General and Comparative Endocrinology 316 (2022) 113947

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Molecular characterization and expression patterns of glucocorticoid receptors in the viviparous black rockfish *Sebastes schlegelii*

Xiaojie Wang [1], Wei Meng [1], Xin Qi, Yun Li, Jifang Li, Likang Lyu, Jianshuang Li, Yijia Yao, Shaojing Yan, Chenpeng Zuo, Songyang Xie, Haishen Wen [*]

Key Laboratory of Mariculture (Ocean University of China), Ministry of Education, Ocean University of China, Qingdao 266003, PR China

ARTICLE INFO

Keywords:
Black rockfish
Viviparity
Glucocorticoid receptor
Steroid 11β-hydroxylase
Parturition
Gene expression profile
Localization
In vitro treatment

ABSTRACT

Glucocorticoid receptors (GRs) are ligand-activated transcription factors associated with anti-inflammation, stress, metabolism and gonadal development. In this study, two *gr* genes (*gr1* and *gr2*) were cloned and analyzed from a viviparous teleost, black rockfish (*Sebastes schlegelii*). The phylogenetic analysis of GRs showed that GR1 and GR2 clustered into teleost GR1 and GR2 separately and differed from the GRs of tetrapods or basal ray-finned fishes. Black rockfish GRs possess four modular domains of the nuclear receptor superfamily: an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region (HR) and a ligand-binding domain (LBD). Nine conserved amino acid inserts were found in the GR1 DBD, and the ligand cavity-related amino acids of GR1 and GR2 LBD were slightly different. Tissue distribution analysis revealed that *grs* was widely expressed in various tissues, while *cyp11b* was mainly expressed in the testis and head kidney. The *cyp11b* transcripts were localized in the interrenal glands of the head kidney, the main source of cortisol; *grs* transcripts were detected in oocytes, the follicle layer and the ovarian wall. Histologically, significant blood vessel dilation was observed in the fetal membrane during or after parturition of black rockfish. The highest levels of serum cortisol and ovarian *cyp11b* mRNA were detected in parturition. In addition, the relative expression level of *gr1* was upregulated significantly after delivery, while the levels of *gr2* showed no significant change. In addition, *in vitro* GC treatment inhibited the expression of *il1b* but significantly upregulated the transcription of *il1r1*. These data provide evidence that GRs are likely to work as anti-inflammatory factors by inhibiting the functions of pro-inflammatory factors in the parturition of black rockfish.

1. Introduction

Glucocorticoids (GCs) are a class of steroid hormones that are involved in several physiological processes, such as inflammation, metabolism, development and stress response (Alberto et al., 2020; Milla et al., 2009). In mammals and teleosts, cortisol is the most important endogenous GC (Arterbery et al., 2011; Li et al., 2012), whose biosynthesis is catalyzed by cytochrome P450 steroid 11β-hydroxylase (CYP11B) in the zona fasciculata of the adrenal cortex (in mammals) or the interrenal gland of the head kidney (in teleosts) (Alsop and Vijayan, 2009; Sanders et al., 2016; Schiffer et al., 2015).

Glucocorticoid receptor (GR), also known as NR3C1 (nuclear receptor subfamily 3, group C, member 1), is the receptor for cortisol and other GCs (Weikum et al., 2018). Nuclear receptors belong to a superfamily that evolved from a common ancestral steroid receptor. After the whole genome duplication (WGD) of jawed vertebrates, corticosteroid receptor (CR) duplicated and eventually evolved into GR and MR (mineralocorticoid receptor) (Thornton, 2001; Thornton et al., 2003). Most teleost fishes have two copies of the GR gene (*gr1* and *gr2*) as a result of teleost-specific WGD (T-WGD) 320–350 MYA (million years ago), but only one MR has been retained in teleosts (Arterbery et al., 2011; Bury, 2017). Similar to other nuclear receptors, GR is composed of four modular domains: an N-terminal domain (NTD or A/B domain), a DNA-binding domain (DBD or C domain), a hinge region (HR or D domain) and a ligand-binding domain (LBD or E domain) (Weikum et al., 2018). GR works as a ligand-activated transcription factor and regulates the transcription of downstream genes (Alberto et al., 2020).

