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Molecular cloning of PEPCK and stress response of black porgy (*Acanthopagrus schlegeli*) to increased temperature in freshwater and seawater

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Abstract

Stress responses to increased temperature in black porgy reared in freshwater (FBP) and seawater (SBP) were examined via endocrinological and blood physiological methods. A rise in temperature increased plasma cortisol levels, which were significantly higher in FBP compared to SBP. The stimulated expression of phosphoenolpyruvate carboxykinase (PEPCK) mRNA in liver might result from the high cortisol level, and this explains the observed higher plasma glucose levels in FBP versus SBP. Full-length cDNA sequence for PEPCK was determined by 3′ and 5′ RACE procedures. PEPCK cDNA clone was found to contain 2563 nucleotides including an open reading frame that encodes 624 amino acids. While aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of FBP increased with temperature, there was no change in SBP. In FBP, T_3 were 2.3 ± 0.3 ng/ml at 20 °C and significantly decreased to 1.0 ± 0.3 ng/ml at 30 °C. On the other hand, in SBP, it were 3.1 ± 0.5 ng/ml at 20 °C but significantly increased to 5.2 ± 0.4 ng/ml at 30 °C. When comparing osmolality at the temperature of 30 °C and of 20 °C, the difference was found to be greater for FBP than SBP. Accordingly, the results suggest that FBP suffers greater stress than SBP with increased temperature, and provide stress responses and osmoregulatory abilities against stressors in black porgy that could differ depending on salinities.
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Keywords: Black porgy; *Acanthopagrus schlegeli*; Temperature rise; Stress response; Cortisol; PEPCK mRNA; RACE; Osmoregulation

1. Introduction

Water temperature is one of the most important factors that affect the growth and production of fish. In aquaculture, rapid temperature changes cause stress in fish, in a manner similar to transportation, confinement, size-grading or high stocking density. When fish are exposed to a stressor, activity in the hypothalamus–pituitary–interrenal axis is increased, resulting in secretion of cortisol into the blood (Perry and Reid, 1993; Wendelaar Bonga, 1997; Chang and Hur, 1999). Secondary responses include imbalance concerns blood ion levels, increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by destruction of hepatocytes, kidney and heart, increase in cardiac impulse, increase in oxygen consumption and energy stimulus, i.e. increased blood glucose (Tomasso et al., 1980; Eddy, 1981; Carmichael et al., 1984; McDonald and Milligan, 1997). Gluconeogenesis is known to occur through catalysis of phosphoenolpyruvate carboxykinase (PEPCK) via stimulation of glucocorticoids in the liver (Hanson and Reshef, 1997; Radziuk and Pye, 2001).

Thyroid hormones, such as L-thyroxine (T_4) and triiodo-L-thyronine (T_3), are known to stimulate metabolism and growth in most vertebrates (McDonald and Milligan, 1992), and plasma thyroid hormone increases during metamorphosis in flatfish (Inui et al., 1995) and smoltification of salmon (Brown and Bern, 1989). In addition, changes in

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thyroid hormone levels are used as a stress index (Vijayan and Leatherland, 1989). However, in the case of changes in T_4 and T_3 blood concentrations, the response pattern was found to differ depending on the type of stressor life stage (Yoo et al., 2003).

Black porgy (*Acanthopagrus schlegeli*) is a euryhaline marine teleost with a broad scope for water salinity. We have been studying this freshwater aquaculture species for its excellent osmoregulatory ability (Chang et al., 2002; Min et al., 2003, 2005). Therefore, to obtain fundamental data for freshwater aquaculture of black porgy, this study examined the effect of a temperature rise on blood cortisol, as the primary stress response, in black porgy reared in freshwater and seawater, as well as plasma osmolality, the levels of ions, AST, ALT and glucose in the blood, and PEPCK expression, as secondary stress responses. The study also examined how stressor affects plasma T_3 concentrations.

2. Materials and methods

2.1. Fish and acclimation

Black porgy (average length: 14.3 ± 0.4 cm; weight: 51.0 ± 6.0 g), from the culture cages of the Marine Science Technology Center (Pukyong National University, Korea), were placed into 4 U of 220 L circulation filter tanks in the laboratory at 40 fish per tank and held for 2 weeks. Two tanks were converted to freshwater according to the method of Min et al. (2003), i.e. spring water was added to the tank to give 10% seawater and the fish acclimatized for 3 days. Then, more spring water was added to give pure freshwater and the fish reared for 35 days. The remaining fish were held in the seawater tanks. Fish were kept at 20 ± 1 °C before experimentation, on a 12L:12D cycle and fed a commercial diet (42% protein, 7% fat, 4% fiber, 17% ash, and 2.7% phosphorus) twice a day.

2.2. Water temperature rise and sampling

Black porgy were acclimated in seawater or freshwater in 4 U of 40 L square circulating filter tanks with automatic temperature regulation systems (JS-WBP-170RP, Johnsam Co., Korea), 20 fish per tank, and acclimated for 24 h. Blood and tissue samples were taken from three fish in two tanks (a total of six fish) when water temperature reached 20, 25, and 30 °C, with an increase in temperature of 1 °C every day from 20 to 30 °C. Survival rate was examined with regard to temperature rise in the remaining two tanks. Fish were anesthetized with 200 mg/L solution of tricaine methan sulphonate (MS-222) and blood taken from the caudal vasculature using a 3 ml syringe coated with heparin. After centrifugation (10,000 rpm, 4 °C, 5 min), the plasma was stored in an ultra-low freezer at −80 °C before analysis.

Experimental fish were decapitated and brain, liver, kidney, gonad, and intestine frozen immediately in liquid nitrogen and stored at −80 °C until analysis by RT-PCR. No food was given during the experimental period (10 days, from 20 to 30 °C).

