

Molecular cloning and differential expression of the glucocorticoid receptor gene in the estuarine tapertail anchovy *Coilia nasus*

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ABSTRACT. To understand the regulation of the glucocorticoid receptor gene (*Gcr*) during loading and transport stress in fish, the *Gcr* gene of *Coilia nasus* was cloned. *Gcr* in *C. nasus* is expressed strongly in the liver and muscle, and less strongly in the gills, brain, spleen, intestine, trunk kidney, and head kidney. *Gcr* expression in both the liver and muscle was increased by loading and transport stress. NaCl reduced the death rate caused by loading and transport stress, and the expression of *Gcr* in liver and muscle differed significantly between the NaCl and non-NaCl groups. To investigate whether the elevated *Gcr* transcripts were translated into protein, proteins extracted from the liver and muscle were analyzed. In both tissues, *C. nasus* GCR protein expression patterns paralleled those of *Gcr* mRNA during stress.

Key words: *Coilia nasus*; Glucocorticoid receptor; Stress; NaCl; Hypothalamus-pituitary-interrenal; Rapid amplification of cDNA ends

INTRODUCTION

The hypothalamus-pituitary-interrenal (HPI) axis, or the stress axis, regulates the stress responses and is highly conserved across vertebrates (Wendelaar Bonga, 1997; Barton et al., 2002). The activation of the HPI axis begins in the hypothalamus (Wunderink et al., 2011), which secretes cortisol releasing factor (CRF) and stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary. This pituitary peptide binds to the melanocortin 2 receptor (MC2R) on steroidogenic cells in the interrenal tissue, leading to corticosteroidogenesis (Aluru and Vijayan, 2008, 2009). Cortisol represents the end product of the HPI axis and influences the expression of responsive genes (Gower, 1993; Dekloet et al., 1998), which in turn influence many functions of the central nervous system in mammals, including arousal, cognition, mood, sleep, metabolism, cardiovascular tone, and the immune and inflammatory reactions (Kino, 2007; Chrousos and Kino, 2009; Srinivasan et al., 2013). In teleost fish, cortisol can be significantly increased by stress, therefore, this hormone is always regarded as a stress marker. Cortisol can enhance the mobilization of liver glycogen, resulting in increased production of endogenous glucose (Lopez-Patino et al., 2014). The biological function of cortisol is mediated by its receptors, and the glucocorticoid receptor (GCR) is one of the most important. As a result, the glucocorticoid receptor gene (*Gcr*) is a vital gene in responding to stress.

The estuarine tapertail anchovy *Coilia nasus* (Temminck and Schlegel, 1846) is widely distributed in Korea, Japan, and the Yangtze River and coastal waters of China (Jiang et al., 2012). Its nutritive value and taste make it a commercially important species. However, *C. nasus* is highly susceptible to stress, which often results in death, causing significant losses to the industry. Although *Gcr* is an important gene in the stress response, little is known about the regulation of the *Gcr* gene during loading and transport stress in this fish. We found that the number of deaths caused by stress in *C. nasus* can be reduced by NaCl, therefore, in this study, we have examined in detail how the cortisol, *Gcr* and plasma osmolality levels respond to stress and NaCl treatment.

MATERIAL AND METHODS

Animal welfare

Animal welfare and experimental procedures were carried out in accordance with the guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006), and were approved by the animal ethics committee of the Chinese Academy of Fishery Sciences (2014JBFR06).

Experimental animals

C. nasus were adapted to a 7.0 x 5.0 x 1.0 m³ aquarium with a water temperature of 24.5° ± 1.0°C, pH 7.8, and dissolved oxygen concentration of 9.2 ± 0.5 mg O₂/L in dechlorinated, aerated water. The fish were fed twice daily, at 07:00 hours and 17:00 hours. At the outset of the experiments, all the fish appeared healthy.