Black rockfish (*Sebastes schlegelii*) belongs to the Sebastidae family and has a viviparity reproductive strategy (He et al., 2019). The sexual maturity of male and female black rockfish is asynchronous: male fishes

* Corresponding author at: Key Laboratory of Mariculture, Ocean University of China, No. 5 Yushan Road, Shinan District, Qingdao 266003, PR China.
E-mail address: wenhaishen@ouc.edu.cn (H. Wen).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ygcen.2021.113947
Received 15 March 2021; Received in revised form 8 November 2021; Accepted 18 November 2021
Available online 27 November 2021

reach sexual maturity from November to December; after mating, sperm are dispersed in the ovary for approximately four months until oocyte maturation (around March); after internal fertilization, pregnancy of female fishes lasts for approximately one month, and then parturition occurs (Mori et al., 2003; Wang et al., 2021). In mammals, GRs play a role in gestation, fetal development and parturition (Roberts et al., 2017; Wang et al., 2020). However, GC functions in the parturition of viviparous teleosts have not been reported.

In this study, we analyzed the protein domains, evolutionary relationships and mRNA expression patterns of *gr1* and *gr2* during the parturition process in female black rockfish. Moreover, we also stimulated ovarian cells *in vitro* using cortisol and dexamethasone (DXMS) and analyzed the expression levels of related genes.

2. Materials and methods

2.1. Ethics statement

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Ocean University of China. The protocol for animal care and handling used in this study was approved by the Committee on the Ethics of Animal Experiments of Ocean University of China. Before sacrificing and handling, experimental fish were anesthetized with 100 ng/ml ethyl 3-aminobenzoate methanesulfonic acid (MS222), and all efforts were made to minimize the suffering of the animals. The field studies did not involve endangered or protected species.

2.2. Fish and sampling

Female black rockfish were obtained from marine cages located offshore of Rushan, Shandong, China (36.92°N, 121.54°E) in mid-April. Fishes were then raised in indoor cement pools with a culvert system. Subsequently, ovarian tissues and sera were collected from five pregnant fishes as samples before parturition. In approximately late April, the black rockfish were ready for delivery. Once delivery proceeded, the fish were sampled immediately during parturition, and a total of five fish were gathered. Similarly, another five fishes were sampled 24 h after parturition.

2.3. Total RNA extraction and reverse transcription

Total RNA was extracted from ovaries and other tissues using TRIzol reagent (Vazyme, China) according to the manufacturer's instructions. RNA quantity and purity were assessed by a Biodropsis BD-1000 nucleic acid analyzer (OSTC, China) and by electrophoresis using a 1% agarose gel. cDNA was prepared using the HiScript® III SuperMix (+gDNA wiper) kit (Vazyme, China) according to the manufacturer's instructions.

2.4. Molecular cloning and sequencing of gr1, gr2 and cyp11b

Based on the gonadal transcriptome (PRJNA573572) data and genome (unpublished) data of black rockfish, the *gr1*, *gr2* and *cyp11b* sequences were cloned and verified. All primers used in the present study are listed in Table S1. Head kidney cDNA was used as the template for cloning. The PCR product was purified, cloned into the pCE2 TA/Blunt-Zero vector (Vazyme, China) and sequenced.

2.5. Multiple alignments, phylogenetic tree construction and homologous modeling

Multiple alignments of amino acid sequences were performed based on GR amino acid sequences of several species using ClustalX 2.1 and ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). A phylogenetic tree was reconstructed from the multiple alignments of deduced amino acid sequences with the neighbor-joining method using MEGA-X. Values on the trees represent bootstrap scores of 1000 iterations, indicating the credibility of each branch. The tertiary structures of GR DBD and LBD were modeled by homology modeling methods using SWISS-Model (https://swissmodel.expasy.org/), and pictures were generated in PyMOL 2.4.

