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Profiles of Glucocorticoid Receptor mRNA Expression and Physiological Changes in Response to Osmotic and Thermal Stress Conditions in Black Porgy (*Acanthopagrus schlegeli*)

*By Kwang Wook An, Hyun Suk Shin, Byung Hwa Min[1], Gyung-Suk Kil[2] and Cheol Young Choi**

Division of Marine Environment & Bioscience, Korea Maritime University, Busan 606-791, Korea
[1]Genetics and Breeding Research Center, National Fisheries Research & Development Institute, Geoje, Gyeongnam 656-842, Korea
[2]Division of Electrical & Electronic Engineering, Korea Maritime University, Busan 606-791, Korea

ABSTRACT The present study investigated the expression of glucocorticoid receptor (GR) mRNA as a stress response during salinity changes (35, 10, and 0 psu) and water temperature changes (from 20°C to 30°C, 1°C/day) in black porgy. We cloned the full-length GR cDNA from the kidney and examined its expression in the gill, kidney, and intestine by quantitative real-time PCR (QPCR) during salinity and water temperature changes. During salinity changes, the levels of GR mRNA in the gill, kidney, and intestine were highest at 0 psu, and the levels of plasma cortisol and glucose were elevated, but triiodothyronine (T_3) decreased. Also, during water temperature changes, the levels of GR mRNA in the gill, kidney, and intestine increased at 30°C. Plasma parameters also increased with an increase in water temperature. Therefore, this upregulation of GR mRNA was a good indicator of stress, such as those resulting from changes in salinity and water temperature.

Key words : Black porgy, glucocorticoid receptor, salinity change, water temperature change

INTRODUCTION

Stress factors in fish can be divided into physical factors, such as salinity, culture density, temperature, and dissolved oxygen, and chemical factors, such as parasiticides and disinfectants under culture conditions (Beckmann *et al.*, 1990). Physical factors, such as salinity and temperature change, affect growth, reproduction, metabolism, osmoregulation, and immune ability, causing negative effects under physiological conditions such as a disturbance in growth and reproduction (Ackerman *et al.*, 2000). To protect against stress caused by changes in the environment, fish have stress response systems to maintain homeostasis (Barton and Iwama, 1991).

When fish are under stress, the hypothalamus-pituitary-interrenal axis is activated and stimulates the synthesis and release of cortisol, a glucocorticoid hormone, via adrenocorticotropic hormone released from the pituitary (Specker *et al.*, 1989). Therefore, cortisol is an index of stress (Wendelaar Bonga, 1997). Primary stress responses induce secondary responses, such as electrolyte changes in plasma and tissue (Carmichael *et al.*, 1984), and changes in thyroxine (T_4) and triiodothyronine (T_3) levels (Wendelaar Bonga, 1993). Cortisol released into plasma regulates various physiological processes and affects cells directly by diffusion or by binding to the cortisol (glucocorticoid) receptor (GR), which is a specific receptor expressed on the cell membrane (Beato *et al.*, 1996). GR has AB-domain, DNA-binding domain (DBD), and hormone-binding domain (HBD) and shows a high degree of similarity among fish species (Greenwood *et al.*, 2003). In fish, GR has been cloned from many studies in teleost fish (Acerete *et al.*, 2007; Filby and Tyler, 2007; Vizzini *et al.*, 2007), and several studies have examined GR mRNA-related stress, *e.g.*, tilapia (*Oreochromis mossambicus*) treated with vitellogenin (Park *et al.*, 2007) and Arctic char (*Salvelinus alpinus*) treated with polychlorinated biphenyl (PCB) (Aluru *et al.*, 2004). The expression of GR mRNA and plasma cortisol levels were reported in Atlantic salmon (*Salmo salar*) (Kiilerich *et al.*, 2007) and tilapia (Takahashi *et al.*, 2006) adapted to seawater. Vijayan *et al.* (2003) reported that cortisol increased the GR mRNA level in rainbow trout (*Oncorhynchus mykiss*)

*Corresponding author: Cheol Young Choi Tel: 82-51-410-4756, Fax: 82-51-404-4750, E-mail: choic@hhu.ac.kr

after treatment with cortisol, and Tomy *et al.* (2009) demonstrated that cortisol in plasma and GR mRNA increased with changes in salinity.

