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Noise-induced Stress Response on Cortisol, Glucose, albumin and Glucocorticoid Receptor Expression in the Japanese eel, *Anguilla japonica*[1a]

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소음스트레스에 대한 뱀장어의 코티졸, 글루코스, 알부민과 Glucocorticoid Receptor 유전자 발현 연구[1a]

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ABSTRACT

We measured blood plasma parameters(cortisol, glucose, albumin) and glucocorticoid receptor(*GCR*) gene expression level of the Japanese eel(*Anguilla japonica*) exposed to an explosion noise for an hour in order to evaluate the effects of noise stress and to explore the possibility of these parameters as biomarkers on noise stress for one of this valuable aquaculture species. Plasma cortisol and glucose reached high levels with significant differences compared to the control group, whereas albumin showed a low value after 1 h of exposure. In addition, tissue distribution of *GCR* gene expression was studied by real-time RT-PCR of ten organs(brain, eye, gill, gonad, heart, intestine, kidney, liver, muscle and skin). Liver showed the highest level of expression in the control followed by gill, muscle and intestine. A time-course study revealed induction in liver, gill, muscle and intestine after 30 min or 1 h of noise exposure.

KEY WORDS: BLOOD PARAMETERS, BIOMARKER, AQUACULTURE, REAL-TIME RT-PCR

요 약

소음 스트레스로 인한 뱀장어(*Anguilla japonica*)의 영향을 파악하기 위하여 스트레스 지표로 사용되는 코티졸, 포도당, 알부민 및 glucocorticoid receptor(*GCR*) 유전자의 발현 양을 측정, 분석하여 노출되지 않은 대조구와 비교하였다. 그 결과, 알부민은 노출 1시간 후에 낮은 값을 보인 반면 코티졸과 포도당은 대조구에 비해 매우 큰 차이를 보이며 높게 나타났다. *GCR* 유전자의 조직 발현 결과 간, 아가미, 근육 및 소장에서 많이 발현하였다. 소음 노출에 따른 시간의 변화에서 간과 아가미 근육과 소장에서 발현이 감소하는 양상을 나타내었다. 실험결과 뱀장어의 glucocorticoid receptor 유전자의 발현변화가 소음 스트레스로 인한 영향을 파악하는데 유용한 지표가 될 수 있음을 확인하였다.

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주요어: 혈액 지수, 바이오마커, 양식, 실시간 정량 분석

INTRODUCTION

It has become increasingly apparent that anthropogenic noise such as explosion, pile driving and excavation work for construction in estuaries and coastal regions has the potential to affect the health of aquatic mammals, birds, fishes, amphibians, reptiles and perhaps even invertebrates (e.g. NRC, 1994, 2000, 2003, 2005; Richardson *et al*., 1995; Popper *et al*., 2003, 2004; Hastings, 2008; Popper and Hastings, 2009). Despite the concerns raised by increased presence of anthropogenic sound in the aquatic environment, very little is known about the effects of exposure to such sounds on fishes(NRC, 1994, 2000, 2003; Popper *et al*., 2003, 2004; Hastings, 2008; Popper and Hastings, 2009).

Fish in stressful situations recognize the condition and simultaneously secrete cortisol(Sturmhofel and Bartke, 1998). Cortisol is a kind of glucocorticoid hormone and cortisol goes into the tissues via the blood stream, then cortisol combines with the glucocorticoid receptor(*GCR*) within the tissues. This combined cortisol - *GCR* complex degrades the glycogen and releases the glucose into the blood as an energy source in order to deal with a stressful situation and to maintain homeostasis. Through the determination of *GCR* mRNA combined with cortisol, a physiological stress tolerance and effect can be estimated at the gene level(Wendelaar Bonga, 1997). Although extensive research effort has been allocated to aspects of stress handling, smoltification and reproduction, and the effects of increased cortisol levels on *GCR* expression (Maule and Schreck, 1991; Basu *et al*., 2003), physiological research on the role of fish *GCR* upon noise stress is as yet scarce. Only a few studies focused on *GCR* activities with respect to noise stress(Shin, 1995, 2000; Smith *et al*., 2004; Popper *et al*., 2005; Shim and Han, 2008).