2.6. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed to determine the expression levels of *gr1*, *gr2* and other genes in black rockfish ovaries and other tissues. qPCR was conducted using the ChamQ^{TM} SYBR® Color qPCR Master Mix (High Rox Premixed) kit (Vazyme, China) following the manufacturer's instructions. The qPCR program was as follows: 95 °C for 30 s; 40 cycles of 95 °C for 10 s and 60 °C for 30 s; and melting curves: 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. The threshold cycle (C_T) values were measured for each sample, and *18S* rRNA (GenBank accession number: KF430619.1) was selected as the reference gene (Liman et al., 2013). The relative mRNA expression levels of the genes were calculated using the $2^{-\Delta\Delta C_T}$ method from triplicate samples. qPCR was run in triplicate to confirm the results.

2.7. Preparation of DIG-labeled riboprobes and in situ hybridization (ISH)

Primers with recognition sequences of T7 or SP6 RNA polymerase (Table S1) were designed to prepare the DNA templates for RNA probes by means of PCR. Anti-sense or sense probes of *gr1*, *gr2* and *cyp11b* mRNA were transcribed *in vitro* using a DIG RNA Labeling Kit (SP6/T7) (Roche, Switzerland) according to the manufacturer's instructions.

The ovaries and head kidney were collected from black rockfish. The tissues were fixed in 4% paraformaldehyde (in PBS, pH 7.4) for 4–6 h. Then, they were embedded in paraffin and sectioned (7 μm). Tissue sections were then deparaffinized, rehydrated, and immersed in the following RNase-free solutions successively: 0.2 M HCl (8 min), PBS (5 min × 2), 10 μg/mL protease K (in PBS) (37 °C, 5 min), PBS (5 min), 0.1 M pH 8.0 triethanolamine-HCl with 0.25% acetic oxide (10–15 min) and 2 × SSC (5 min). Sections were then incubated in prewarmed hybridization buffer without probe (50% deionized formamide, 5 × SSC, 5 × Danhats, 1 mg/ml yeast tRNA, 10% dextran sulfate sodium) inside a humidified box in a hybridization oven at 55℃ for 1 h. Probes were then diluted in hybridization buffer (1.5 μg/mL) and added to slides and placed in a humidified box at 55℃. After overnight hybridization (12–16 h), the following washes were performed in preheated 2 × SSC, 1 × SSC, 0.2 × SSC, and 0.1 × SSC (55 °C, 15–30 min for each step) to remove nonspecific adsorption of probes. The DIG-labeled probes were detected using alkaline-phosphatase-conjugated anti-DIG Fab fragments (1:400 dilution) and nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (Roche, Switzerland) according to the manufacturer's instructions. Sections were dehydrated and coverslipped with mounting medium for microscopic observation.

2.8. Radioimmunoassay of serum cortisol

The serum cortisol levels before/during/after parturition were measured using a Cortisol Iodine-125 [^{125}I] Radioimmunoassay Kit (product No., D10PZB, Beijing Northern Institute, China). The assay sensitivity reached 0.15 ng/mL, and detection ranged from 10 to 500 ng/mL according to the kit protocol.

2.9. In vitro GC treatment

Cortisol (Yuanye, China) was dissolved in absolute ethanol at concentrations of 10^{-5}, 10^{-4} and 10^{-3} mol/L, and the stock solution of DXMS (Yuanye, China) was prepared in the same way. The ovaries of black rockfish were washed with PBS with antibiotics (penicillin and

streptomycin) and transferred into Trypsin-EDTA Solution (Servicebio, China). Tissue was cut up and digested for 5–10 min at room temperature, and then the same amount of Leibovitz's L-15 complete medium (with antibiotics and 10% fetal calf serum) (Biological Industries, Israel) was added to stop digestion. The suspension was filtered with a cell strainer (40 μm), centrifuged and resuspended in L-15 complete medium. The ovarian cells were then inoculated into a 12-well cell culture plate and cultured for 24 h in a CO_2-free incubator at 25 °C. After 2 h of serum starvation, complete medium was added, the cortisol or DXMS stock solutions were diluted into medium (1:1000) at final concentrations of 10^{-8}, 10^{-7}, and 10^{-6} mol/L, and the control groups were treated with 0.1% ethanol. Each treatment was performed in triplicate. After 3 h/6h of stimulation, TRIzol reagent was added to each well for cell RNA extraction and qPCR.