2.3. Isolation of PEPCK cDNA

The degenerate primers were designed from highly conserved regions of phosphoenolpyruvate carboxykinase (PEPCK) from the zebrafish (*Danio rerio;* Accession No. BC053122), Atlantic salmon (*Salmo salar;* Accession No. DQ144903) and rainbow trout (*Oncorhynchus mykiss;* Accession No. AF246149). The sequence of the forward primer (5′-ATG T[CG]A TCC CCT TCA G[CT]A TGG-3′) was based on nucleotides 532–552; the sequence of the reverse primer (5′-CGC CAG TTA AA[CGT] G[AC]C TCG TAC-3′) was based on nucleotides 1443–1465. Total RNA was extracted using a Trizol kit (Gibco/BRL, Grand Island, NY, USA). Reverse transcription and PCR amplification were conducted using the *AccuPower* RT/PCR PreMix (Bioneer, Seoul, Korea), according to manufacture's instructions. A single PCR product of the expected size (934 bp) was obtained. The amplified DNA was extracted using a plasmid purification kit (NucleoGen, Seoul, Korea) and sequencing performed by COSMO (Seoul, Korea).

2.4. Rapid amplification of cDNA 3′ and 5′ ends (RACE)

For RACE reactions, total RNA was isolated from liver as described above. With 2 μg of total RNA as template, 5′-RACE-ready cDNA and 3′-RACE-ready cDNA were generated using protocols and reagents provided in a CapFishing full-length cDNA premix kit (Seegene, Seoul, Korea). Gene-specific primers were selected from the 934 bp PCR product obtained by RT-PCR. For 5′-RACE, the 50 μl PCR reaction mixture was as follows: 5 μl of 5′-RACE-ready cDNA, 1 μl of 10 mmol l^{-1} 5′ target primer, 1 μl of 10 mmol l^{-1} 5′ RACE gene-specific primer (5′-GSP: 5′-GAT TAC AGG GCC AGT TGT TTA CCA AGG GCT-3′), and 25 μl of SeeAmp Taq Plus master mix. PCR was carried out for 35 cycles as follows: denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s and an extension at 72 °C for 90 s, followed by 1 cycle of 5 min at 72 °C for extension. The PCR product was amplified, cloned into a pGEM-T Easy Vector, and sequenced.

For 3′-RACE, the 50 μl PCR mixture contained 3′-RACE-ready cDNA, 3′-gene-specific primer (3′-GSP: 5′-ACC TCC TGG AAG AAC AAA CCC TGG AGC A-3′), 3′ target primer, and SeeAmp Taq Plus master mix, as described above. PCR conditions were as previously described. The PCR product was amplified, cloned into pGEM-T Easy Vector, and sequenced. DNA and deduced amino acid sequence data were analyzed using GENETYX-WIN (Software Develop. Co., Tokyo, Japan).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from brain, liver, gonad, kidney, and intestine. PEPCK-specific primer for RT-PCR was designed as follows: forward primer: [5′-GAT TAC AGG GCC AGT TGT TTA CCA AGG GCT-3′], reverse primer: [5′-ACC TCC TGG AAG AAC AAA CCC TGG AGC A-3′]. One microgram of total RNA was reverse transcribed for 30 min at 50 °C using oligo(dT) primer and SuperScript™ reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacture's instructions. Equal amounts of RNA (as determined spectrophotometrically) from the various tissues were used for reverse transcription. RT was performed using an *AccuPower* RT/PCR PreMix (Bioneer, Korea). As a control for loading, black porgy β-actin mRNA, also amplified for each RT reaction product, was used. The densitometric process from ethidium bromide-stained gel was optimized for linearity as described previously (Choi and Habibi, 2003).

2.6. Plasma parameters analysis

Plasma cortisol levels were analyzed via radioimmunoassay (RIA) using a cortisol RIA kit (DSL, USA). Plasma T_3 was analyzed with enzyme immuno assay (EIA) using T_3 EIA Kit (Biosewoom, Korea). Plasma glucose, AST, ALT, Na^+, and Cl^- were measured with Biochemistry Autoanalyzer (Hitach 7180, Hitach Co., Japan), plasma osmolality was measured with Vapor Pressure Osmometer (Vapro 5520, WESCOR Co., USA).

2.7. Statistical analysis

Results were presented as means ± SD (or means ± SE) and data were analyzed using SPSS (version 10.0) software. For plasma parameter, significant interactions between salinity (freshwater and seawater) and temperature (20, 25, and 30 °C) were compared using two-way ANOVA. Post hoc multiple comparison were made using a Tukey's test (significance

level $P < 0.05$). Differences between FBP and SBP were analyzed by Student's t-test for each water temperature. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Identification of PEPCK cDNA

A PCR-based cloning strategy (RT-PCR followed by 3′- and 5′-RACE) was used to clone a cDNA encoding a putative PEPCK from black porgy liver. The 2563 base pair (bp) cDNA includes an open reading frame (ORF) of 1872 bp that began with the first ATG codon at position 111 bp and ended with a TAA stop codon at position 1983 bp (Accession No. AY929162). A comparison of the amino acid sequence of black porgy PEPCK to previously reported amino acid PEPCK sequences are shown in Fig. 1. The deduced amino acid sequence of black porgy PEPCK had the highest identity with zebrafish (*D. rerio*) PEPCK (80.2%) (Accession No. BC053122), followed by