The Japanese eel(*Anguilla japonica*) is a popular fish in Korea and Japan and fetches a premium price owing to its high-quality meat. Because of the great economic importance of Japanese eel aquaculture, artificial reproduction has been attempted for many years, so far unsatisfactorily (Sang *et al*., 1994). So, most elvers used in artificial aquaculture are caught as fry eel in estuaries or near shore. Frequently, eel farms are located close to shore or near rivers; therefore, various noise sources and vibrations from construction sites may become a stress factor for aquaculture farms as noise generated from machinery like pumps and combustion engines. In addition will the study of noise effects on fish physiology, biochemistry and gene expression be of very general scientific interest.

We measured plasma parameters(cortisol, glucose and albumin) and *GCR* gene expression level of the Japanese eel(*Anguilla japonica*) exposed to explosion noise in order to evaluate the effects of noise stress and to demarcate a molecular biomarker for noise stress.

MATERIALS AND METHODS

1. Experimental Conditions

The Japanese eel(total length, 37.8 ± 8.3 ㎝; body mass, 164.29 ± 44.60 g) were obtained from a commercial supplier and transported to the laboratory avoiding any physical stress. They were acclimatized to laboratory conditions for three days at 23°C and to a photoperiod of 12 h light - 12 h dark in an 1,000 L tank containing well-aerated water(pH 7.90, dissolved oxygen, DO 5.8 mg/L). During the acclimation period, fish were fed a commercial diet and were fasted from a day before the experiment. For the exposure of noise, an experimental aquarium(acrylic plastic, length 3 m, diameter 65 cm, volume 1.03 m^3; speakers mounted on one side that generated the noise) was manufactured after the consultation of an expert for radio engineering science(Figure 1). Acclimatized fish(n=25) were transferred the aquarium containing freshwater(21 ± 1.0°C, pH were exposed to the explosion noise(average maximum sound pressure, 212 dB/ uPa; maximum sound pressure of shock wave, 254 dB/ uPa) recorded underwater at the tidal power plant construction site of Shihwa-lake area for an hour. A control group(n=25) was maintained without exposure to