Black porgy (*Acanthopagrus schlegeli*) is a widely distributed marine protandrous hermaphrodite that is of particular interest in commercial aquaculture in various parts of Asia, including Korea. Culture of black porgy in a hypoosmotic environment has been reported (Min *et al.*, 2003, 2006). The present study was performed to isolate the full-length GR cDNA and to investigate the changes in GR mRNA expression, plasma cortisol, glucose, and T_3 concentrations of fish transferred sequentially to 0 psu to obtain biological base data for culturing black porgy under hypoosmotic environmental conditions. In addition, we investigated changes in GR mRNA expression, plasma cortisol, glucose, and T_3 concentrations in black porgy exposed to high water temperatures sequentially from 20°C to 30°C to measure stress levels for use as physiological base data in this species.

MATERIALS AND METHODS

1. Experimental fish

The study was performed with 1-year-old black porgy (n=60, 14.3±0.4 cm, 51.0±6.0 g) reared in 220-L circulating filter tanks in the laboratory. Before the experiment, water temperature and photoperiod were 20±1°C and 12L:12D, respectively.

2. Salinity change

Transfer of black porgy to a hypoosmotic environment was performed according to the methods of Min *et al.* (2003). Briefly, underground water was poured into two square 40-L circulating filter tanks and the water was adjusted at 10 psu and then the fish were exposed for 24 h. After then, underground water was again added up to 0 psu and the fish were exposed for 24 h. The water temperature and photoperiod were maintained at 20±1°C and 12L : 12D, respectively. No fish died during salinity changes.

3. Increasing water temperature

Black porgy was reared in seawater in two square 40-L circulating filter tanks with automatic temperature regulation systems (JS-WBP-170RP; Johnsam Co., Seoul, Korea) at a density of 10 fish per tank and allowed to acclimatize to the conditions for 24 h. The water temperature was then increased from 20°C to 30°C in daily increments of 1°C. No fish died during the increase in water temperature.

4. Sampling

Five fish from each group (35 psu seawater, 10 psu seawater, 0 psu freshwater and 30°C water temperature) were selected randomly for tissue and blood sampling and anesthetized with tricaine methanesulfonate (200 mg/L, MS-222; Sigma, St. Louis, MO); blood was taken from the caudal vasculature using a 3-mL heparinized syringe. After centrifugation (10,000 rpm, 4°C, 5 min), the plasma was stored in at −80°C before analysis, and fish were killed by spinal transection for collection of the tissues (gills, kidney, and intestine). Immediately after sample collection, the tissues were frozen in liquid nitrogen and stored at −80°C until total RNA extraction was performed.

5. Identification of GR cDNA

The primers used for GR amplification were designed using highly conserved regions of gilthead seabream (*Sparus aurata*, GenBank accession no. DQ486890) and European seabass (*Dicentrarchus labrax*, AY619996): GR forward primer (5′-CTG TTT CTC ATG TCT TTC GG-3′) and GR reverse primer (5′-TTT CGG TAA TTG GTT GCT GAT GAT-3′). Total RNA was extracted from the black porgy kidney using a TRIzol kit (Gibco/BRL, Grand Island, NY).