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bPEPCK   1:MPPQLQSQNQSGPRVLQGDLSALSPAVKEFLDANVTLCQPDSIHICDGSDEENRTILAQLEEQGLIKKLS  70
zPEPCK   1:MPPQLQSQDRSCPRVLQGDLASLSASVREFIDSSVSLCQPDALHICDGSEQENSTILSLLEEQGAIKRLR  70
hPEPCK   1:MPPQLQNGLNLSAKVVQGSLDSLPQAVREFLENNAELCQPDHIHICDGSEEENGRLLGQMEEEGILRRLK  70
cPEPCK   1:MAPELKTEVNIISKVIQGDLESLPPQVREFIESNAKLCQPESIHICDGSEEENKKILDIMVEQGMIKKLS  70

bPEPCK  71:KYENCWLARTDPRDVARVESKTVIVTPDQRDTVPTPLDGGVSQLGRWMSPEEFDKAMTQRFPGCMKGRTM 140
zPEPCK  71:KYSNCWLARTDPRDVARVESKTVIVTAEQRDTVPTPTGGGVSQLGRWMCPEEWDKAMNLRFPGCMKGRVM 140
hPEPCK  71:KYDNCWLALTDPRDVARIESKTVIVTQEQRDTVPIP-KTGLSQLGRWMSEEDFEKAFNARFPGCMKGRTM 139
cPEPCK  71:KYENCWLALTNPRDVARIESKTVIITQEQRDTIPIP-KTGSSQLGRWMSEEDFEKAFNTRFPGCMQGRTM 139

bPEPCK 141:YVIPFSMGPVGSPLSKIGVELTDSPYVVASMRVMTRMGKAVLSALGTGDFVRCLHSVGCPLPLKKPLVNN 210
zPEPCK 141:YVIPFSMGPVGSPLSKIGVELTDSPYVVASMRIMTRMGKTVLSALGNGEFVRCLHSVGCPLPLKKPLVNN 210
hPEPCK 140:YVIPFSMGPLGSPLSKIGIELTDSPYVVASMRIMTRMGTPVLEALGDGEFVKCLHSVGCPLPLQKPLVNN 209
cPEPCK 140:YVIPFSMGPIGSPLAKIGIELTDSPYVVASMRMMTRMGTAALKALGNGEFVKCLHSVGCPLPLKEPLINN 209

                                PEPCK-specific domain
bPEPCK 211:WPCNHEQTLIAHIPDRRQIVSFGSGYGGNSLLGKKCFALRIASRIAKEEGWLAEHMLILGVTNPAGEKKY 280
zPEPCK 211:WPCNPELTLVAHIPDQRKIVSFGSGYGGNSLLGKKCFALRIASRIAKEEGWLAEHMLILGITNPAGQKKY 280
hPEPCK 210:WPCNPELTLIAHLPDRREIISFGSGYGGNSLLGKKCFALRMASRLAKEEGWLAEHMLILGITNPEGEKKY 279
cPEPCK 210:WPCNPELTLIAHLPDRREIISFGSGYGGNSLLGKKCFALRIASRIAKEEGWLAEHMLILGITNPEGEKKY 279

                    Kinase-1                  Kinase-2
bPEPCK 281:MAAAFPSACGKTNLAMLCPTLPGWKVECVGDDIAWMKFDDQGNLRAINPENGFFGVAPGTSAKTNPNAMK 350
zPEPCK 281:FAAAFPSACGKTNLAMLKPSLPGWKVECVGDDIAWMKFDKEGNLRAINPENGFFGVAPGTSSKTNPNAMS 350
hPEPCK 280:LAAAFPSACGKTNLAMMNPSLPGWKVECVGDDIAWMKFDAQGHLRAINPENGFFGVAPGTSVKTNPNAIK 349
cPEPCK 280:FAAAFPSACGKTNLAMMNPSRPGWKIECVGDDIAWMKFDELGNLRAINPENGFFGVAPGTSIKTNPNAIK 349

bPEPCK 351:TIIKNTVFTNVAETSDGGVYWEGMDQSLPEGVTITSWKNKPWSKEDGEPCAHPNSRFCTPAGQCPIIDPL 420
zPEPCK 351:TISCNTLFTNVAESSDGGVFWEGMDEDLPEGVTLTSWKNQPWTPEDGEPCAHPNSRFCTPAAQCPIIDPQ 420
hPEPCK 350:TIQKNTIFTNVAETSDGGVYWEGIDEPLASGVTITSWKNKEWSSEDGEPCAHPNSRFCTPASQCPIIDAA 419
cPEPCK 350:TIFKNTIFTNVAETSDGGVYWEGIDEPLPPGVTLTSWKNKDWTPDNGEPCAHPNSRFCTPASQCPIMDPA 419

bPEPCK 421:WESPEGVPIEAIIFGGRRPEGVPLVYEAFNWRHGVFVGAAMRSEATAAAEHKGKMIMHDPFAMRPSFGYN 490
zPEPCK 421:WESPEGVPIEAIIFGGRRPQGVPLVYEAFDWAHGVFVGASMRSEATAAAEHKGKVIMHDPFAMRPFFGYN 490
hPEPCK 420:WESPEGVPIEGIIFGGRRPAGVPLVYEALSWQHGVFVGAAMRSEATAAAEHKGKIIMHDPFAMRPFFGYN 489
cPEPCK 420:WESPEGVPIEGIIFGGRRPAGVPLVYEAFNWQHGVFIGAAMRSEATAAAEHKGKIIMHDPFAMRPFFGYN 489

bPEPCK 491:FGQYLSHWLSMADRPGAKLPKIFHVNWFRKSPTAGFLWPGFGDNIRVLDWMFRRVNGAAGAMPSVVGYLP 560
zPEPCK 491:FGQYLSHWLSMEQRPGAKLPKIFHVNWFGRSSSGRFLWPGFGENIRVLEWMFGRLSGGAEARTTAVGLVP 560
hPEPCK 490:FGKYLAHWLSMAQHPAAKLPKIFHVNWFRKDKEGKFLWPGFGENSRVLEWMFNRIDGKASTKLTPIGYIP 559
cPEPCK 490:FGKYLAHWLSMAHRPAAKLPRIFHVNWFRKDSQGKFLWPGYGENSRVLEWMFNRIQGKASAKSTAIGYIP 559

