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Environmental stress-related gene expression and blood physiological responses in olive flounder (*Paralichthys olivaceus*) exposed to osmotic and thermal stress

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We isolated warm temperature acclimation-related protein 65-kDa (Wap65) cDNA from the liver of olive flounder and investigated the mRNA expression of Wap65 and HSP70 in olive flounder exposed to osmotic (17.5, 8.75, and 4 psu) and thermal stress (25 and 30°C). The mRNA expression of Wap65 and HSP70 was increased by thermal stress. The mRNA expression of HSP70 was also increased by osmotic stress, whereas no significant change in Wap65 expression was detected. These results indicate that Wap65 mRNA expression occurs specifically in response to increases in water temperature, but not in response to osmotic stress. Plasma cortisol levels were also increased by osmotic and thermal stress. We also utilized the stress hormone cortisol to examine whether Wap65 expression is thermal-stress-specific. Cortisol treatment increased HSP70 mRNA expression *in vitro*, but had no significant effect on Wap65 mRNA expression. Thus, thermal stress, but not osmotic stress, induces Wap65 expression.

Keywords: Wap65; HSP70; salinity; temperature; stress; cortisol

Introduction

Physical factors such as changes in salinity and temperature can negatively affect the growth, reproduction, metabolism, osmoregulation, and immune ability of fishes (Bly and Clem 1992; Bowden 2008). Water temperature is a particularly important factor affecting growth and reproduction (Schreck et al. 1989), whereas salinity affects mainly metabolism, growth, survival, and immunity (Britoa et al. 2000). Recent studies have shown that changes in water temperature affect the mRNA expression of heat shock protein 70 (HSP70) in silver sea bream (*Sparus sarba*) (Deane and Woo 2005) and warm temperature acclimation-related protein 65kDa (Wap65) in black porgy (*Acanthopagrus schlegeli*) (Choi et al. 2008), whereas changes in salinity affect the mRNA expression of Na^+/K^+-ATPase and osmotic stress transcription factor 1 in black porgy (Choi and An 2008).

It was previously shown that Wap65 is expressed in response to high water temperatures and that it is involved in the adaptation of fishes to high water temperatures (Kikuchi et al. 1995). Wap65 was first detected as a glycoprotein in the plasma of goldfish (Kikuchi et al. 1993) and has since been identified in medaka (*Oryzias latipes*), channel catfish (*Ictalurus punctatus*), swordtail fish (*Xiphophorus hellerii*), and fugu (*Takifigu rubripes*) (Kikuchi et al. 1995; Kinoshita et al. 2001; Hirayama et al. 2003, 2004; Nakaniwa et al. 2005; Aliza et al. 2008). Teleost Wap65 shares significant sequence identity with mammalian hemopexin and has been suggested to play roles in iron homeostasis, antioxidant activity, bacteriostatic defense, nerve regeneration, transfer of heme, and the expression of genes known to promote cell survival (Delanghe and Langlois 2001).

Similarly, heat shock proteins (HSPs) are induced by environmental stressors such as heat, salinity, contaminants, heavy metals, and oxidative stress (Cara et al. 2005). HSP70 has been shown to protect cells from damage caused by these stressors (Padmini and Usha 2008). In accordance with this, cells exposed to stressful environments (e.g., changes in water temperature or salinity) show increased HSP70 expression (Wang et al. 2007).

Environmental stressors such as changes in water temperature and salinity act on the nervous system, causing the release of corticoid stress hormones via humoral responses through the hypothalamus-pituitary-interrenal axis (Wendelaar Bonga 1997; Mommsen et al. 1999). The primary corticoid released in response to stress is cortisol, which is known to affect various physical functions, including metabolism, immune ability, and osmoregulation (Wendelaar Bonga 1997; Mommsen et al. 1999).