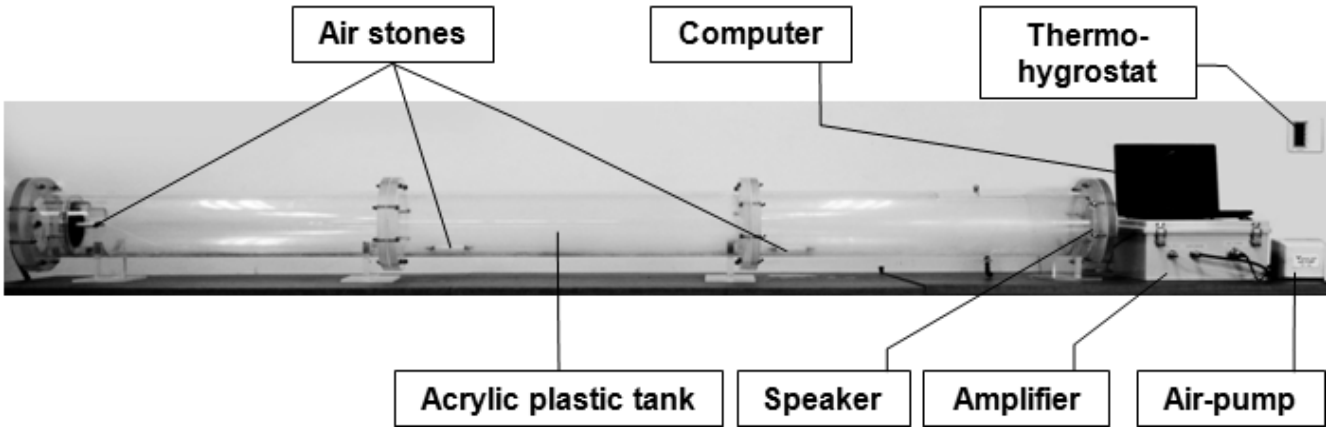

Figure 1. A picture of experimental aquarium used for noise exposure

noise. Sampling(n=3) from exposure and control group was performed at 30 min and an hour after the exposure to noise, and at 1, 2, 4 and 12 hours after the termination of noise exposure. Fish were fasted during the experiment.

2. Hematological Index

Sampled fish were anesthetized by immersion in buffered tricaine methanesulfonate of 800 mg/L(MS-222; pH 7.0; Sigma) and gathered blood from a vascular of caudal peduncle by using a syringe(3 ㎖-23 G; BD Diagnostics, UK) treated with 2 IU/㎖ heparin sodium within 2 min after anesthesia. Cortisol was analyzed by a radio-immunoassay(RIA)(Guy *et al*., 1972) and the hexokinase method using the absorbance of NADH was used for measuring glucose(Slein, 1963). Albumin concentration was measured with a colorimetric determination using bromcresol green(BCG)(Webster, 1974).

3. Total RNA Extraction and cDNA Synthesis

For molecular biological analysis, ten organs(brain, eye, gill, gonad, heart, intestine, kidney, liver, muscle and skin) were dissected after anesthesia with MS-222(800 mg/L). Each organ was stored into RNA later(Sigma- Aldrich, MO), and was frozen at -70°C until analysis. Fish tissue was homogenized in TRIZOL® reagent(Molecular Research Center, Inc., Cincinnati, OH, USA). Total RNAs were extracted according to the manufacturers' protocol. RNA qualities were confirmed spectrophotometrically. Single-stranded cDNA was synthesized from 1 ug total RNA using an oligo$(dT)_{20}$ primer and PrimeScript™ 1st strand cDNA synthesis Kit(Takara, Japan) by reverse transcription according to the manufacturers' protocol.

4. Real time Quantitative Reverse Transcription Polymerase Chain Reaction(qRT-PCR)

Expression of *GCR* transcripts was studied using quantitative real time RT-PCR. Real time RT-PCR was performed using oligo$(dT)_{20}$ primer and SuperScript™ III reverse transcriptase(Invitrogen) according to the manufacturers' instructions. The PCR conditions were as follows: 95°C/5 min; 35 cycles of 95°C/15 s, 50°C/15 s, 72°C/33 s; 72°C/10 min. The *A. Japonica* β -actin gene(GenBank Accession no. GU001950) was used as a reference to normalize the expression levels between the samples after comparison test with GEPDH gene(GenBank Accession no. AB075021). For real time RT-PCR, primers were designed using the Primer express® software(Ver. 3.0; Applied Biosystems) as followed, *GCR*, F: 5'- CAAGA TCCGGCGGAAAAAC, R: 3'- CCTCGCTTCCAGGTT CATTC; β -actin, F: 5'- TCGTGCGTGACATCAAGGA, R: 3'- GGCGGCGGTGCTCAT. QuantiTect® SYBR® Green PCR Kit(QIAGEN) was used to detect specific PCR products. Amplification and detection of SYBR® Green were performed with the 7500 Real-Time PCR system (Applied Biosystems, Lincoln, CA, USA). Real time RT-PCR data were obtained as threshold cycle(C_T) value and used to calculate ΔC_T values(ΔC_T is the C_T of the target gene subtracted from the C_T of the reference gene) of each sample. Fold change for the relative gene

expression to the control was determined by the $2^{-\Delta\Delta CT}$ method(Livak and Schmittgen, 2001). All experiments were done in triplicate.

5. Statistical Analysis

One-way ANOVA followed by Tukey's multiple range comparison tests was used to check for differences among the ten tissues. Student's t-test was used to determine significances between the control and the exposed groups in each time. Differences were considered significant at $p < 0.05$. All data were expressed as means ± S.D.