bPEPCK 561:CRDSLNLQGLKEEVDLDELFSLDQEFWQREVEDVRKYFDTQVNDDLPNEVARQLDLLQQRVKQM------ 624
zPEPCK 561:ADGALNLHGLPDVEPL-ELFRVSQEFWMQELQEIREYFSRELNRDLPQEMQRQLELLEHRLTHTHVSSKH 630
hPEPCK 560:KEDALNLKGL-GHINMMELFSISKEFWEKEVEDIEKYLEDQVNADLPCEIEREILALKQRISQM------ 622
cPEPCK 560:ADTALNLKGL-EDINLTELFNISKEFWEKEVEEIKQYFEGQVNADLPYEIERELLALEMRIKQL------ 622
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Fig. 1. Comparison of amino acid sequence of black porgy (*Acanthopagrus schlegeli)* PEPCK, zebrafish (*Danio rerio*) PEPCK, human (*Homo sapiens*) PEPCK, and chicken (*Gallus gallus*) PEPCK optimally aligned to match identical residues, indicated by shaded box. The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. The PEPCK sequences used for alignment are black porgy PEPCK (bPEPCK, AY929162), zebrafish PEPCK (zPEPCK, BC053122), human PEPCK (hPEPCK, AY794987), and chicken PEPCK (cPEPCK, NM_205471). To aid comparison, functional motifs, as described by Matte et al. (1987), are indicated; oxaloacetate-binding domain, kinase-1 and kinase-2 motifs. Gaps in the sequences are indicated as dashes.

human (*Homo sapiens*) PEPCK (75.8%) (Accession No. AY794987), and chicken (*Gallus gallus*) PEPCK (74.7%) (Cook et al., 1986).

3.2. Tissue distribution of the PEPCK mRNA by RT-PCR

The tissue distribution of the PEPCK transcript was assessed by RT-PCR using primers designed from the sequence of black porgy PEPCK. At 20 °C, PEPCK mRNA of FBP and SBP were not expressed in other organs, except kidney. At 30 °C, FBP showed the highest expression in the liver and first expression in the intestine. However, the degree of expression in kidney showed no difference from that at 20 °C. For SBP, PEPCK mRNA was expressed first in the liver but not in the intestine, which differed from FBP (Fig. 2).

3.3. Plasma cortisol and glucose levels

P-values resulting from the Two-way ANOVA of the plasma parameters assessed are presented in Table 1.

Plasma cortisol levels in both FBP and SBP increased significantly with temperature rise. In addition, cortisol concentrations in FBP were significantly higher than in SBP from 25 °C. In fact, at 30 °C, the levels in FBP were twice as high as in SBP. Plasma glucose levels in FBP and SBP were 49.5 ± 6.5 and 54.6 ± 2.5 mg/dl, respectively, at 20 °C, but increased significantly to 91.0 ± 6.2 and 70.8 ± 2.3 mg/dl, respectively, at 30 °C ($P < 0.05$). However, there was no difference in the level of plasma glucose in FBP or SBP up to 25 °C, but FBP recorded significantly higher levels than SBP at 30 °C (Fig. 3).

FBP began dying at 28 °C and showed a survival rate of 40% at 30 °C, whereas, all SBP survived the experimental period.

Table 1
P-values from two-way ANOVAs of plasma parameters in freshwater- and seawater-adapted black porgy (*Acanthopagrus schlegeli*) with water temperature rise (20 → 30 °C)

Parameter	Salinity	Temperature	Salinity × temperature
Cortisol	0.004	<0.001	0.004
Glucose	0.223	<0.001	0.022
AST	0.048	0.001	0.001
ALT	0.181	<0.001	0.014
T_3	<0.001	0.006	<0.001
Na^+	<0.001	<0.001	<0.001
Cl^-	<0.001	<0.001	<0.001
Osmolality	<0.001	0.009	<0.001

3.4. Plasma AST, ALT, and T_3 levels

AST levels of FBP were 33.0 ± 4.7 IU/L at 20 °C but increased three times to 101.3 ± 21.1 IU/L at 30 °C. ALT levels showed a similar tendency due to temperature rise. During the experimental period, AST and ALT levels

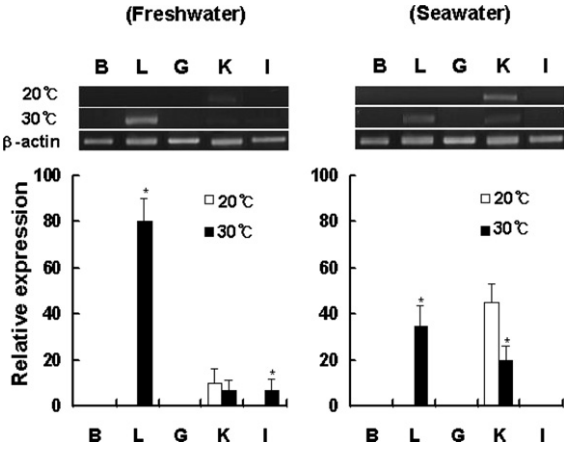

Fig. 2. Expression of PEPCK mRNA in tissues of freshwater- and seawater-adapted black porgy (*Acanthopagrus schlegeli*) with water temperature rise (20 → 30 °C). One microgram of total RNA prepared from brain (B), liver (L), gonad (G), kidney (K), and intestine (I) was reverse transcribed and amplified using black porgy PEPCK-specific primer. Tissue distribution of black porgy PEPCK was analyzed by RT-PCR. The expression of β-actin mRNA was evaluated in each RT reaction product as a control. The expression level of each tissue was normalized with respect to the β-actin signal and expressed as relative expression level. Value represented as means ± SE ($n = 3$). *Significant difference between fish from 20 and 30 °C ($P < 0.05$).