Cloning and sequencing of *C. nasus Gcr*

TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from

the liver. A PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Osaka, Japan) was used to synthesize the first-strand cDNA. ClustalX 1.83 multiple-alignment software was used to align the *Gcr* nucleotide sequences from a variety of species. Primers were designed based on the nucleotides conserved in the sequences of 11 species reported previously: *Homo sapiens*, *Pan troglodytes*, *Macaca mulatta*, *Canis lupus familiaris*, *Gallus gallus*, *Mus musculus*, *Rattus norvegicus*, *Danio rerio*, *Takifugu rubripes*, *Oryzias latipes* and *Oncorhynchus mykiss* (Table 1). A 562-bp fragment of the *Gcr* gene was amplified from the *C. nasus* liver cDNA using the primer pair 10S/14A (Table 2). The 5' and 3' ends of the *C. nasus Gcr* cDNA were obtained by rapid amplification of cDNA ends (RACE) (Zhuan Dao, Wuhan, China).

Table 1. GenBank accession numbers of GCR sequences used in this study.

Species	Accession No.
<i>Homo sapiens</i>	NP_001019265.1
<i>Pan troglodytes</i>	XP_001155243.1
<i>Macaca mulatta</i>	XP_001097126.1
<i>Bos Taurus</i>	NP_001193563.1
<i>Canis familiaris</i>	XP_535225.2
<i>Gallus gallus</i>	NP_001032915.1
<i>Mus musculus</i>	NP_032199.3
<i>Rattus norvegicus</i>	NP_036708.2
<i>Oncorhynchus mykiss</i>	NP_001117954.1
<i>Oryzias latipes</i>	NP_001156605.1
<i>Danio rerio</i>	NP_001018547.2

Table 2. Sequences of primers used in this study.

Primer	Sequence	Usage
R844-1	GGACGAAGCACAGCAT	5'RACE
R844-3	GCAGCAGGGTCATCTGGTCG	5'RACE
502-1	TGGCAGAGGTTCTACCAGCTCACCA	3'RACE
502-2	CATGACATGGTGGGCGGCTGCTGA	3'RACE
10S	AAATGGGCTAAAGCTCTGCCAG	Homologous cloning forward primer
14A	TGGTAACTGGTTGCTGATGATTTC	Homologous cloning reverse primer
25S	TGCTCCTGGCTTCTCCTCATG	Forward primer for Cn <i>GCR</i> RT-qPCR
25A	TGCAGACGCACAAAACCTCGTTG	Reverse primer for Cn <i>GCR</i> RT-qPCR
42S	TGATTGGGACTGGGATTGAA	Forward primer for 18sRNA RT-qPCR
42A	TAGCGACGGGCGGTGTGT	Reverse primer for 18sRNA RT-qPCR
B1	AACGGATCCGGTATGTGCAAAGC	Forward primer for <i>actb1</i> RT-qPCR
B2	GGGTCAGGATACCTCTCTTGCTCTG	Reverse primer for <i>actb1</i> RT-qPCR

Reverse transcription-quantitative PCR (RT-qPCR) analysis of Cn*Gcr* mRNA expression profiles

Total RNA was extracted and first-strand cDNA was synthesized as described above. RT-qPCR was used to determine the *C. nasus Gcr* expression profiles, using *18sRNA* as the reference gene for detection of expression levels in different tissues, and *actb1* (encoding β -actin) was used for stress response detection. 25S/25A was used to amplify *Gcr*, 18sRNA (42S/42A) and *actb1* (B1/B2) (Table 2) had similar melting temperatures (T_m) and were designed to amplify 136- and 179-bp fragments, respectively. SYBR[®] Green Real time PCR Master Mix (Takara) was used to perform the RT-qPCR, with the following cycling parameters: 94°C for 2 min, followed by 40 cycles of 15 s at 94°C, 34 s at 60°C, and 45 s at 72°C. All the samples were analyzed in triplicate and the expression of the target genes was calculated (fold

change) with the $2^{-\Delta\Delta Ct}$ method. The expression levels of each gene were normalized to the reference gene, and then the data were expressed as the ratio of the CnGCR mRNA expression in the tissue to its expression in muscle.