We used 2.5 μg of total RNA for cDNA synthesis and performed reverse transcription using oligo-d $(T)_{15}$ anchor primer and M-MLV reverse transcriptase (Bioneer, Seoul, Korea) according to the manufacturer's instructions. PCR was conducted using 2× Taq Premix I (Solgent, Daejeon, Korea) and amplification was performed according to the manufacturer's instructions. The PCR products were purified and cloned into the pGEM-T Easy Vector (Promega, Madison, WI). The transformed colonies were cultivated in DH5α (RBC Life Sciences, Seoul, Korea), and the plasmid DNA was extracted using a Labopass Plasmid DNA Purification Kit (Cosmo, Seoul, Korea); then the GR was cleaved from the plasmid DNA with *Eco*RI (Fermentas, Glen Burnie, MD). The GR sequences were analyzed using an ABI DNA sequencer (Applied Biosystems, Foster City, CA).

6. Rapid amplification of GR cDNA of the 3′- and 5′-ends (3′- and 5′-RACE)

For PCR, total RNA was extracted from the kidney using a TRIzol kit (Gibco/BRL). 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, Seoul, Korea). First-strand cDNA synthesis was conducted using an oligo-d $(T)_{18}$ anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA T $(T)_{18}$-3′) and CapFishing™ adaptor (Seegene).

Gene-specific primers were selected from the PCR products obtained by RT-PCR. For 3′-RACE, PCR reaction mixtures contained 5 μL of 3′-RACE cDNA, 1 μL of 10 μM 3′-RACE primer, 1 μL of 10 μM 3′-RACE GR-