In this study, we isolated the full-length Wap65 cDNA from the livers of olive flounder and compared the mRNA expression levels of Wap65 and HSP70 following exposure to increased temperatures and changes in salinity. Wap65 was specifically expressed

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in response to changes in the thermal environment. Furthermore, the mRNA expression of Wap65 and HSP70 was increased in hepatocytes cultured in the presence of the stress hormone cortisol. We also analyzed plasma cortisol concentrations in olive flounder exposed to changes in temperature and salinity, to deepen our understanding of how changes in temperature and salinity alter the physiology of this species.

Materials and methods

Experimental fish and conditions

Olive flounder ($n = 800$; length, 10 ± 0.5 cm; weight, 19.9 ± 1.3 g) were obtained from a commercial fish farm and allowed to acclimate to the experimental conditions for 2 weeks in three 300-L circulation filter tanks in the laboratory. During the experiments, the water temperature and photoperiod were maintained at 20 ± 1°C and 12 h light: 12 h dark, respectively. The fish were given a commercial feed twice daily (09:00 and 17:00).

Thermal stress and osmotic stress

The water temperature increased by 1°C every day from 20°C to 30°C (1°C/day) using automatic temperature-regulation systems (Johnsam Co., Boocheon, Korea) and the experimental fish were kept in seawater (35 psu) for 24 h and then transferred sequentially to tanks with salinities of 17.5, 8.75, and 4 psu, which were produced by adding underground water. The fish were maintained at each salinity for 24 h. Four fish from each group (20°C and 35 psu, control group; 25°C and 30°C, experimental groups; and 17.5, 8.75 and 4 psu, experimental groups) were randomly selected for blood and tissue sampling. The fish were anesthetized with 200 mg L^{-1} tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO, USA) prior to blood collection. Blood was collected from the caudal vasculature using a 3-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4°C; 10,000 rpm; 5 min) and stored at −80°C until analysis. To collect liver tissue samples, the fish were euthanized by spinal transection. Immediately after collection, the samples were frozen in liquid nitrogen and stored at −80°C until total RNA was extracted for analysis.

Identification of Wap65 cDNA

Primers for Wap65 were designed using highly conserved regions of gilthead seabream, *Sparus aurata* Wap65 (GenBank accession no. ACN54269) and black porgy, *Acanthopagrus schlegeli* Wap65 (ABL74446): Wap65 forward primer (5′-CAA GGT GTT CAG CTA TCA CC-3′) and Wap65 reverse primer (5′-TGT GAG CGA TGT GAT GAT CC-3′). Total RNA was extracted from the livers using a TRIzol kit (Gibco/BRL, USA). Reverse transcription (RT) was performed using M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed using a 2x Taq Premix I (Solgent, Korea). PCR was carried out as follows: initial denaturation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 40 s, and extension at 72°C for 1 min; followed by 7 min at 72°C for the final extension.

The amplified PCR product was separated on 1% agarose gels. The PCR product was purified and ligated into pGEM-T Easy Vector (Promega, USA). The colony formed by transformation was cultivated in DH5α (RBC Life Sciences, Korea), and plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Korea). Based on the plasmid DNA, Wap65 cDNA sequence data was analyzed using an ABI DNA Sequencer (Applied Biosystems, USA).

Wap65 rapid amplification of cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the Wap65 RACE reaction, total RNA was extracted from the livers using a TRIzol kit (Gibco/BRL, USA). Using 2.5 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, 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™ adaptor (Seegene, Korea).

Wap65-specific primers were selected from the PCR product by RT-PCR. For 3′ RACE, the 50-μL PCR reaction mixture contained 5 μL of 3′ RACE cDNA, 1 μL of 10 mM 3′ RACE target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL of 10 mM 3′ RACE Wap65-specific primer (5′-CAC ATC CGA TCA CGG TGG AAA CAT GTA C-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was performed as follows: initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 1 min; followed by 5 min at 72°C for the final extension.