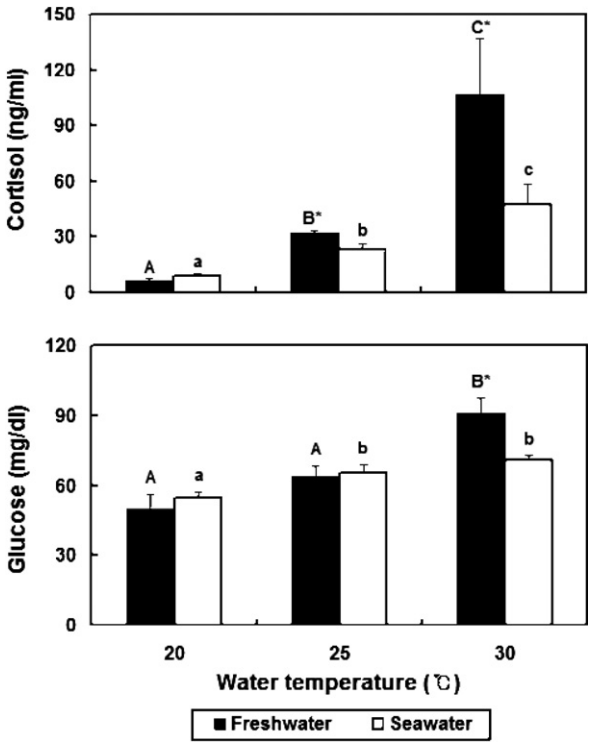

Fig. 3. Levels of plasma cortisol (up) and glucose (down) in freshwater- and seawater-adapted black porgy (*Acanthopagrus schlegeli*) with water temperature rise (20 → 30 °C). Each value represents means ± SD ($n = 6$). Different capital letters indicate significant difference between FBP values, and small letters between SBP values ($P < 0.05$). *Significant difference between FBP and SBP ($P < 0.05$).

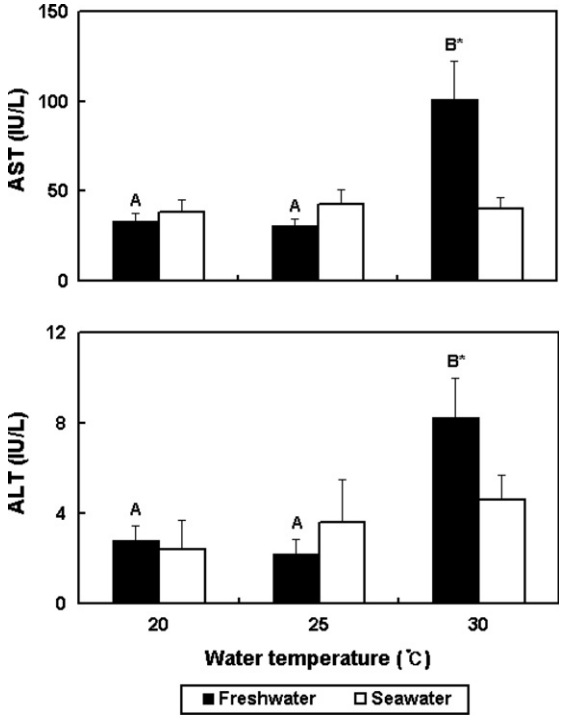

Fig. 4. Levels of AST (up) and ALT (down) in freshwater- and seawater-adapted black porgy (*Acanthopagrus schlegeli*) with water temperature rise (20 → 30 °C). Each value represents means ± SD ($n = 6$). Different capital letters indicate significant difference between FBP values, and small letters between SBP values ($P < 0.05$). *Significant difference between FBP and SBP ($P < 0.05$).

in SBP were 38.0 ± 7.3–42.7 ± 8.2 and 2.4 ± 1.3–4.6 ± 1.1 IU/L, respectively, showing no change due to temperature rise (Fig. 4).

Plasma T_3 levels in FBP were 2.3 ± 0.3 ng/ml at 20 °C, but significantly decreased to 1.0 ± 0.3 ng/ml at 30 °C. On the other hand, levels in SBP were 3.1 ± 0.5 ng/ml at 20 °C, but significantly increased to 5.2 ± 0.4 ng/ml at 30 °C ($P < 0.05$) (Fig. 5).

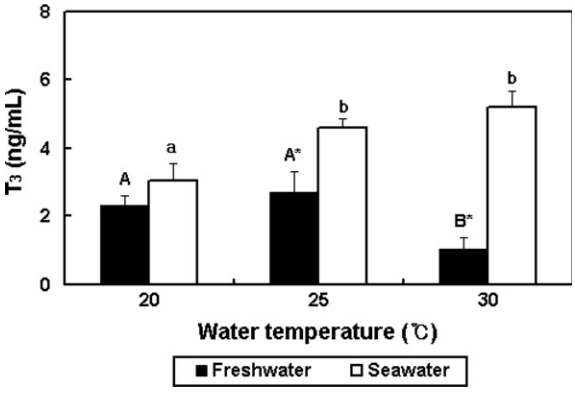

Fig. 5. Levels of T_3 in freshwater- and seawater-adapted black porgy (*Acanthopagrus schlegeli*) with water temperature rise (20 → 30 °C). Each value represents means ± SD ($n = 6$). Different capital letters indicate significant difference between FBP values, and small letters between SBP values ($P < 0.05$). *Significant difference between FBP and SBP ($P < 0.05$).