RESULTS

1. Plasma Parameters

Plasma parameters of noise exposed *A. japonica* are shown in Figure 2. Albumin concentration of the control group did not show a significant difference during the experimental period, whereas the albumin concentration of the exposure group had a significant lower value than the control after an hour of exposure and recovered similar to the concentration of the control 4 h after termination of exposure(Figure 2a).

Glucose concentration of the control group showed no significant change during the experimental period, while the fish of the exposure group showed a sudden surge of 158.5 ± 13.5 mg/dL at 30 min after exposure, and a significant difference was observed from 4 h onward (Figure 2b).

The changing pattern of cortisol was similar to glucose, and the concentration of the experimental group was consistent throughout the experimental period. A peak value(4.02 ± 0.26 ug/dL) was observed at 30 min after exposure and significant differences were maintained until 4 h after termination of exposure(Figure 2c).

2. Expression of the Glucocorticoid Receptor mRNA

1) Tissue Distribution Patterns of *A. japonica GCR* Genes

To check whether *A. japonica GCR* genes were ubiquitously expressed in the tissues, we measured mRNA expression in ten different tissues. As shown in Figure 3,

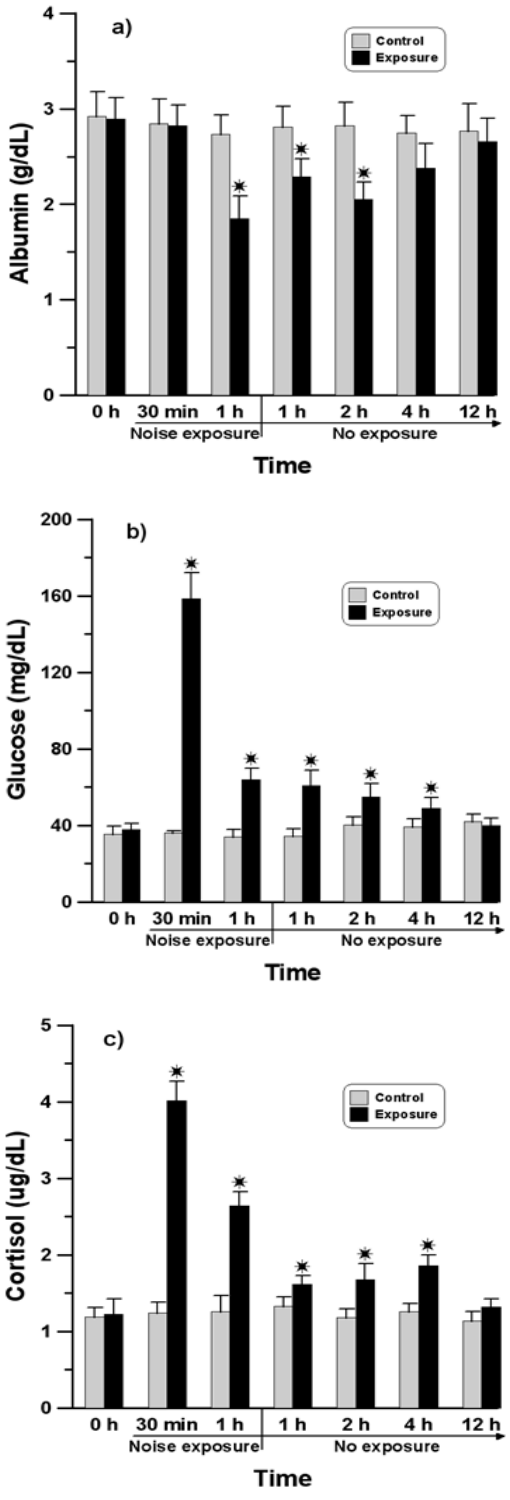

Figure 2. Plasma parameters(a: albumin, b: glucose, c: cortisol) levels of Japanese eel, *Anguilla japonica*