For 5′ RACE, the 50-μL PCR reaction mixture contained 5 μL of 5′ RACE cDNA, 1 μL of 10 mM 5′ RACE target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 mM 5′ RACE Wap65-specific primer (5′-TGA ACG TGG CTG CAG CGT TCT CTC TC-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out as follows: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94 °C for 40 s, annealing at 62°C for 40 s, and extension

at 72°C for 1 min; followed by 5 min at 72°C for the final extension. Amplified PCR products were processed by electrophoresis using 1% agarose gels containing ethidium bromide (0.5 μg/μl). The transformation and sequencing were the same as described above.

Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of Wap65 and HSP70 mRNA in total RNA extracted from the liver. Primers for QPCR were designed with reference to the known sequences of olive flounder as follows (accession no. GQ396265 (Wap65); DQ662230 (HSP70); EU090804 (β-actin)): Wap65 forward primer (5′-AAC CAA GGC TGT GGA GAA GAA AGA G-3′), Wap65 reverse primer (5′-GTG TCC GTG GAA GCA GTA GTA GTG-3′), HSP70 forward primer (5′-GGA CCT GCT GCT TCT GGA TGT C -3′), HSP70 reverse primer (5′-TCT GCT TAG TAG GAA TGG TGG TGT TG -3′), β-actin forward primer (5′-AAA TGG GAA CCG CTG CCT C-3′), and β-actin reverse primer (5′-TTC CTT CTG CAT ACG GTC AG-3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA) according to the manufacturer's instructions. QPCR was performed as follows: denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s. As an internal control, experiments were duplicated with β-actin, and all data were expressed as change with respect to corresponding β-actin calculated threshold cycle (CT) levels.

Plasma cortisol analysis

Plasma cortisol was measured by radioimmunoassay using a radioimmunoassay kit (Diagnostic System Laboratories, Webster, Texas).

In vitro experiment (tissue culturing by treatment cortisol)

After liver tissues were removed from five olive flounders, the liver tissues were placed in a 24-well culture plate (SPL Life Sciences, Korea). The liver tissues were treated with cortisol (hydrocortisone 21-hemisuccinate; Sigma, USA) 0.0001 μg/ml and 0.001 μg/ml in medium 199 (Invitrogen, USA) according to the manufacturer's instructions, and cultured for 0, 6, 12, 24, and 48 h. Liver was cultured under this condition for 48 h in an incubator at 18°C, 100% humidity, and 5% CO_2 in air. Following the incubation period, each sample was centrifuged (20°C, 10000 *g*, 15 s) and supernatant was removed, and stored in individual micro-centrifuge tubes at −80°C until assay.

Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One way ANOVA followed by Dunnett's post-hoc test was used to compare the differences to control in the data ($P < 0.05$).

Results

Identification of full-length Wap65 cDNA

A single PCR product of the expected size (728 base pairs (bp)) was obtained by RT-PCR. A PCR-based cloning strategy (RT-PCR followed by 3′ and 5′ RACE) was used to clone the full-length cDNA encoding Wap65. The full-length cDNA contained 1308 nucleotides, including an open reading frame (ORF) predicted to encode a protein of 435 amino acids (GQ396265). The amino acid sequence of olive flounder Wap65 was compared with those deduced from the cDNAs of other teleost species, revealing the following amino acid similarities: 74% with gilthead seabream Wap65 (ACN54269), 73% with black porgy Wap65 (ABL74446), 70% with fugu rubripes Wap65 (NP_001027856), and 68% with Japanese medaka Wap65 (NP_001098173). These five fish species have 10 cysteines and at least two N-linked glycosylation sites in common (NES, NSS, NCT, and NDT; Figure 1).

Wap65 and HSP70 mRNA expression

Using a quantitative PCR assay, we examined changes in Wap65 and HSP70 mRNA expression in olive flounder exposed to thermal stress and osmotic stress. Wap65 mRNA expression was significantly increased in the fish at 25°C, but was decreased at 30°C. In contrast, in the livers of the fish in the osmotic stress experimental group, weak Wap65 mRNA expression was detected. HSP70 mRNA expression was significantly increased in the fish at 25°C, followed by a decrease at 30°C; an increase was also noted at 8.75 psu and was maintained down to 4 psu (Figure 2).