Stress experiment

The *C. nasus* juveniles used in the experiment had been cultured in three 7.0 x 5.0 x 1.0 m³ ponds for one year. When the fish were 15 months old, five fish were removed from each pond and euthanized with 70 mg/L buffered tricaine methanesulfonate (MS-222). These 15 fish were the non-stressed controls. Another 75 fish were netted from each pond and 15 were loaded into each of 15 tanks (volume: 75 x 55 x 33 cm³). These 15 tanks were divided into five groups of three tanks each, from which fish were sampled at different time points. The tanks were shaken once every 5 min over the course of 0, 2, 4, 6, or 8 h to simulate transport, and five fish from each tank were euthanized. The mean length of all the fish (N = 90) sampled in this experiment was 136.98 mm \pm 9.26 (S.M.) and their mean mass was 8.86 g \pm 1.76 g.

Another 75 fish were netted from the same ponds and subjected to the same experimental procedure, except that 1.0% NaCl was included in the water. The mean length of all the fish (N = 90) sampled in this experiment was 141.46 mm \pm 11.99 and their mean mass was 9.93 g \pm 5.06.

Blood was collected into CAD (citric acid) capillary tubes after the caudal fin was severed. All fish appeared healthy during dissection, and the tissues were removed and placed in TRIzol Reagent. The plasma was separated by centrifugation, and the plasma and tissue samples were stored at -80°C until analysis. Total RNA extraction, cDNA synthesis, and RT-qPCR were performed as described above.

All mRNA levels are reported as means \pm SD and were subjected to two-way analysis of variance (ANOVA). Differences were considered significant at $P \leq 0.05$.

Cortisol, glucose, and plasma osmolality

Plasma cortisol was quantified using the radioimmunoassay described by Redding et al. (1984). Plasma glucose was quantified using the protocol described by Admin and Tasd (1977), and it was measured at 635 nm on a Beckman Cx-4 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Plasma osmolality was quantified using an Osmomat030 osmometer (Gonotec, Berlin, Germany).

Two-way ANOVA was used to analyze the differences among the times-post-transport in the same group, and the differences between the with-NaCl and without-NaCl treatment groups. The survival rates were tested by Chi-square tests ($P < 0.05$).

Western blotting

Western blotting was conducted according to Du et al. (2016). The KeyGEN Whole Cell Lysis Assay (KeyGEN, Nanjing, China) was used to extract tissue protein and the bicinchoninic acid (BCA) method (Pierce, Bonn, Germany) was used to determine protein content. The anti-GCR antibody (Bioss, Beijing, China) and the anti-actin antibody (Aviva, London, England) were diluted 1:5000 with 10 mM PBS (pH 7.4). The horseradish-peroxidase-labeled goat anti-rabbit IgG antibody (Aviva, London, England) was diluted 1:5000. The bands were visualized with Pierce ECL Plus (Thermo Fisher Scientific, Waltham, MA, USA).

RESULTS

Cloning and sequence characterization of the *C. nasus* Gcr gene

A 562-bp cDNA fragment was amplified from *C. nasus* liver cDNA. A BLASTx analysis of the sequence showed that it shared strong similarity with other *Gcr* genes. Based on this conserved sequence, the full-length cDNA of *C. nasus* *Gcr* (GenBank accession No.: KJ747634) was obtained with RACE. Its full length was 3175 bp and it contained a 2379-bp open reading frame (ORF) encoding a protein of 792 amino acids. The 5' and 3' untranslated regions were 89 and 707 bp, respectively. One polyadenylation signal (AATAAA) was present 13 nucleotides upstream from the polyA tail. The identities shared by the deduced *C. nasus* *Gcr* amino acid sequence and its homolog used in other species were: *D. rerio* 52%, *G. gallus* 42%, *R. norvegicus* 41%, *M. musculus* 41%, *B. taurus* 41%, *C. lupus familiaris* 41%, *M. mulatta* 41%, *P. troglodytes* 40% and *H. sapiens* 40% (Figure 1A). The *C. nasus* GCR protein was predicted to contain a GCR domain (350 residues), a ZnF-C4 domain (81 residues), and a HOLI domain (165 residues) (Figure 1B).