3.5. Plasma ions and osmolality

While plasma Na^+ of FBP were 167.0 ± 3.3 mEq/L at 20 °C, it significantly decreased to 131.0 ± 16.0 mEq/L at 30 °C. But there was no change in SBP due to temperature rise. In addition, while plasma Cl^- of FBP decreased at 30 °C , that of SBP were 156.4 ± 1.7 mEq/L at 20 °C but increased to 159.8 ± 1.4 mEq/L at 25 °C and continued to increase up to 30 °C. During temperature rise, there was no difference in plasma osmolality between FBP and SBP up to 25 °C. However, FBP showed decrease, while SBP showed increase at 30 °C ($P < 0.05$) (Table 2).

4. Discussion

This study compared the stress response of black porgy acclimated in freshwater (FBP) with that of black porgy reared in seawater (SBP) to an artificial stressor, i.e. a temperature rise. The results can be applied as baseline data for the freshwater aquaculture of black porgy. We examined plasma cortisol levels in FBP and SBP, as the primary stress response, when the temperature of ambient water was increased and found that hormone concentrations increased with temperature rise. Basic levels of plasma cortisol in fish may differ depending on species, size, sex, and rearing conditions; however, given that it was 30–40 ng/ml for salmonid (Pickering and Pottinger, 1989), 5–50 ng/ml for tilapia (*Oreochromis niloticus*) (Auperin et al., 1997), and 10–35 ng/ml for black porgy (Chang et al., 2002; Min et al., 2003), the experimental fish in this study, it is believed that the values up to 25 °C were basic levels. However, at 30 °C, FBP and SBP recorded plasma cortisol levels of 107 and 47 ng/ml, respectively, showing that the level in FBP was twice as high as in SBP. This result demonstrates that FBP has a lower resistance to high water temperature than SBP and, thus, suffers from more stress. In general, when fish are under stress, glucose concentrations increase with increased plasma cortisol (Barton and Iwama, 1991; Davis et al., 1985). Cortisol and catecholamines (CAs) play a role of mediating stress-related gluconeogenesis and hyperglycemia, respectively (Vijayan et al., 1997; Fabbri et al., 1998). Hyperglycemia is known to satisfy the increased energy requirements due to stress (Vijayan et al., 1997). When Atlantic salmon (*S. salar*) (Olsen et al., 1995) and red drum (*Sciaenops ocellatus*) (Robertson et al., 1988) were subject to acute stress, their plasma cortisol and glucose levels increased in tandem. In addition, hyperglycemia has been reported in chinook salmon (*Oncorhynchus tshawytscha*) (Barton and Schreck, 1987), coho salmon (*Oncorhynchus kisutch*) (Wedemeyer, 1973), and red seabream (*Pagrus major*) (Ishioka, 1980) as a stress response to temperature rise. This study has also shown that temperature rise increased plasma glucose levels in black porgy. Plasma CAs were not measured in this study, since they are very sensitive to sampling disturbance (Barton and Iwama, 1991). Nevertheless, the trend of glucose elevations during temperature rise in our study most likely reflects CA-induced glycogenolysis. Higher glucose responses to

Table 2
Levels of plasma Na^+, Cl^- and osmolality in freshwater- and seawater-adapted black porgy (*Acanthopagrus schlegeli*) with water temperature rise (20 → 30 °C)

Water temperature (°C)	Na^+ (mEq/L)		Cl^- (mEq/L)		Osmolality (mOsm/kg)	
	FW	SW	FW	SW	FW	SW
20	$167.0 \pm 3.3^{A*}$	179.0 ± 2.0	$144.7 \pm 1.1^{A*}$	156.4 ± 1.7^{a}	349.3 ± 2.2^{A}	355.0 ± 5.0^{a}
25	$170.0 \pm 2.4^{A*}$	181.2 ± 2.6	$151.8 \pm 3.0^{A*}$	159.8 ± 1.4^{b}	354.7 ± 7.6^{A}	362.0 ± 4.5^{a}
30	$131.0 \pm 16.0^{B*}$	180.3 ± 1.1	$110.3 \pm 17.8^{B*}$	159.3 ± 1.0^{b}	$286.3 \pm 27.6^{B*}$	372.5 ± 6.3^{b}

Each value represents means ± SD ($n = 6$). Different capital letters indicate significant difference between FBP values, and small letters between SBP values ($P < 0.05$).
* Significant difference between fish from freshwater and seawater ($P < 0.05$).

stressors at higher temperatures are considered to be a reflection of stimulated release of CAs due to a greater metabolic activity in fish acclimated to a higher temperature (Barton and Schreck, 1987; Davis and Parker, 1990). In particular, FBP recorded higher concentrations of glucoses than SBP during temperature rise, it is also suggested that CAs levels in FBP are higher than those SBP. Stressor causes the gluconeogenesis as cortisol improves the gluconeogenic capacity of PEPCK, the gluconeogenic enzyme of the liver (Hanson and Reshef, 1997). The high identity (up to 74.7%) between the amino acid sequence of PEPCK in black porgy and that in other vertebrates reflects that this sequence corresponds to a functional enzyme. PEPCK catalyzes the conversion of oxaloacetate to phosphoenolpyruvate on pathways of gluconeogenesis. Thus, black porgy PEPCK appears to possess the PEPCK-specific domain to bind oxaloacetate, as well as kinase-1 and kinase-2 motifs (Fig. 1) to bind the GTP chain and Mg^{2+}, respectively, specific for all known PEPCKs (Matte et al., 1987). Finally, the main gluconeogenic tissues (liver and kidney) and the intestine express the black porgy PEPCK mRNA, as in mammals (Hanson and Reshef, 1997). These data also confirm previous data on PEPCK activities, which are highly specific to liver and kidney in fish (Suarez and Mommsen, 1987). As a result of examining the expressed distribution of PEPCK mRNA in gluconeogenic tissues due to temperature rise using PEPCK cDNA information from black porgy, it was shown to be highly expressed in the liver. Specifically, FBP recorded higher value than SBP. Accordingly, this study concludes that FBP suffers greater stress due to temperature rise than SBP, and that higher levels of plasma cortisol in FBP activate liver PEPCK, much more so than in SBP, to secrete glucose into the blood.