Plasma cortisol levels

In the thermal stress group, the mean plasma cortisol concentration was increased to 13.5 ± 0.42 ng/mL at 25°C and then decreased as the temperature was increased. In the osmotic stress group, the mean plasma cortisol concentration was 0.7 ± 0.23 ng/mL in the controls. It was significantly increased to 2.5 ± 0.5 ng/mL at 17.5 psu and was maintained down to 4 psu (Figure 3).

*Quantification of Wap65 and HSP70 mRNA expression by cortisol treatment (*in vitro*)*

The changes observed in the mRNA expression of Wap65 and HSP70 in cultured liver tissues are shown

Figure 1. Comparison of the primary sequences of Wap65 from olive flounder, *Paralichthys olivaceus*, gilthead seabream (*Sparus aurata*), black porgy (*Acanthopagrus schlegeli*), fugu rubripes (*Takifugu rubripes*), and Japanese medaka (*Oryzias latipes*). The sequences shown are aligned to match identical residues. The sequences were taken from the GenBank/EMBL/DDBJ database. The GenBank accession numbers for the sequences used in our alignment are as follows: olive flounder (ofWap65, GQ396265), gilthead seabream (gsWap65, ACN54269), black porgy (bpWap65, ABL74446), fugu rubripes (frWap65, NP_001027856), and Japanese medaka (jmWap65, NP_001098173). Shaded regions indicate identical amino acids. The five pairs of cysteine residues are indicated by asterisks. Potential N-linked glycosylation sites are boxed.

in Figure 4. In the 0.0001 μg/ml cortisol group, HSP70 mRNA expression was increased to about 9.7 times the control level at 6 h and then decreased until 48 h. In the 0.001 μg/ml cortisol group, HSP70 mRNA expression was increased to about 36.2 times the control level within 6 h and then decreased until 48 h. No significant difference in Wap65 mRNA expression was noted in the cortisol treatment groups.

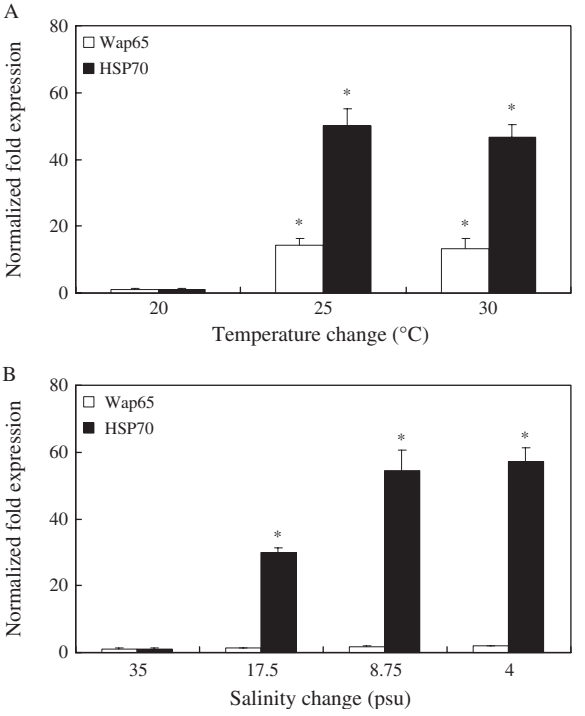

Figure 2. mRNA expression of Wap65 and HSP70, as measured by quantitative real-time PCR, in olive flounder exposed to thermal (25 and 30°C; A) and osmotic stress (17.5, 8.75, and 4 psu; B). Total liver RNA (2.5 μg) was reverse-transcribed and amplified using gene-specific primers. The results are expressed as the normalized fold-expression with respect to β-actin in the same sample. Asterisks indicate a significant difference compared with the controls ($P < 0.05$). The values represent the mean ± SD ($n = 5$).