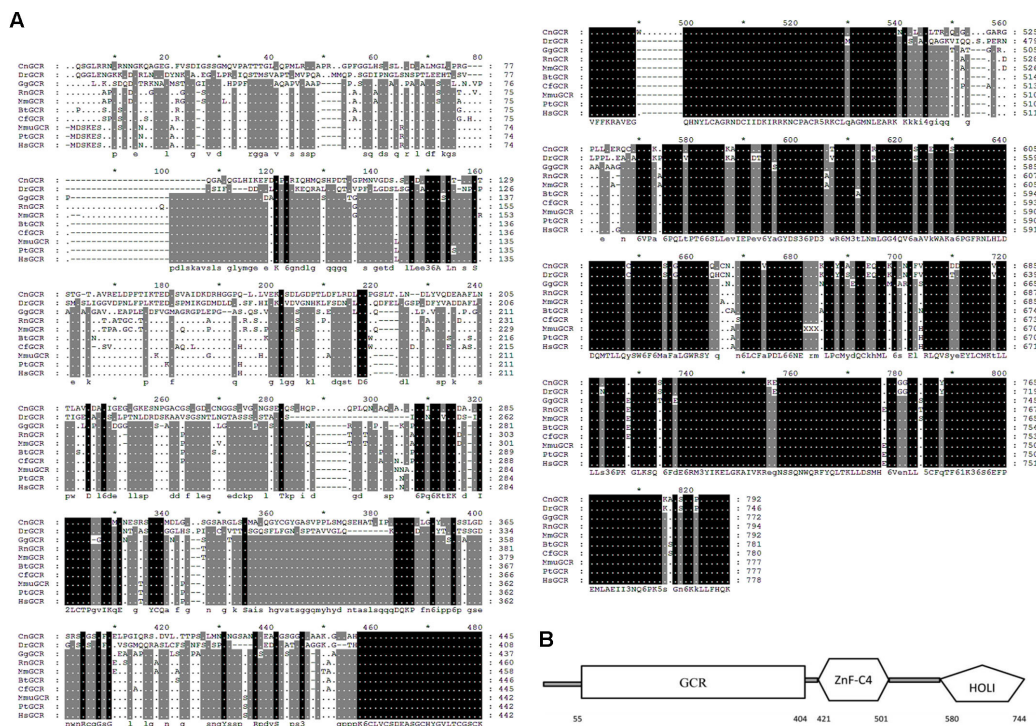


Figure 1. Deduced amino acid sequence and predicted domain structure of CnGCR. **A.** Alignment of the deduced amino acid sequence of CnGCR and other reported GCR sequences. The residues completely conserved across all species are aligned and shaded in black. Cn, *Coilia nasus*; Dr, *Danio rerio*; Gg, *Gallus gallus*; Rn, *Rattus norvegicus*; Mm, *Mus musculus*; Bt, *Bos taurus*; Cf, *Canis lupus familiaris*; Mmu, *Macaca mulatta*; Pt, *Pan troglodytes*; Hs, *Homo sapiens*. **B.** Schematic diagram of the structure of CnGCR, which comprises a GCR domain, a ZnF-C4 domain, and a HOLI domain.

Tissue expression profiles of CnGcr

The expression profiles of the *C. nasus Gcr* transcripts were assessed in different tissues with RT-qPCR, using *18sRNA* as the reference gene. In healthy fish, *C. nasus Gcr* was strongly expressed in liver and muscle, and less strongly in the gill, brain, spleen, intestine, kidney, and head kidney (Figure 2).