* Indicates significance($P < 0.05$) change over control values). Bars indicate S.D

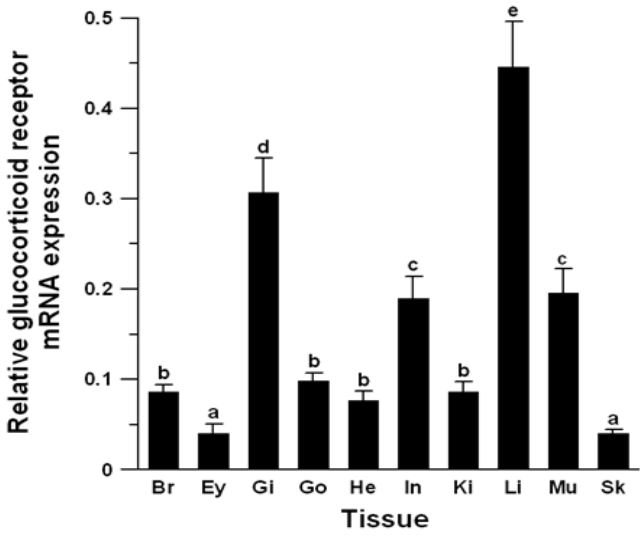

Figure 3. Expression of Japanese eel, *Anguilla japonica* *GCR* gene in different tissues(Br: brain, Ey: eye, Gi: gill, Go: gonad, He: heart, In: intestine, Ki: kidney, Li: liver, Mu: muscle, Sk: skin). Results are expressed as relative expression to β-actin used as housekeeping reference gene. Values are means of triplicate samples for each fish and tissue. Differences between letters indicate significantly different means($P < 0.05$) and bars indicate S.D.

a high level of expression of the *GCR* gene was observed in the liver followed by gill. Muscle and intestine also showed a relatively higher level of expression. Skin showed the lowest level of expression.

2) The Noise Exposure due to Changes in *GCR* mRNA

In order to study whether noise-induces expression of *GCR*, we analyzed *GCR* levels in different tissues(Figure 4). *GCR* gene expression in liver showed a lower level at 30 min after exposure($P < 0.05$). Such significant differences were observed even 4h after termination of exposure(Figure 4a). In gill, the highest expression of the *GCR* gene became apparent at 30 min after exposure. Subsequently significant differences were maintained until the end of the experiment(Figure 4b). In case of the intestine, significant differences of the *GCR* gene expression appeared at 30 min after exposure and were retained until 2 h after termination of exposure. Expression pattern of *GCR* gene in muscle were not similar to other tissues, namely up-regulation was observed at 30 min after exposure then decreased until 4 h after termination of exposure. A higher level of expression was shown at 12 h compared to the control group(Figure 4d).

DISCUSSION

Stress in fish may be induced by various abiotic environmental factors(such as changes in water temperature, pH, dissolved oxygen, pollution), biotic interactions(such as predator, parasite pressure and competition), and by human activities related to fish rearing and harvesting (manipulation, transport, crowding)(Witeska, 2005). Stress reaction involves various physiological changes including alterations in blood composition and immune function. These changes include osmotic disturbances, increase in energy substrate concentrations(glucose, fatty acids), increase in the activity of certain enzymes(lactate dehydrogenase, transaminases, in toxic stress also cytochrome P-450 and glutathione transferase), increase in stress protein levels (HSP, ubiquitin, metallothionein), and a decrease in humoral immune factors(lysozyme, antibodies) (Wendelaar Bonga, 1997; Svoboda, 2001).

In case of light stress the inner balance is usually restored but under severe or prolonged stress conditions compensatory abilities of the organism may be exhausted which results in physiological disturbances or even death (Witeska, 2005).