2.10. Statistical analysis

All data is shown as mean ± standard error of the mean (SEM). Graphs were generated by GraphPad Prism 9 (GraphPad Software, USA). Data of serum cortisol level and gene expression were analyzed using an analysis of variance (ANOVA) using GraphPad Prism 9. The data of *in vitro* GC treatment were subjected to two-way ANOVA followed by Dunnett's multiple comparison test, with individual variances computed for each comparison and other data were subjected to one-way ANOVA Tukey's multiple range test with a single pooled variance. P values$<$0.05 were considered to be significant.

3. Results

3.1. Molecular cloning and sequence analysis

In total, two *gr* genes (*gr1* and *gr2*) were identified in black rockfish. The ORF of *gr1* is 2343 bp (GenBank accession number: MZ441154), encoding a precursor protein of 780 amino acids **(Fig. S1 A)**. The ORF of *gr2* is 2349 bp (GenBank accession number: MZ441155), encoding a protein of 782 amino acids **(Fig. S1 B)**. In addition, only one copy of the *cyp11b* gene was found in black rockfish (GenBank accession number: MZ441156), with an ORF of 1639 bp and a translated protein of 545 aa **(Fig. S1 C)**.

Based on the amino acid alignment, the vertebrate GR proteins were determined to possess four modular domains: an NTD, a DBD, an HR and an LBD **(**Fig. 1**,** Fig. 2**A)**. The overall amino acid identity of the two GRs of black rockfish was 49.34%, with particularly high sequence identities for the DBD (83.72%) and the LBD (87.45%) and a relatively low identity for the NTD (24.49%) and the HR (63.64%) **(**Fig. 1**)**. Most teleosts had two copies of GR genes, while basal ray-finned fishes, robe-finned fishes and tetrapods had only one copy. Furthermore, GR1 and GR2 of black rockfish were clustered with teleost GR1 and GR2 separately and differed from the GRs of other vertebrates **(**Fig. 2**)**. The amino acid sequence of black rockfish GR1 was homologous to those of Nile tilapia GR1 (77.81%), medaka GR1 (71.39%), chicken GR (57.43%), mouse GR (59.13%) and human GRα (57.55%). In addition, black rockfish GR2 was determined to be homologous to Nile tilapia GR2 (81.01%), medaka GR2 (76.80%), zebrafish GRα (64.79%), chicken GR (48.23%), mouse GR (58.28%) and human GRα (56.37%) **(**Fig. 1**)**. One other point worth emphasizing was that Salmoniformes species (rainbow trout, Atlantic salmon, etc.) had two copies of GR1 genes as well as two GR2 genes **(**Fig. 2**)**.

The DBD of vertebrate GRs had two highly conserved zinc fingers (C4-type) **(**Fig. 1**,** Fig. 3 **B, C)**, and each one possessed four cysteines (C) forming coordination bonds with zinc ions (Zn^{2+}). Remarkably, an additional helix (including 9 amino acids WRARQNTDG) was found between the two zinc fingers of teleost GR1 **(**Fig. 3 **A, B)**. In addition, LBD was highly conserved, especially the ligand cavity-related amino acids. LBD is capable of binding ligands (e.g., cortisol) by means of hydrophobic interactions, hydrogen bonds and water bridges **(**Fig. 1**,** Fig. 3**)**.

3.2. Tissue distribution of grs and cyp11b mRNA in black rockfish

The mRNA tissue distribution indicated that *gr1* and *gr2* were widely expressed in the ovary and other tissues. Unlike the *grs* gene, *cyp11b* exhibited tissue-specific expression **(**Fig. 4 **A, B)**. The highest expression level was detected in testis followed by the head kidney, while the levels of *cyp11b* transcripts in other tissues were relatively low **(**Fig. 4 **C)**.