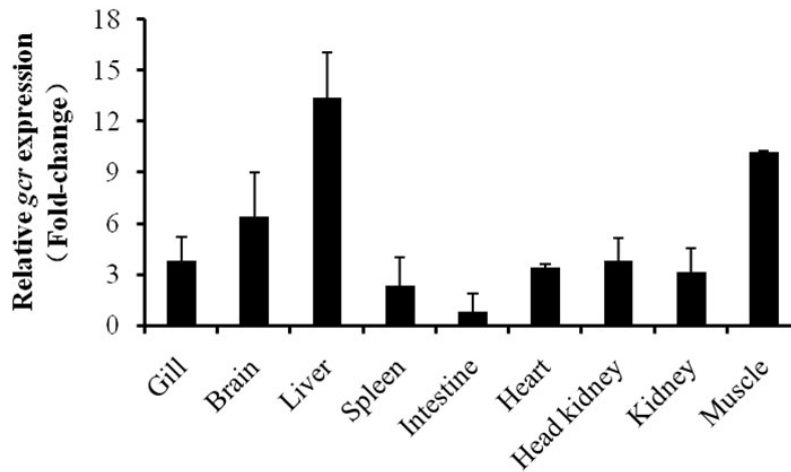


Figure 2. CnGCR mRNA expression in different tissues. RT-qPCR analysis of the expression profiles of CnGCR mRNA in different *C. nasus* tissues. Data are expressed as the ratio of CnGCR mRNA expression in the tissue to its expression in muscle (means \pm SD).

Survival rate and plasma osmolality

After simulated transport, the survival rate decreased to 73.0% in the non-NaCl group (Figure 3A). NaCl (1.0%) was then added to the transport tanks to reduce the death rate caused by stress, and there were no dead fish in the 1.0% NaCl group (Figure 3A). When the plasma osmolality was determined in both groups (Figure 3B), it differed significantly between the non-NaCl and NaCl groups ($P < 0.05$). Plasma osmolality was significantly elevated after loading (0 h), but then decreased significantly in the non-NaCl group. Unlike the non-NaCl group, plasma osmolality increased significantly in the NaCl group compared with the control fish, but was maintained at a level similar to the control level after loading.

Plasma cortisol and glucose responses to stress

In the non-NaCl group, plasma cortisol was significantly elevated in the stressed fish ($P < 0.05$) compared to the control fish ($187.76 \text{ ng/mL} \pm 9.07$; $N = 15$) (Figure 4A). There were no significant differences between the control replicates ($P > 0.05$). In the NaCl group, plasma cortisol in the stressed fish was also significantly elevated ($P < 0.05$) compared to the control fish (Figure 4A), and it was significantly higher in the NaCl group than in the non-NaCl group at 4 h ($P < 0.05$).

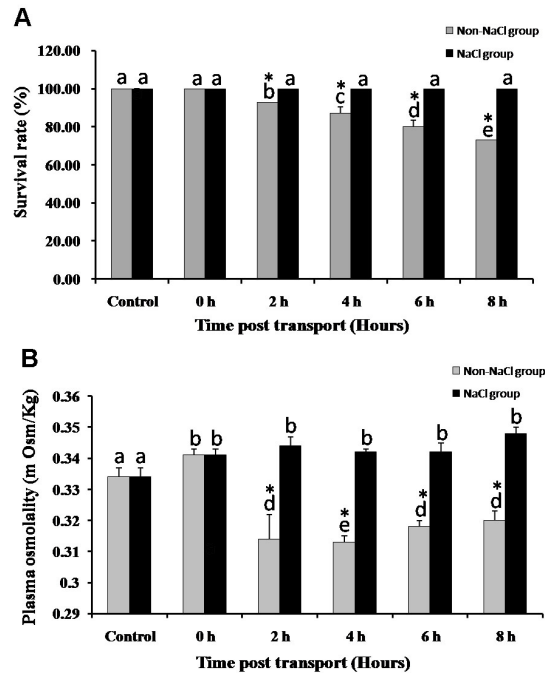


Figure 3. Survival rate and plasma osmolality of fish during loading and transport stress. Bars are the means \pm SD of replicate experiments. ^{a,b,c,d,e}Different letters represent significant differences between the control and different times after stress ($P < 0.05$). *Significant difference ($P < 0.05$) between the non-NaCl group and NaCl group at the same time point. **A.** Survival rate during loading and transport stress in the non-NaCl group and NaCl group. **B.** Plasma osmolality levels during loading and transport stress.