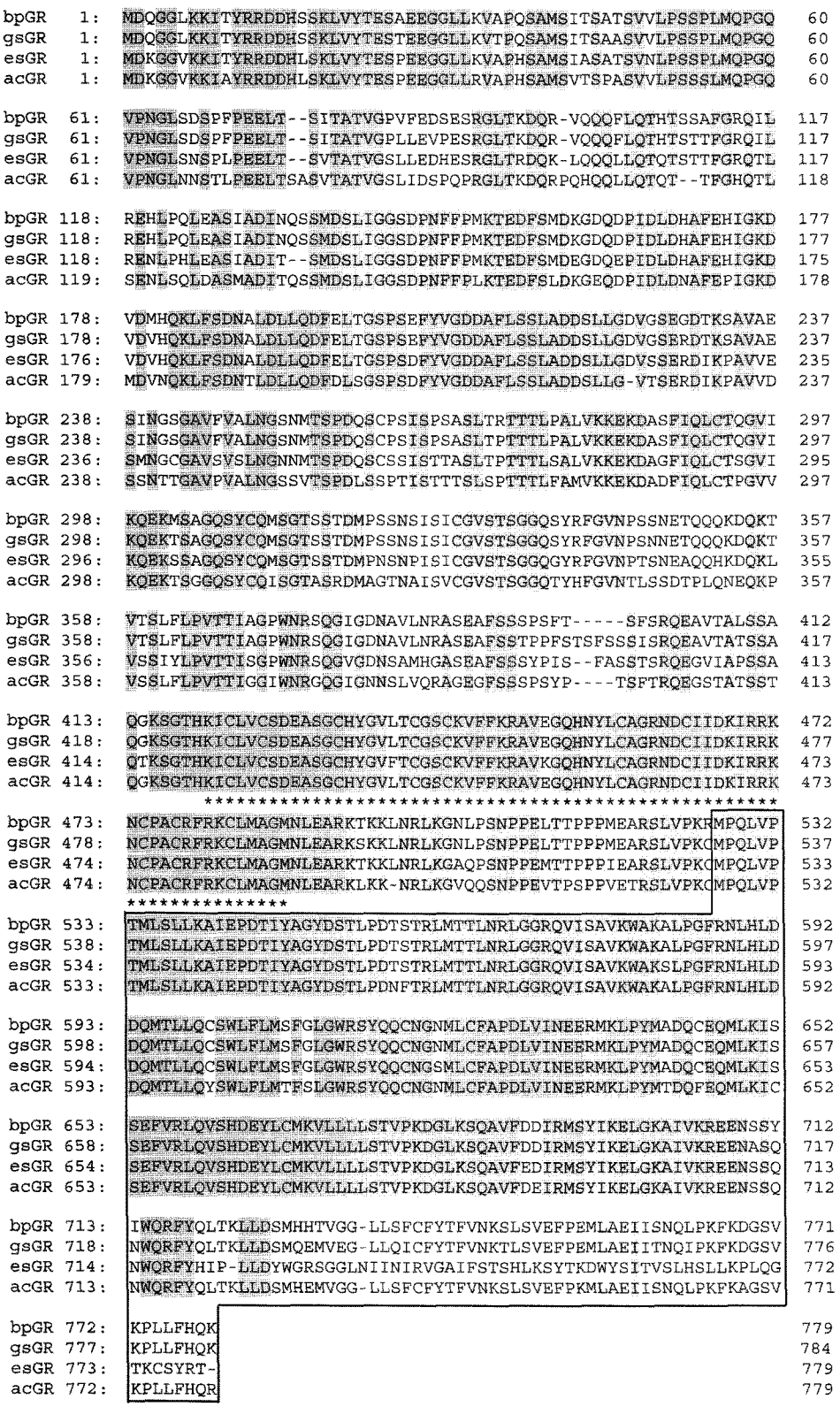

Fig. 1. Comparison of the amino acid sequence of black porgy GR, gilthead sea bream (gsGR) GR, European sea bass (esGR) GR, and African cichlid (acGR) GR optimally aligned to match identical residues indicated by the shaded boxes. The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. The GR amino acid sequences used for alignment are black porgy GR (bpGR, GenBank accession no. AAX 18925), gilthead sea bream GR (gsGR, ABF30967), European sea bass GR (esGR, AAT-41627), and African cichlid GR (acGR, AAM27887). The DNA-binding domain is indicated by an asterisk and the hormone-binding domain is boxed.

specific primer (5′-GTG GAG TTC CCG GAG ATG CTG GCC GAG-3′), and 25 µL of SeeAmp^{TM} Taq Plus Master Mix (Seegene) in a total volume of 50 µL. For 5′-RACE, PCR reaction mixtures contained 18 µL of H_2O, 5 µL of 5′-RACE cDNA, 1 µL of 10 µM 5′-RACE primer, 1 µL of 10 µM 5′-RACE GR-specific primer (5′-

CAT GTT GCC GTT GCA CTG CTG GTA AGA C-3′), and 25 μL of SeeAmp™ Taq Plus Master Mix (Seegene) in a total volume of 50 μL. PCR was carried out according to the manufacturer's instructions (Seegene). After PCR, ligation and transformation were performed using the methods described above.

7. Quantitative real-time PCR (QPCR)

QPCR was performed to determine the relative expression levels of GR mRNA using the total RNA extracted from the gills, kidney, and intestine of black porgy during salinity changes and water temperature rise. The primers for QPCR were designed based on known sequences of black porgy GR: GR forward primer (5′-CGT TGA TGC AGC CAG GAC AGG-3′), GR reverse primer (5′-GGA ACT GCT GCT GAA CCC TCT G-3′), β-actin forward primer (5′-TCG AGC ACG GTA TTG TGA CC-3′), and β-actin reverse primer (5′-ACG GAA CCT CTC ATT GCC GA-3′). PCR amplification was performed using an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The QPCR conditions were 1 cycle of denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and extension at 70°C for 20 s. Each experimental group was tested three times. As an internal control, experiments were duplicated with β-actin, and all data were normalized to the β-actin calculated threshold cycle (Ct) level.

8. Plasma parameters

Plasma cortisol was analyzed by radioimmunoassay (RIA) using a commercial RIA kit (Diagnostic System Laboratories, Webster, TX). Plasma glucose was analyzed using a Biochemistry Auto Analyzer (model 7180; Hitachi, Tokyo, Japan). Plasma T_3 was analyzed by enzyme immunoassay (EIA) using a T_3 EIA Kit (BioSewoom, Seoul, Korea).