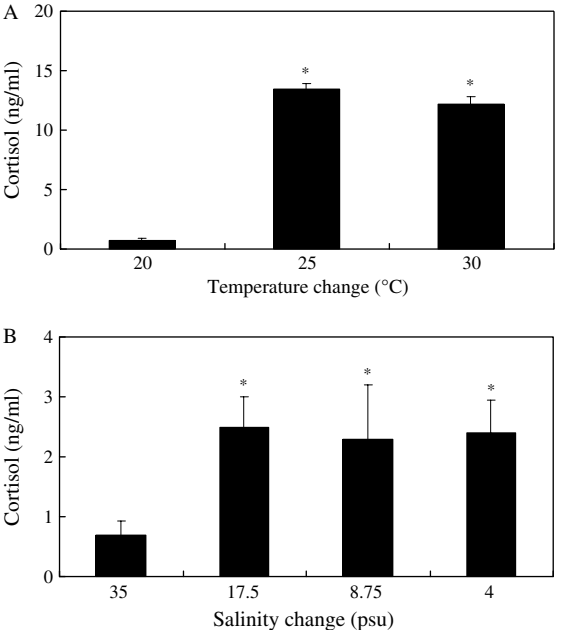

Figure 3. Plasma cortisol concentrations in olive flounder exposed to thermal (25 and 30°C) and osmotic stress (17.5, 8.75, and 4 psu). Asterisks indicate a significant difference compared with the controls ($P < 0.05$). The values represent the mean ± SD ($n = 5$).

Discussion

We examined the mRNA expression of two environmental stress-related genes, Wap65 and HSP70, in response to changes in the thermal environment and salinity. We also measured the mRNA expression of Wap65 and HSP70 in cultured hepatocytes exposed to cortisol, to examine whether Wap65 is specifically expressed in response to thermal stress, unlike HSPs. In addition, we measured the plasma cortisol concentration and compared it with the expression of environmental stress-related genes, to gauge the stress levels in olive flounder exposed to changes in temperature and salinity.

Using the BLAST algorithm (Blastp) of the National Center for Biotechnology Information, we found that the full-length primary sequence of olive flounder Wap65 displays high identity with that from other species: 74% with gilthead seabream, 73% with black porgy, 70% with fugu, and 68% with Japanese medaka (Figure 1). In addition, olive flounder Wap65 typically has ten cysteines and three N-linked glycosylation sites, similar to other members of the Wap65 family (Kinoshita et al. 2001); therefore, we named the gene olive flounder Wap65. Kinoshita et al. (2001) reported that the conserved cysteine residues in Wap65 probably participate in disulfide bridges and that the N-linked glycosylation sites in the protein are likely to bind oligosaccharides. The oligosaccharides attached to this protein promote glycoprotein folding, contribute to protein–protein interactions, regulate other ligand recognition processes, and stabilize the protein, making it resistant to proteolysis (Helenius and Aebi 2004; Ohtsubo and Marth 2006). Up to now, the role of the oligosaccharide in this glycoprotein has been unclear; however, Satoh et al. (1994) reported that N-linked glycosylation is an essential part of the high affinity of human hemopexin for heme, and Hirayama et al. (2004) reported that fish Wap65 binds heme.