In the non-NaCl group, plasma glucose concentration (Figure 4B) was significantly elevated ($P < 0.05$) after the fish were netted (6.31 ± 0.20 mM; $N = 15$) and transported for 2 h (8.38 ± 0.81 mM; $N = 15$), and it decreased after 6 h (4.96 ± 0.44 mM; $N = 15$) and 8 h (4.81 ± 0.78 mM; $N = 15$) of transport, although it was still significantly higher than in the control (4.03 ± 0.66 mM; $P < 0.05$, $N = 15$). After transport for 4 h, the plasma glucose concentration (4.53 ± 0.49 mM; $N = 15$) was not significantly different from the control. In the NaCl group (Figure 4B), a similar pattern of change was observed, but the plasma glucose concentrations after treatment for 2, 6, and 8 h were significantly higher in the non-NaCl group than in the NaCl group ($P < 0.05$, $N = 15$).

CnGcr responses to stress

In the non-NaCl group, RT-qPCR revealed a 3.3-6.3-fold increase in the expression of *Gcr* in the livers of the stressed *C. nasus* ($P < 0.05$; Figure 5A). Similarly, a 6.5-10.2-fold increase in the expression of *C. nasus Gcr* was observed in muscle (Figure 5B). In the NaCl group, RT-qPCR revealed a 6.3-11.0-fold increase in the expression of *Gcr* in the livers of the stressed *C. nasus* ($P < 0.05$; Figure 5A). Similarly, a 6.0-8.4-fold increase in the expression of *C. nasus Gcr* was observed in muscle (Figure 5B). Therefore, the expression of *C. nasus Gcr* in both liver and muscle differed significantly between the non-NaCl and NaCl groups.

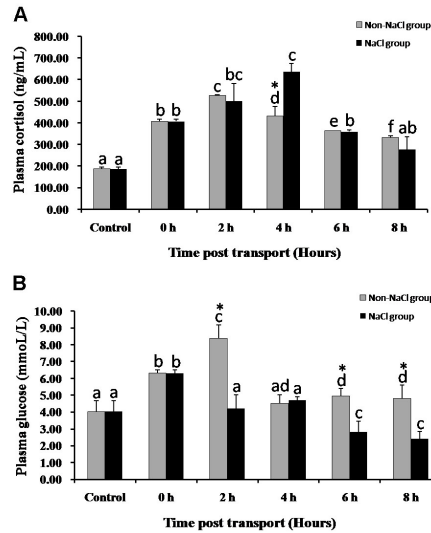


Figure 4. Plasma cortisol and plasma glucose concentrations during loading and transport stress. Bars are the means \pm SD of replicate experiments. ^{a,b,c,d,e,f}Different letters represent significant differences between the control and different times after stress ($P < 0.05$). *Significant difference ($P < 0.05$) between the non-NaCl group and NaCl group at the same time point. **A.** Plasma cortisol concentrations during loading and transport stress in the non-NaCl group and NaCl group. **B.** Plasma glucose levels during loading and transport stress in the non-NaCl group and NaCl group.

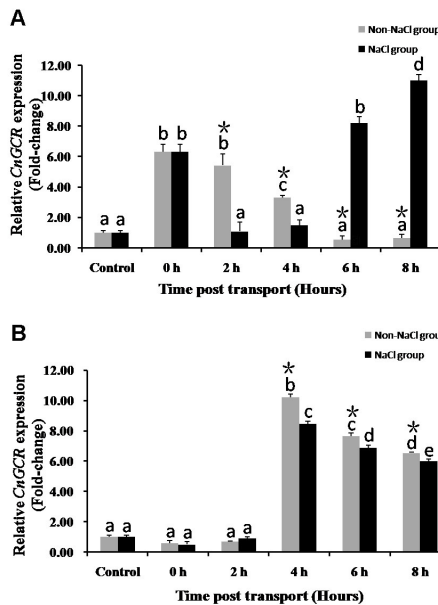


Figure 5. CnGCR mRNA responses to stress. Bars are the means \pm SD of replicate experiments. ^{a,b,c,d}Different letters represent significant differences between the control and different times after stress ($P < 0.05$). *Significant difference ($P < 0.05$) between the non-NaCl group and NaCl group at the same time point. **A.** Expression profiles of liver CnGCR mRNA during stress in the non-NaCl group and NaCl group. **B.** Expression profiles of muscle CnGCR mRNA during stress in the non-NaCl group and NaCl group.