Ovaries of black rockfish were paired and bursiform and surrounded by thin elastic ovarian walls. In the ovary, *gr1* and *gr2* mRNA was mainly localized in oocytes and the ovarian follicle layer, while interstitial tissues were nearly negative **(Fig. 4 D1-D5)**. In addition, transcripts of *gr1* and *gr2* were also detected in the inner columnar epithelium and smooth muscle layer of the ovarian wall **(Fig. 4 E1-E5)**. Strongly positive signals of *cyp11b* mRNA were found in interrenal glands, which were composed of columnar epithelioid tissues located in the central part of the head kidney **(Figs. F1-F5)**.

3.3. Serum cortisol level and gene expression patterns around parturition

In the gestation period, the fetus distributed in ovaries was surrounded by a highly vascular connective tissue layer, an organization originating from the ovarian follicle layer **(Fig. 5 A1, A2)**. When fetuses mature, the connective tissue layer ruptures; as a result, the fetuses are released from ovarian tissue and born. After delivery, the broken connective tissue layers were left in the ovaries accompanied by dilated blood vessels **(Fig. 5 B1, B2)**. The serum cortisol levels during delivery were higher than the levels before or after the process but with no significant difference **(**Fig. 5 **C)**, and the relative *cyp11b* mRNA levels in ovaries showed no significant difference **(**Fig. 5 **D)**. The highest relative *gr1* mRNA levels were detected in regressed ovaries **(**Fig. 5 **E)**, while *gr2* mRNA levels had no significant changes **(**Fig. 5 **F)**.

3.4. The ovarian cells stimulated with cortisol and DXMS

After stimulation with cortisol or DXMS (10^{-6} mol/L), the relative expression level of interleukin 1 β (*il1b*) was downregulated, while interleukin-1 receptor type 1 (*il1r1*) was upregulated significantly **(**Fig. 6 **A, B, I, J)**. However, there was no significant difference in prostaglandin-endoperoxide synthase 2 (*ptgs2*), prostaglandin E_2 receptor 2 (*ptger2*) or matrix metallopeptidase 2/9 (*mmp2/9*) gene expression between the control and cortisol treatment groups **(**Fig. 6 **C-F)**. The expression levels of *ptgs2* and *mmp9* were downregulated by DXMS stimulation, but the latter was not significant **(**Fig. 6 **K, N)**. In addition, the expression levels of *gr1* or *gr2* were not significantly different **(**Fig. 6 **G, H, O, P)**.

4. Discussion

The existence of the two teleost GR genes provides strong evidence for T-WGD. Duplicated glucocorticoid receptors (GRs) have been found in most teleost fish (including the Asian arowana of Osteoglossiformes, a basal teleost group), while those non-T-WGD clades (including cartilaginous fish, basal ray-finned fish, lobe-finned fish and tetrapods) have one GR gene (Glasauer and Neuhauss, 2014; Pasquier et al., 2016; Weikum et al., 2018). There are exceptions; of course, zebrafish have only one *gr* gene with two alternative splicing events, GRα and GRβ (GRβ is unable to bind GCs and is thought to be a competitive inhibitor of GRα) (Alberto et al., 2020). Zebrafish GRα is clustered into teleost GR2, but the zebrafish GR1 gene is missing. In addition, rainbow trout and Atlantic salmon have two GR1 and two GR2 genes as a result of Salmoniformes-specific WGD (Sa-WGD) 50–80 MYA (Glasauer and Neuhauss, 2014; Romero et al., 2020). In this study, phylogenetic analysis revealed that black rockfish GR1/GR2 clustered into clades of teleost GR1/GR2.