9. Data analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL). Differences in the data were compared by one-way ANOVA followed by *post hoc* multiple comparison test (Tukey's test).

RESULTS

1. Identification of full-length GR cDNA

The full-length GR cDNA contained 2,340 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 779 amino acids (AY921612). Using the BLAST algorithm (blastp) at the National Center for Biotechnology Information (NCBI), we found that the GR sequences displayed a high similarity with those of other teleosts; the amino acid similarities were as follows: 94% with gilthead sea bream GR (gsGR, ABF30967), 86% with European sea bass GR (esGR, AAT41627), and 81% with African cichlid (*Astatotilapia burtoni*) GR (acGR, AAM27887). We also found that black porgy GR contained the DBD (residues 420~487) and HBD (527~779) and showed a high degree of similarity with those of other species (Greenwood *et al.*, 2003) (Fig. 1).

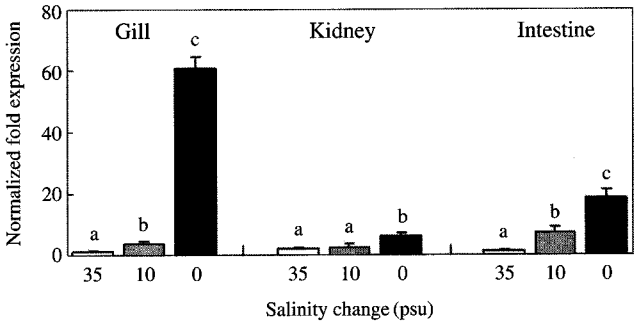

Fig. 2. Expression of GR mRNA in the gill, kidney, and the intestine during salinity change in black porgy. The results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Values with different letters are significantly different ($P<0.05$). Values are means ± SD ($n=5$).

Table 1. Plasma cortisol, glucose and T_3 levels during salinity changes in black porgy

Ambient	Cortisol (ng/mL)	Glucose (mg/dL)	T_3 (ng/mL)
35 psu	5.3 ± 1.3^{a}	53.0 ± 2.0^{a}	3.0 ± 0.5^{b}
10 psu	15.1 ± 4.7^{b}	58.0 ± 5.0^{ab}	2.75 ± 0.5^{a}
0 psu	30.5 ± 7.5^{c}	64.0 ± 2.6^{b}	2.65 ± 0.4^{a}

Values with different letters are significantly different ($P<0.05$). Values are means ± SD ($n=5$).

2. Physiological responses during osmotic stress

1) Expression of GR mRNA

The level of GR mRNA expression was highest at 0 psu in the gills (about 60-fold higher than at 35 psu), kidney (about 6-fold higher than at 35 psu), and intestine (about 18-fold higher than at 35 psu) (Fig. 2).

2) Plasma parameters

Plasma cortisol levels were 5.3 ± 1.3 ng/mL at 35 psu and then increased to 15.1 ± 4.7 ng/mL at 10 psu, peaking at 0 psu (30.5 ± 7.5 ng/mL). Plasma glucose levels were 53.0 ± 2.0 mg/dL at 35 psu and then increased to 58.0 ± 5.0 mg/dL at 10 psu, peaking at 0 psu (64.0 ± 2.6 mg/dL). In contrast, the levels of T_3 in plasma were decreased gradually at 10 psu (2.75 ± 0.5 ng/mL) and 0 psu (2.65 ± 0.4 ng/mL) (Table 1).