To investigate whether the elevated *Gcr* transcripts were translated into protein, proteins extracted from liver and muscle were analyzed with western blotting. The antibody-specific detection of these two protein extracts are shown in Figure 6A and B. In both tissues, the *C. nasus* GCR protein expression patterns were similar to the mRNA expression patterns during stress. In the non-NaCl group, liver GCR increased and peaked after 0 and 2 h of stress, after which it decreased (Figure 6C). However, in the NaCl group, GCR expression was significantly down-regulated after 2 and 4 h of stress and significantly up-regulated after 6 and 8 h of stress. GCR protein expression in the muscle also peaked 4 h after stress, and then decreased, although it remained high (Figure 6D). In the NaCl group, GCR protein expression was significantly down-regulated in muscle relative to the non-NaCl group (Figure 6D).

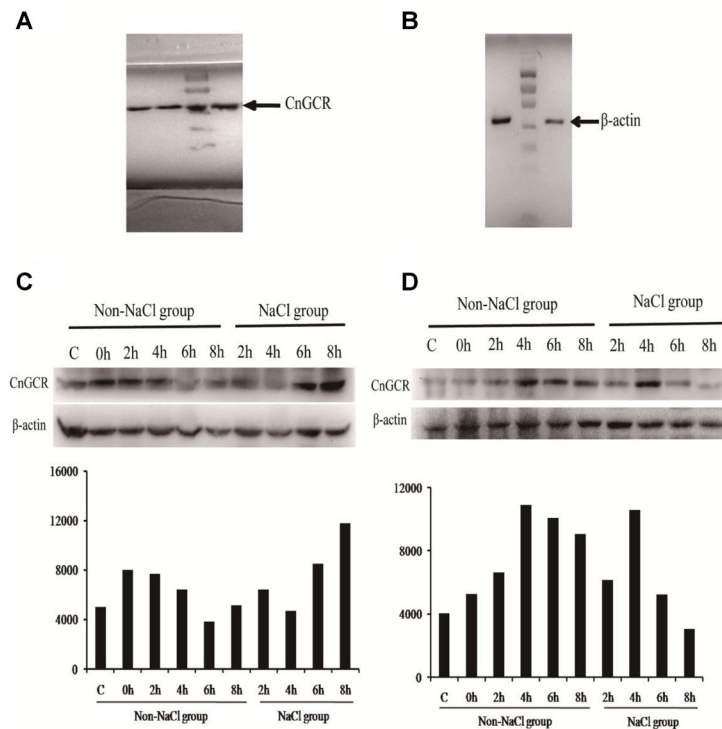


Figure 6. CnGCR protein responses to stress. **A.** Specific detection of CnGCR antibody. **B.** Specific detection of β -actin antibody. **C.** Expression profiles of liver CnGCR protein during stress in the non-NaCl group and NaCl group. **D.** Expression profiles of muscle CnGCR protein during stress in the non-NaCl group and NaCl group.

DISCUSSION

A comparison of the amino acid sequences of *C. nasus* GCR and other GCRs from a variety of species (Figure 1A), including *D. rerio*, *G. gallus*, *R. norvegicus*, *M. musculus*, *B. taurus*, *C. familiaris*, *M. mulatta*, *P. troglodytes*, and *H. sapiens*, revealed that *C. nasus* GCR has the three conserved domains found in all GCRs: a GCR domain at the N-terminus, a ZnF-C4 domain, and a HOLI domain at the C-terminus (Figure 1B). The sequence identities of these domains were high across species, especially for the ZnF-C4 and HOLI domains,

at more than 80% relative to the proteins of other fishes and mammals. All the sequence characteristics of GCR indicate that *C. nasus* GCR, together with the GCRs of other fish, have similar functions to those observed in mammals.