The mRNA expression of Wap65 increases with a rise in water temperature (Kinoshita et al. 2001; Choi et al. 2008). We observed an increase in Wap65 mRNA expression as the water temperature was raised, in agreement with data from goldfish (Kikuchi et al. 1995), carp (Kinoshita et al. 2001), and black porgy (Choi et al. 2008). HSP70 mRNA expression also increased as the water temperature was raised, in agreement with data from rainbow trout (Basu et al. 2003) and silver sea bream (*S. sarba*) (Deane and Woo 2005) exposed to acute thermal stress. In comparison, HSP70 mRNA expression was increased under

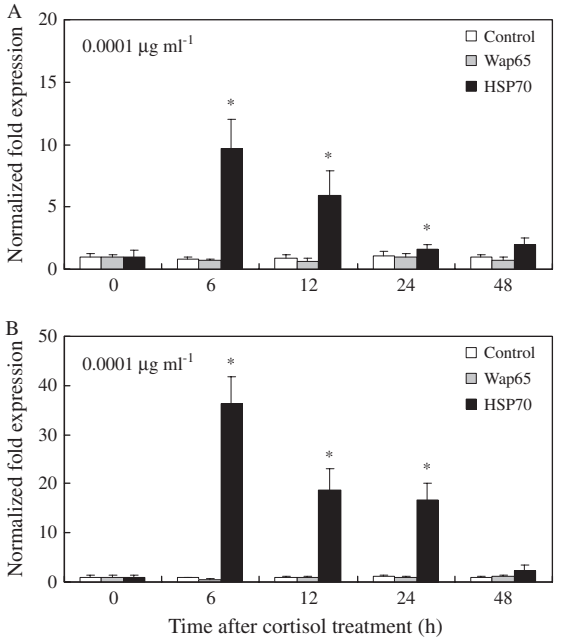

Figure 4. mRNA expression of Wap65 and HSP70, as measured by quantitative real-time PCR, in the livers of cortisol-treated olive flounder (*in vitro*). Olive flounder livers were incubated for 0, 6, 12, 24, and 48 h with medium alone (control), or with medium containing 0.0001 or 0.001 μg/ml cortisol. Total liver RNA (2.5 μg) was reverse-transcribed and amplified using gene-specific primers. The results are expressed as the normalized fold-expression with respect to β-actin in the same sample. Asterisks indicate a significant difference compared with the controls ($P < 0.05$). The values represent the mean $\pm$ SD ($n = 5$).

conditions of low salinity (Figure 2). Dong et al. (2008) reported that HSP70 mRNA expression was significantly increased when sea cucumbers (*Apostichopus japonicus* Selenka) that were adapted to 32 psu were transferred to a low-salinity environment (20 psu). Lindquist (1986) and Feder and Hofmann (1999) reported that high levels of inducible HSP70 corresponded to high levels of protein damage as well as increased tolerance to subsequent stressors, including changes in salinity.

Overall, HSP70 mRNA expression was significantly increased in response to thermal and low-salinity stresses, whereas Wap65 mRNA expression was significantly increased in response to thermal stress only. In the case of black porgy, Choi et al. (2008) reported that Wap65 was expressed specifically under conditions of thermal stress, which indicates that Wap65 expression is thermal-stress-specific and is unaffected by other common types of stress, such as changes in salinity. In terms of the difference in mRNA expression between Wap65 and HSP70 in response to thermal stress, Kikuchi et al. (1993) reported that the 10 N-terminal-most amino acids of Wap65 were clearly different from those in HSP70, suggesting that Wap65 and HSPs are expressed in different ways in response to increases in water temperature. Thus, we hypothesize that these 10 amino acids perform an unknown function related to thermal stress-induced expression, and Wap65 may have functions in self defense mechanisms that acclimation to warm temperature.

In this study, the plasma cortisol concentrations of the fish were significantly increased by thermal and salinity stresses (Figure 3), in agreement with previous data from a variety of teleosts, including olive flounder (Chang et al. 2002; Davis 2004; Choi et al. 2007). Therefore, a rapid change in water temperature or salinity generates stress in fish, primarily by activating the hypothalamus-pituitary-interrenal axis, resulting in the secretion of cortisol into the blood (Wendelaar Bonga 1997).