Typically, increased plasma cortisol concentrations(in between 30 ~ 300 ng/㎖) can result in response to acute stress that are rapidly secreted within 0.5 to an hour after exposure(Espelid *et al*., 1996; Wendelaar Bonga, 1997). In the present experiment, the rapid increased cortisol shown initially to the exposure, indicates that noise can be considered as an acute stress source for Japanese eel. In addition, Ortuno *et al*.(2001) reported that increased cortisol concentrations through acute stress are usually obtained after 6 - 24 hours of exposure. In our experiment, increased cortisol concentrations recovered as the control levels off at 12 h after termination of exposure.

Plasma glucose seems to have a similar tendency as cortisol under stress conditions(Mommsen, 1999; Caruso *et al*., 2005). In our experiment the concentration of glucose showed similar trends. In addition, plasma glucose levels of stressed fish were rapidly increasing with

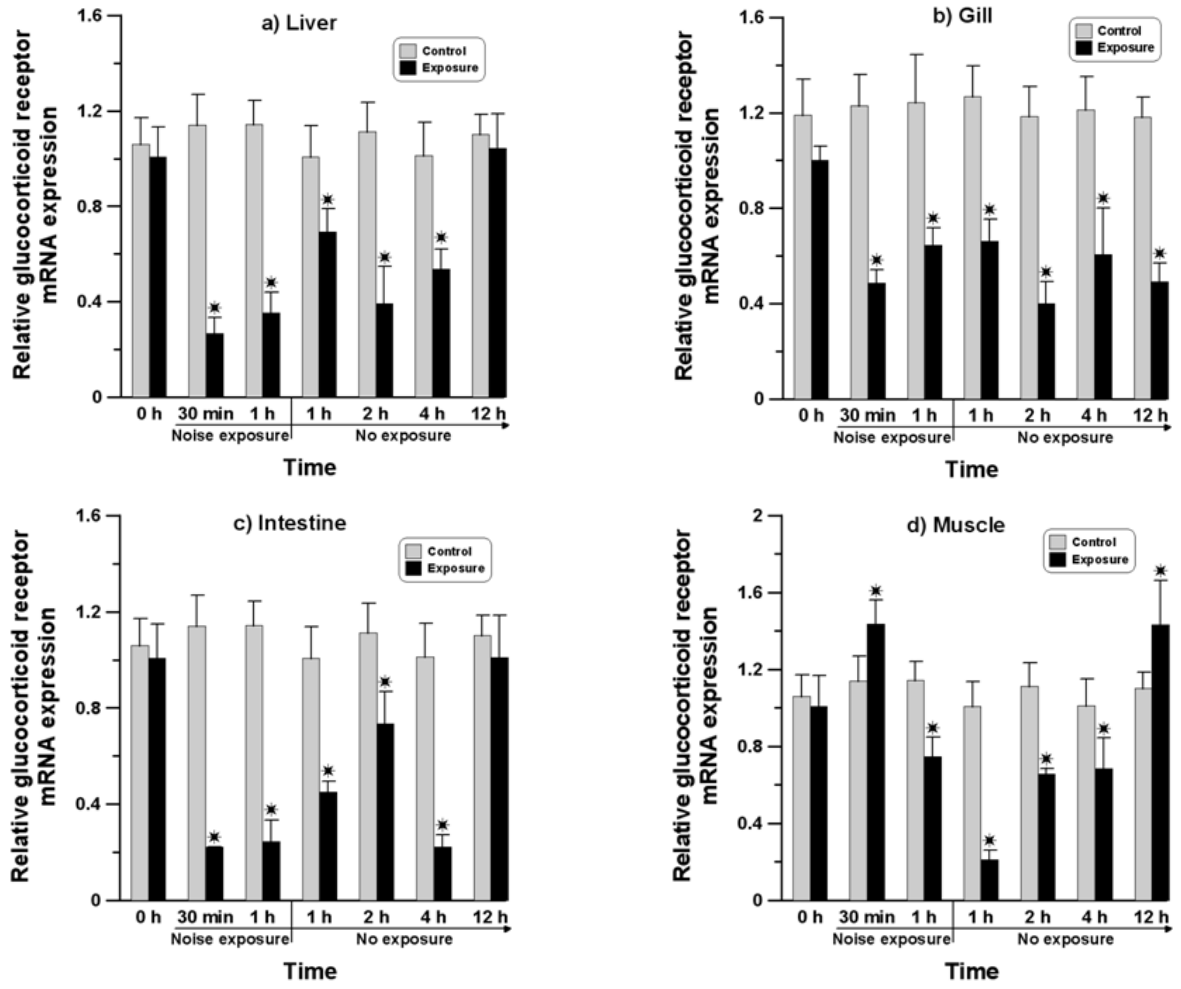

Figure 4. Relative mRNA expression of Japanese eel, *Anguilla japonica GCR* gene in different tissues after exposure to noise for 12 h. (*) Indicates significance($P < 0.05$) change over control values). Results are expressed as relative expression to β-actin used as housekeeping reference gene. Values are means of triplicate samples for each fish and tissue. Bars indicate S.D.

exposure, and recovered to the control level within one or two days(Ortuno *et al*., 2001).

According to Rabanal *et al*.(2003), is plasma albumin playing a role in physiological functions such as in the synthesis, transport, and removal of several components that are important as a source of protein supply for cells. Therefore, decreased albumin could serve as a suitable stress indicator. During the present experiment, the level of albumin decreased significantly at 1 h after exposure and was maintained until 2 h after termination of the noise exposure.

In all vertebrates, glucocorticosteroids play a key regulatory role in stress responses, growth, general metabolism, reproduction and immunity(Mommsen *et al*., 1999). In fish, cortisol is intimately involved in the regulation of water and minerals(Gilmour, 2005).