As noted above, GCRs are crucial to the regulation of cardiovascular, metabolic, immunologic, and homeostatic functions. GCRs have an established link to physical and mental stress (Lee et al., 2013), and they are involved in regulating metabolic rates, skeletal muscle protein metabolism, liver gluconeogenesis, and hyperglycemia (Brillon et al., 1995). So GCRs are expressed quite ubiquitously in fish, especially in liver (Vijayan et al., 2003), gills (Kuo et al., 2013), and muscle (Rotllant et al., 1997). *C. nasus Gcr* mRNA is highly expressed in liver and muscle (Figure 2), which is consistent with the critical functions of cortisol in these tissues (Dean et al., 2003; Fontainhas et al., 2003; Marshall, 2003; Tripathi and Verma, 2004). Cortisol is a key mediator of stress-induced hyperglycemia, and importantly, it supports the increased energy demand associated with stress (Mommensen et al., 1999; Sapolsky et al., 2000). Cortisol is also a vital osmoregulatory factor, and both gluconeogenic metabolism and osmoregulation are mediated by *Gcr* (Hanson and Reshef, 1997; Vijayan et al., 2003). The liver is an important gluconeogenic tissue and muscle is one of the tissues involved in osmotic balance, so *C. nasus Gcr* is highly expressed in both these tissues.

C. nasus is highly susceptible to stress, (Du et al., 2014a,b). In our experiment, the death rate caused by loading and transport stress was reduced by 1.0% NaCl (Figure 3A). In aquaculture practice, netting- and transport-induced stresses are reduced by adding salt to freshwater, a procedure that affects glucose concentrations and osmoregulation. The HPI axis is also known as the stress axis (Fryer et al., 1984; Bernier et al., 1999), and it consists of a cascade of hormones that regulate cortisol secretion from the interrenal tissue (Abraham et al., 2009). This hormone mediates stress-induced hyperglycemia and osmoregulation (Henderson and Jones, 1967; Mayer and Maetz, 1967). In our study, plasma cortisol was significantly elevated after stress in the non-NaCl group (Figure 4A), as it is in other fish. Plasma glucose concentrations were also significantly increased in the non-NaCl group (Figure 4B), but plasma osmolality decreased after stress (Figure 3B). Therefore, loading and transport stress altered the plasma glucose concentrations and osmolality of the fish. Osmoregulatory adjustments are very important for fish, because without appropriate regulation, death may result (Takei et al., 2006; Kammerer et al., 2010; Gilmour et al., 2012). As a result, some of the fish in the non-NaCl group died. However, no fish died in the NaCl group (Figure 3A). As reported, adding salt to an almost isotonic level creates an iso-osmotic environment that helps fish to osmoregulate more easily and is less energy demanding and less stressful compared to a hypo-osmotic freshwater environment (Zhou et al., 2003). When the cortisol levels in the two groups were compared, NaCl had no significant regulatory effect on the plasma cortisol levels in most samples (Figure 4A), whereas it regulated plasma glucose concentrations and maintained plasma osmolality at a stable level (Figure 3B and Figure 4B).

In the early stage of the *C. nasus* stress response, NaCl reduced liver GCR expression (Figure 5A and Figure 6C), but it stimulated GCR expression in the later stage of the stress response. This result indicates that negative feedback regulation is involved in this process. High plasma glucose may induce NaCl to inhibit GCR expression, which then reduces the glucose levels. Similarly, low plasma glucose induces NaCl to stimulate GCR expression, thus increasing the glucose levels. In short, NaCl maintains plasma glucose at a stable level during the stress process. NaCl ameliorated CnGCR expression in muscle after 4 h of stress, and also maintained the osmotic pressure at a stable level.

In conclusion, as in other reported studies, we found that cortisol was significantly increased by stress, and this altered the plasma glucose concentrations and osmolality of the fish. NaCl increased the survival rate of the fish by regulating plasma glucose concentrations and maintaining plasma osmolality at a stable level. There may be a negative feedback pathway involved in this process that warrants further study.

Conflicts of interest

The authors declare no conflict of interest.

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