In our previous study, no difference in plasma osmolality (347–357 mOsm/kg) was found between FBP and SBP for 90 days (Min et al., 2005). In this study, when FBP and SBP were subjected to temperature rise, normal osmoregulation was achieved up to 25 °C, i.e. 349–362 mOsm/kg. At 30 °C however, apparently osmoregulation was disturbed, as is indicated by the decrease in osmolality for FBP and an increase in SBP. When compared to osmolality at 20 °C, it was found that the difference was greater in FBP, which suffered more stress than SBP, as expected.

AST and ALT are amino transfer enzymes and their blood concentrations are a general index of liver function in vertebrates. They are used to evaluate the stress response caused by temperature change, low oxygen, pH, ammonia or heavy metals (Pan et al., 2003). In this study, AST and ALT levels in FBP significantly increase with temperature rise, while there was no change in SBP. This suggests that hepatocytes in FBP are damaged due to temperature rise, which will be led to a decrease in liver function.

Thyroid hormones, such as T_3 and T_4 , are secondary indices of stress and are influenced by the level of cortisol (Wendelaar Bonga, 1993). It has been reported that both T_4 and T_3 concentrations increased with an increase in plasma cortisol as a stress response to transformation in flatfish (Tanaka et al., 1995). In this study, an increase in T_3 and plasma cortisol was expected with temperature stress, but T_3 increased in SBP only and decreased in FBP. There are two possible reasons for this. First, hypothalamus–pituitary–thyroid gland activity may differ between FBP and SBP for the same stress factor (temperature rise is the stressor causing stress). Second, given that increased plasma cortisol is harmful to fish, with delayed sexual maturation and growth suppression (Pickering et al., 1987), it can be inferred that T_3 levels decrease, since high cortisol levels in FBP due to temperature rise limit hypothalamus–hypophysis–thyroid gland activity and decrease secretion of T_4 (or inhibit the activity of 5′-deiodinase required for conversion from T_4 to T_3). Recently, the effect of thyroid hormone on osmoregulation has been disclosed in tilapia (*O. mossambicus*) (Peter et al., 2000). Accordingly, this study suggests that decreased plasma T_3 in FBP may be one cause of osmoregulation failure in fish, finally resulting in death.

In conclusion, this study shows that FBP suffers more stress than SBP due to temperature rise, resulting in normal osmoregulation failure. It seems that FBP consumes more energy in osmoregulation than SBP and have weaker resistance to external stress. Our results suggest black porgy suffer greater temperature-related stress in freshwater than in seawater.

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References

Auperin, B., Baroiller, J.F., Ricordel, M.J., Fostier, A., Prunet, P., 1997. Effect of confinement stress on circulation levels of growth hormone and two prolactins in freshwater-adapted tilapia, *Oreochromis niloticus* (L). Gen. Comp. Endocrinol. 108, 35–44.

Barton, B.A., Schreck, C.B., 1987. Influence of acclimation temperature on interrenal and carbohydrate stress responses in juvenile chinook salmon(*Oncorhynchus tshawytsc*). Aquaculture 62, 299–310.

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of *corticosteroids*. Annu. Rev. Fish. Dis. 1, 3–26.

Brown, C.L., Bern, H.A., 1989. Hormones in early development with special reference to teleost fishes. In: Hormones in Development, Maturation and Senescence of Neuroendocrine Systems. Academic Press, New York, pp. 289–306.

Carmichael, G.J., Tomasso, J.R., Simco, B.A., Davis, K.B., 1984. Characterization and alleviation of stress associated with hauling largemouth bass. Trans. Am. Fish. Soc. 113, 778–785.

Chang, Y.J., Min, B.H., Chang, H.J., Hur, J.W., 2002. Comparison of blood physiology in black seabream (*Acanthopagrus schlegeli*) cultured in converted freshwater from seawater and seawater from freshwater. J. Korean Fish. Soc. 35, 595–600.

Chang, Y.J., Hur, J.W., 1999. Physiological responses of grey mullet (*Mugil cephalus*) and Nile tilapia (*Oreochromis niloticus*) by rapid changes in salinity of rearing water. J. Korean Fish. Soc. 32, 310–316.

Choi, C.Y., Habibi, H.R., 2003. Molecular cloning of estrogen receptor and expression pattern of estrogen receptor subtypes in male and female goldfish. Mol. Cell. Endocrinol. 204, 169–177.

Cook, J.S., Weldon, S.L., Garcia-Ruiz, J.P., Hod, Y., Hanson, R.W., 1986. Nucleotide sequence of the mRNA encoding the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) from the chicken. Proc. Natl. Acad. Sci. USA 83, 7583–7587.

Davis, K.B., Torrance, P., Parker, N.C., Suttle, M.A., 1985. Growth, body composition, and hepatic tyrosine aminotransferase activity in cortisol fed catfish,*Ictalurus punctatus* Rafinesque. J. Fish. Biol. 27, 177–184.

Davis, K.B., Parker, N.C., 1990. Physiological stress in striped bass: effect of acclimation temperature. Aquaculture 91, 349–358.

Eddy, F.B., 1981. Effects of stress on osmotic and ionic regulation in fish. In: Pickering, A.D. (Ed.), Stress and Fish. Academic Press, London, pp. 77–102.

Fabbri, E., Capuzzo, A., Moon, T.W., 1998. The role of circulating catecholamines in the regulation of fish metabolism: an overview. Comp. Biochem. Physiol. 120C, 177–192.

