the salinity effects on HSP abundance of the osmoregulatory organ, the kidneys, in 3 euryhaline teleosts. Basu et al. (2002) reviewed the cellular stress responses in fish to a wide range of stressors and reported that HSP expression increased in response to a variety of stressors. To examine euryhaline teleosts with the capacity to survive in hypotonic, isotonic, and hypertonic environments, this study detected the protein abundance of HSP90, which is one of the major classes of HSPs, to estimate physiological homeostasis of the 3 euryhaline teleosts from different primary natural habitats. The kidneys, the internal osmoregulatory organ of euryhaline teleosts, are mesonephric and contain renal tubules that might possess glomeruli. The loop of Henle is not found in fish kidneys, and thus fish cannot produce urine that is hypertonic to the blood (Marshell and Grosell 2006). Expression levels of branchial *HSP90* mRNA were elevated when black sea bream (*Acanthopagrus schlegeli*) and juvenile Atlantic salmon (*S. salar*) were respectively exposed to FW and SW for 24 h (Choi and An 2008, Pan et al. 2000). Furthermore, HSP90 protein amounts in gills of *A. schlegeli* changed with changing environmental salinities for 8 mo in a chronic acclimation study (Deane et al. 2002). Elevated ambient nitrite concentrations induce renal HSP90 expression in sea bream (*S. sarba*), and elevated temperatures induce it in Atlantic salmon (Deane and Woo 2007, Pan et al. 2000). However, environmental salinity changes did not result in significant alterations in the levels of the renal *HSP70* multigene family or heat shock factor (*HSF*) 1 of euryhaline sea bream in a long-term acclimation experiment (Deane and Woo 2004). Short-term experiments also revealed constant expression levels of the renal *HSP90* gene in Atlantic salmon after transfer from FW to SW (Pan et al. 2000). Similarly, renal protein amounts of HSP90 in our studied species did not significantly differ among the different salinity groups (Figs. 2-4). Unchanging levels of renal HSPs indicate that the kidneys do not face to the stressful environments and reflect that euryhaline teleosts have the ability to maintain physiological homeostasis upon a salinity challenge.

Under various conditions, organisms develop strategies to survive in a gradient between homeostasis and death (De Jong et al. 2006). Experiments with chronic acclimation of 3 euryhaline teleosts in this study showed stable MWCs and renal HSP90 protein levels, illustrating the potential as a bioindicator to point out osmotic homeostasis of the internal environment in euryhaline teleosts even from different primary natural habitats. Further studies are needed to investigate acute, short-term changes in physiological, biochemical, and stress responses.

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