Finally, we compared the mRNA expression of Wap65 and HSP70 in hepatocytes cultured in the presence of the stress hormone cortisol, to examine whether Wap65 exhibits thermal-stress-specific gene expression. Wap65 was not detected, whereas HSP70 expression was significantly increased in cortisol-treated hepatocytes (Figure 4). In rainbow trout hepatocytes treated with cortisol, HSP90 mRNA expression was increased (Aluru and Vijayan 2007). Basu et al. (2003) found that HSP70 mRNA expression was significantly increased in rainbow trout fed cortisol. These results indicate that HSP70 expression is increased by cortisol-induced stress. We analyzed the mRNA expression of HSP70 in olive flounder hepatocytes treated with cortisol; however, no significant difference in Wap65 mRNA was detected, indicating that Wap65 mRNA expression is not significantly altered by common sources of stress, such as cortisol.

In conclusion, we showed that Wap65 expression is thermal-stress-specific, although its relationship with temperature acclimation is unknown. Thus, additional studies of the roles of Wap65 are required.

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References

Aliza D, Ismail IS, Kuah MK, Shu-Chien AC, Muhammad TST. 2008. Identification of Wap65, a human homologue of hemopexin as a copper-inducible gene in swordtail fish, *Xiphophorus helleri*. Fish Physiol Biochem. 34: 129–138.

Aluru N, Vijayan MM. 2007. Hepatic transcriptome response to glucocorticoid receptor activation in rainbow trout. Physiol Genomics. 31:483–491.

Basu N, Kennedy CJ, Iwama GK. 2003. The effects of stress on the association between hsp70 and the glucocorticoid receptor in rainbow trout. Comp Biochem Physiol A. 134:655–663.

Bly JE, Clem W. 1992. Temperature and teleost immune functions. Fish Shellfish Immunol. 2:159–171.

Bowden TJ. 2008. Modulation of the immune system of fish by their environment. Fish Shellfish Immunol. 25:373–83.

Britoa R, Chimal ME, Rosas C. 2000. Effect of salinity in survival, growth, and osmotic capacity of early juveniles of *Farfantepenaeus brasiliensis* (Decapoda: Penaeidae). J Exp Mar Biol Ecol. 244:253–263.

Cara JB, Aluru N, Moyano FJ, Vijayan MM. 2005. Food-deprivation induces HSP 70 and HSP 90 protein expression in larval and gilthead sea bream and rainbow trout. Comp Biochem Physiol B. 142:426–431.

Chang YJ, Min BH, Chang HJ, Hur JW. 2002. Comparison of blood physiology in black porgy (*Acanthopagrus schlegeli*) cultured in converted freshwater from seawater and seawater from freshwater. J Korean Fish Soc. 35:595–600.

Choi CY, Min BH, Jo PG, Chang YG. 2007. Molecular cloning of PEPCK and stress response of black porgy (*Acanthopagrus schlegeli*) to increased temperature in freshwater and seawater. Gen Comp Endocrinol. 152:47–53.

Choi CY, An KW, Choi YK, Jo PG, Min BH. 2008. Expression of warm temperature acclimation-related protein 65-kDa (Wap65) mRNA, and physiological changes with increasing water temperature in black porgy, *Acanthopagrus schlegeli*. J Exp Zool A. 309: 206–214.

Choi CY, An KW. 2008. Cloning and expression of Na^+/K^+-ATPase and osmotic stress transcription factor 1 mRNA in black porgy, *Acanthopagrus schlegeli* during osmotic stress. Comp Biochem Physiol B. 149:91–100.

Davis KB. 2004. Temperature affects physiological stress responses to acute confinement in sunshine bass (*Morone chrysops x Morone saxatilis*). Comp Biochem Physiol A. 139:433–440.

Deane EE, Woo NYS. 2005. Cloning and characterization of the hsp70 multigene family from silver sea bream: Modulated gene expression between warm and cold temperature acclimation. Biochem Biophys Res Commun. 330:776–783.

Delanghe JR, Langlois MR. 2001. Hemopexin: a review of biological aspects and the role in laboratory medicine. Clin Chim Acta. 12:13–23.