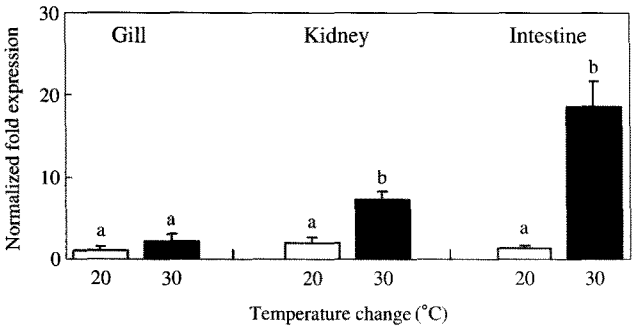

Fig. 3. Expression of GR mRNA in black porgy with increasing water temperature from 20°C to 30°C. Values with different letters are significantly different ($P<0.05$). Values are means±SD ($n=5$).

Table 2. Plasma cortisol, glucose and T_3 levels during temperature changes in black porgy

Ambient	Cortisol (ng/mL)	Glucose (mg/dL)	T_3 (ng/mL)
20°C	5.3 ± 1.3^a	53.0 ± 2.0^a	3.0 ± 0.5^a
30°C	45.0 ± 1.3^b	67.5 ± 0.85^b	5.0 ± 0.5^b

Values with different letters are significantly different ($P<0.05$). Values are means±SD ($n=5$).

3. Physiological responses during water temperature rise

1) Expression of GR mRNA

The GR mRNA at 30°C increased significantly in the kidney (about 7-fold higher than at 20°C) and intestine (about 18-fold higher than at 20°C), but no significant changes were observed in the gills during the experimental period (Fig. 3).

2) Plasma parameters

The plasma cortisol levels were 5.3±1.3 ng/mL at 20°C, which then increased rapidly to 45.0±1.3 ng/mL at 30°C. The plasma glucose levels were 53.0±2.0 mg/dL at 20°C, which then increased to 67.5±0.85 mg/dL at 30°C. And, T_3 levels were 3.0±0.5 ng/mL at 20°C, increasing to 5.0±0.5 ng/mL at 30°C (Table 2).

DISCUSSION

In this study, we cloned the full-length GR cDNA from the kidney of black porgy and investigated the expression pattern of GR mRNA in various tissues (gills, kidney, and intestine) after transfer to a hypoosmotic environment as well as an environment with a high water temperature. In addition, we examined the extent of stress due to changes in osmotic and temperature conditions by analyzing plasma parameters.

Under conditions of hypoosmotic stress, the levels of GR mRNA expression in the gills, kidney, and intestine were highest at 0 psu, and the level of expression was highest in the gills among these tissues. This observation indicated imbalance of osmotic regulation in black porgy with salinity change, which led to activity of the hypothalamus-pituitary-interrenal axis and then increased cortisol levels in the plasma (Barton, 2002). We suggest that the expression of GR mRNA increased in experimental tissues through increased cortisol (Singer *et al.*, 2007; Tomy *et al.*, 2009). In particular, in the present study, the expression of GR mRNA was highest in the gills of the black porgy, suggesting that cortisol levels increased to promote the exchange of ions in the gills and then increased the expression of GR mRNA in the gills (Singer *et al.*, 2007). Plasma cortisol levels increased as the salinity increased gradually, which is peaked at 0 psu, indicating that the fish were stressed after transfer to the hypoosmotic environment. Sathiyaa and Vijayan (2003) also reported that cortisol stimulated the expression of GR mRNA as they treated cultured liver cells of rainbow trout with cortisol. In addition, the increase in expression of GR mRNA with plasma cortisol on transfer of black porgy to a hypoosmotic environment indicated that the increase in GR by cortisol maintains homeostasis against stress by adaptation to the hypoosmotic environment and changes in salinity (Beato *et al.*, 1996; Tomy *et al.*, 2009). Cortisol is known as a seawater adaptation hormone (McCormick, 2001), but the plasma cortisol and glucose levels were the highest at 0 psu in the present study, suggesting that the plasma cortisol and glucose levels are increased by osmotic stress perceived during adaptation in hypoosmotic environments (Arjona *et al.*, 2007; Tomy *et al.*, 2009). In general, when fish are exposed to environmental stresses, plasma cortisol levels increase, followed by an increase in plasma glucose level (Barton and Iwama, 1991). A study has suggested that increases in the cortisol level with stress regulate gluconeogenesis in the liver, and hyperglycemia is known to satisfy the increased energy requirements due to stress (Vijayan *et al.*, 1997).