Hanson, R.W., Reshef, L., 1997. Regulation of phosphoenol pyruvate carboxykinase (PEPCK) gene expression. Ann. Rev. Biochem. 66, 581–611.

Inui, Y., Yamano, K., Miwa, S., 1995. The role of thyroid hormone in tissue development in metamorphosing flounder. Aquaculture 135, 87–98.

Ishioka, H., 1980. Stress reaction in the marine fish. Stress reactions induced by temperature changes. Bull. Jpn. Soc. Sci. Fish. 46, 523–531.

Matte, A., Tari, L.W., Goldie, H., Delbaere, L.T., 1987. Structure and mechanism of phosphoenolpyruvate carboxykinase. J. Biol. Chem. 272, 8105–8108.

McDonald, D.G., Milligan, C.L., 1992. Chemical properties of the blood. In: Hoar, W.S., Randall, D.J., Farrell, A.P. (Eds.), Fish Physiology XIIB. Academic Press, New York, pp. 55–133.

McDonald, D.G., Milligan, C.L., 1997. Ionic, osmotic and acid base regulation in stress. In: Iwama, G.W., Pickering, A.D., Sumpter, J.P., Schreck, C.B. (Eds.), Fish Stress and Health in Aquaculture. University Press, Cambridge, pp. 119–144.

Min, B.H., Choi, C.Y., Chang, Y.J., 2005. Comparison of physiological conditions on black porgy,*Acanthopagrus schlegeli* acclimated and reared in freshwater and seawater. J. Aquaculture 18, 37–44.

Min, B.H., Kim, B.K., Hur, J.W., Bang, I.C., Byun, S.K., Choi, C.Y., Chang, Y.J., 2003. Physiological responses during freshwater acclimation of seawater-cultured black porgy (*Acanthopagrus schlegeli*). Korean J. Ichthyol. 15, 224–231.

Olsen, Y.A., Einarsdottir, I.E., Nilssen, K.J., 1995. Metomidate anaesthesia in Atlantic salmon, *Salmo salar* , prevents plasma cortisol increase during stress. Aquaculture 134, 155–168.

Pan, C.H., Chien, Y.H., Hunter, B., 2003. The resistance to ammonia stress of *Penaeus monodonFabricius juvenile* fed diets supplemented with astaxanthin. J. Exp. Mar. Biol. Ecol. 297, 107–118.

Perry, S.F., Reid, S.D., 1993. β-Adrenergic signal transduction in fish: interactive effects of catecholamines and cortisol. Fish. Physiol. Biochem. 11, 195–203.

Peter, M.C.S., Lock, R.A.C., Wendelaar Bonga, S.E., 2000. Evidence for an osmoregulatory role of thyroid hormones in the freshwater mozambique tilapia *Oreochromis mossambicus*. Gen. Comp. Endocrinol. 120, 157–167.

Pickering, A.D., Pottinger, T.G., 1989. Stress responses and disease resistance in salmonid fish: effect of chronic elevation of plasma cortisol. Fish. Physiol. Biochem. 7, 253–258.

Pickering, A.D., Pottinger, T.G., Carragher, J., Sumpter, J.P., 1987. The effects of acute and chronic stress on the levels of reproductive hormones in plasma of mature male brown trout, *Salmo trutta* L. Gen. Comp. Endocrinol. 68, 249–259.

Radziuk, J., Pye, S., 2001. Hepatic glucose uptake, gluconeogenesis and the regulation of glycogen synthesis. Diabetes Metab. Res. Rev. 17, 250–272.

Robertson, L., Thomas, P., Arnold, C.R., 1988. Plasma cortisol and secondary stress responses of cultured red drum (*Sciaenops ocellatus*) to several transportation procedures. Aquaculture 68, 115–130.

Suarez, R.K., Mommsen, T.P., 1987. Gluconeogenesis in teleost fishes. Can. J. Zool. 65, 1869–1882.

Tanaka, M., Tanangonan, J.B., Tagawa, M., de Jesus, E.G., Nishida, H., Isaka, M., Kimura, R., Hirano, T., 1995. Development of the pituitary, thyroid and interrenal glands and applications of endocrinology to the improved rearing of marine fish larvae. Aquaculture 135, 111–126.

Tomasso, J.R., Davis, K.B., Parker, N.C., 1980. Plasma corticosteroid and electrolyte dynamics of hybrid striped bass (white bass × striped bass) during netting and hauling stress. Proc. World Maricult. Soc. 11, 303–310.

Vijayan, M.M., Pereira, C.E., Grau, G., Iwama, G.K., 1997. Metabolic responses associated with confinement stress in tilapia: the role of cortisol. Comp. Biochem. Physiol. 116C, 89–95.

Vijayan, M.M., Leatherland, J.F., 1989. Cortisol-induced changes in plasma glucose, protein, and thyroid hormone levels and liver glycogen content of coho salmon (*Oncorhynchus kisutch* Walbaum). Can. J. Zool. 67, 2746–2750.

Wedemeyer, G., 1973. Some physiological aspects of sublethal heat stress in the juvenile steelhead trout (*Salmo gairdneri*) and coho salmon (*Oncorhynchus kisutch*). J. Fish. Res. Bd. Can. 30, 831–834.

Wendelaar Bonga, S.E., 1993. Endocrinology. In: Evans, D.H. (Ed.), The Physiology of Fishes. CRC Press Inc., Boca Raton, pp. 469–502.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.

Yoo, J.H., Takeuchi, T., Jeong, K.S., 2003. Effect of thyroid hormones and albinism during metamorphosis of flounder, *Paralichthys olivaceus* under low temperature stress. J. Korean Fish. Soc. 36, 65–67.