Dong Y, Dong S, Meng X. 2008. Effects of thermal and osmotic stress on growth, osmoregulation and Hsp70 in sea cucumber (*Apostichopus japonicus* Selenka). Aquaculture. 276:179–186.

Feder ME, Hofmann GE. 1999. Heat-shock proteins, molecular chaperones, and the stress responses: evolutionary and ecological physiology. Annu Rev Physiol. 61: 243–282.

Helenius A, Aebi M. 2004. Roles of N-linked glycans in the endoplasmic reticulum. Annu Rev Biochem. 73: 1019–1049.

Hirayama M, Nakaniwa M, Ikeda D, Hirazawa N, Otaka T, Mitsuboshi T, Shirasu K, Watabe S. 2003. Primary structures and gene organizations of two types of Wap65 from the pufferfish *Takifugu rubripes*. Fish Physiol Biochem. 29:211–224.

Hirayama M, Kobiyama A, Kinoshita S, Watabe S. 2004. The occurrence of two types of hemopexin-like protein in medaka and differences in their affinity to heme. J Exp Biol. 207:1387–1398.

Kikuchi K, Watabe S, Suzuki Y, Aida K, Nakajima H. 1993. The 65-kDa cytosolic protein associated with warm temperature acclimation in goldfish, *Carassius auratus*. J Comp Physiol B. 163:349–354.

Kikuchi K, Yamasita M, Watabe S, Aida K. 1995. The warm temperature acclimation-related 65-kDa protein, Wap65, in goldfish and its gene expression. J Biol Chem. 270:17087–17092.

Kinoshita S, Itoi S, Watabe S. 2001. cDNA cloning and characterization of the warm-temperature-acclimation-associated protein Wap65 from carp *Cyprinus carpio*. Fish Physiol Biochem. 24:125–134.

Lindquist S. 1986. The heat-shock response. Annu Rev Biochem. 55:1151–1191.

Mommsen TP, Vijayan MM, Moon TW. 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Fish Biol Fisheries. 9:211–268.

Nakaniwa M, Hirayama M, Shimizu A, Sasaki T, Asakawa S, Shimizu N, Watabe S. 2005. Genomic sequences encoding two types of medaka hemopexin-like protein Wap65, and their gene expression profiles in embryos. J Exp Biol. 208:1915–1925.

Nouroozzadeh J, Tajaddinisarmadi J, Wolff SP. 1994. Measurement of plasma hydroperoxide concentrations by ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. Anal Biochem. 200:403–409.

Ohtsubo K, Marth JD. 2006. Glycosylation in cellular mechanisms of health and disease. Cell. 126:855–867.

Padmini E, Usha RM. 2008. Impact of seasonal variation on HSP70 expression quantitated in stressed fish hepatocytes. Comp Biochem Physiol B Biochem Mol Biol. 151:278–285.

Satoh T, Satoh H, Iwahara S, Hrkal Z, Peyton DH, Muller-Eberhard U. 1994. Roles of heme iron-coordinating histidine residues of human hemopexin expressed in baculovirus infected insect cells. Proc Natl Acad Sci USA. 91:8423–8427.

Schreck CB, Bradford CS, Fitzpatrick MS, Patino R. 1989. Regulation of the interregnal of fishes: non-classical control mechanism. Fish Physiol Biochem. 7:259–265.

Wang J, Wei Y, Li X, Cao H, Xu M, Dai J. 2007. The identification of heat shock protein genes in goldfish (*Carassius auratus*) and their expression in a complex environment in Gaobeidian Lake, Beijing, China. Comp Biochem Physiol C. 145:350–362.

Wang F, Yang H, Gao F, Liu G. 2008. Effects of acute temperature or salinity stress on the immune response in sea cucumber, *Apostichopus japonicus*. Comp Biochem Physiol A. 151:491–498.

Wendelaar Bonga SE. 1997. The stress response in fish. Physiol Rev. 77:591–625.