As a result of basal level expression, *GCR* mRNA in liver, gill, muscle and intestine of Japanese eel showed high expression levels. Previous studies on rainbow trout(*Oncorhynchus mykiss*) and European seabass (*Dicentrarchus labrax*) also reported a significantly higher amount of expression in liver, gills and intestinal tissues (Singer *et al*., 2007; Vazzana *et al*., 2008). Chakraborti *et al*.(1987) reported that the cytosol fractions of gills had highest cortisol-binding activity, followed by liver, intestine, and muscle in brook trout(*Salvelinus fontinalis*). The

associated constants for liver, intestine, and muscle were in the same order of magnitude as those for gill. These results are consistent with the concept of nonmembrane steroid receptors of target organs. However, Greenwood *et al*.(2003) showed a higher expression of the *GCR* gene in liver, heart and pancreas in the African cichlid, *Haplochromis burtoni*. These different expression profiles depending on the target tissues seem to be due to sensitivity differences of the cortisol.

Evidence from the literature strongly suggests that the major corticosteroids produced by this gland, cortisol, regulates both glucocorticoid and mineralocorticoid activities in fish(Bern and Madsen, 1992; Wendelaar Bonga, 1997), and that target gene expression is achieved via cortisol binding to corticosteroid receptors which act as ligand-inducible transcription factors(Gronemeyer and Laudet, 1995).

GCR mRNA expression level in the liver, gill and intestine of our study immediately decreased significantly in comparison to the control group after exposure to noise. These results are corresponding with most previous studies on *GCR* regulation in teleost fish. They come along with a down-regulation of *GCR* expression content with elevated cortisol concentrations(Maule and Schreck, 1991; Mommsen *et al*., 1999; Pottinger, 1990; Pottinger *et al*., 1994; Shrimpton and McCormick, 1999; Shrimpton and Randall, 1994). About this negative feedback of the *GCR* mRNA expression have been explained by a few studies(Hontela, 1997; Wendelaar Bonga, 1997; Vijayan *et al*., 2003)

As a result, we explored the possibility of parameters such as cortisol and *GCR* mRNA expression as biomarkers on noise stress for one of this valuable aquaculture species. Therefore, does *GCR* mRNA provide a stress indicator that can be used as a biomarker for noise stress.

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