Thyroid hormone (T_4 and T_3), as an index of secondary stress, is affected by cortisol, and plasma thyroid hormone levels were reported to be increased by cortisol (Wendelaar Bonga, 1993). In the present study, T_3 decreased with transfer to the hypoosmotic environment (Table 1). It can be inferred that T_3 levels decreased due to inhibition of the activity of 5′-deiodinase required for conversion from T_4 to T_3 by osmotic stress (Min *et al.*, 2006).

In addition, we investigated the expression of GR mRNA in the gills, kidney, and intestine of black porgy with increases in water temperature from 20°C to 30°C. The expression of GR mRNA was elevated in all tissues examined. These observations indicate that the plasma cortisol level increased with an elevation in water temperature, which then increased GR mRNA expression (Beato *et al.*, 1996; Sathiyaa and Vijayan, 2003). This

result corresponded to those of previous studies indicating increases in plasma cortisol levels with an elevation of water temperature in green sturgeon (*Acipenser medirostris*; Lankford *et al.*, 2003) and sunshine bass (*Morone chrysops* × *Morone saxatilis*; Davis *et al.*, 2004).

In addition, the increase in glucose seems to satisfy the increased energy requirement associated with high-water temperature stress, similar to osmotic stress in the present study. These results were observed when Chinook salmon (*Oncorhynchus tshawytscha*) (Barton and Schreck, 1987), coho salmon (*Oncorhynchus kisutch*) (Wedemeyer, 1973), and red sea bream (*Pagrus major*) (Ishioka, 1980) were under stress due to rising temperature.

And also, plasma T_3 was significantly increased with increasing water temperature at 30°C (Table 2). This result suggests that increases in T_3 were involved in maintenance of body homeostasis with increasing cortisol levels (Bleau *et al.*, 1996).

The present study revealed the grade of stress with changes in salinity and increases in water temperature through the expression of GR mRNA as well as plasma cortisol, glucose, and T_3 levels.

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염분과 수온 스트레스에 따른 감성돔의 glucocorticoid receptor mRNA 발현 특징과 생리적 변화에 관한 연구

안광욱 · 신현숙 · 민병화[1] · 길경석[2] · 최철영

한국해양대학교 해양환경 · 생명과학부, [1]국립수산과학원 육종연구센터,
[2]한국해양대학교 전기전자공학부

요 약 : 본 연구에서는 감성돔의 염분과 수온 변화에 따른 스트레스 반응을 알아보기 위하여 glucocorticoid receptor (GR) mRNA 발현을 조사하였다. 감성돔 신장으로부터 전장의 GR cDNA를 클로닝하였고, 염분과 수온이 변화하는 동안 아가미, 신장 및 장에서 GR mRNA 발현 변화를 quantitative real-time PCR (QPCR)을 이용하여 조사하였다. 염분 변화시, 아가미, 신장 및 장에서 GR mRNA 발현은 0 psu에서 가장 높게 나타났으며, 혈장 cortisol과 glucose 농도도 증가한 반면, triiodothyronine (T_3) 농도는 감소하였다. 수온 변화시, 아가미, 신장 및 장에서 GR mRNA 발현은 30°C에서 가장 높게 관찰되었다. 혈장 cortisol, glucose 및 T_3 농도 또한 고수온(30°C)에서 증가하였다. GR mRNA 발현의 증가는 염분과 수온 변화와 같은 환경 요인에 대한 좋은 스트레스 지표로 여겨진다.

찾아보기 낱말 : 감성돔, glucocorticoid receptor, 염분 변화, 수온 변화