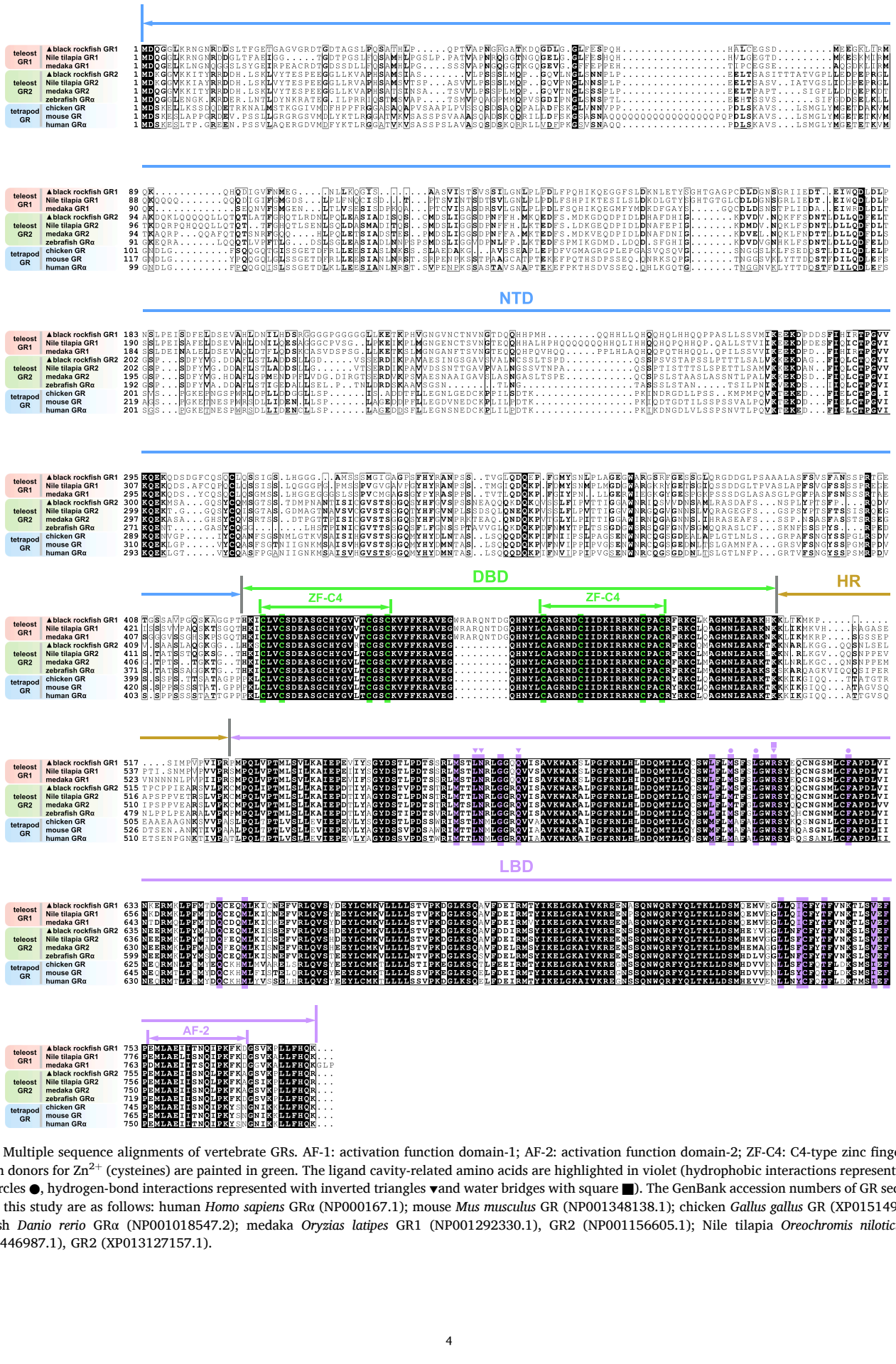

Fig. 1. Multiple sequence alignments of vertebrate GRs. AF-1: activation function domain-1; AF-2: activation function domain-2; ZF-C4: C4-type zinc finger – the electron donors for Zn^{2+} (cysteines) are painted in green. The ligand cavity-related amino acids are highlighted in violet (hydrophobic interactions represented with solid circles ●, hydrogen-bond interactions represented with inverted triangles ▼and water bridges with square ■). The GenBank accession numbers of GR sequences used in this study are as follows: human *Homo sapiens* GRα (NP000167.1); mouse *Mus musculus* GR (NP001348138.1); chicken *Gallus gallus* GR (XP015149519.1); zebrafish *Danio rerio* GRα (NP001018547.2); medaka *Oryzias latipes* GR1 (NP001292330.1), GR2 (NP001156605.1); Nile tilapia *Oreochromis niloticus* GR1 (XP003446987.1), GR2 (XP013127157.1).

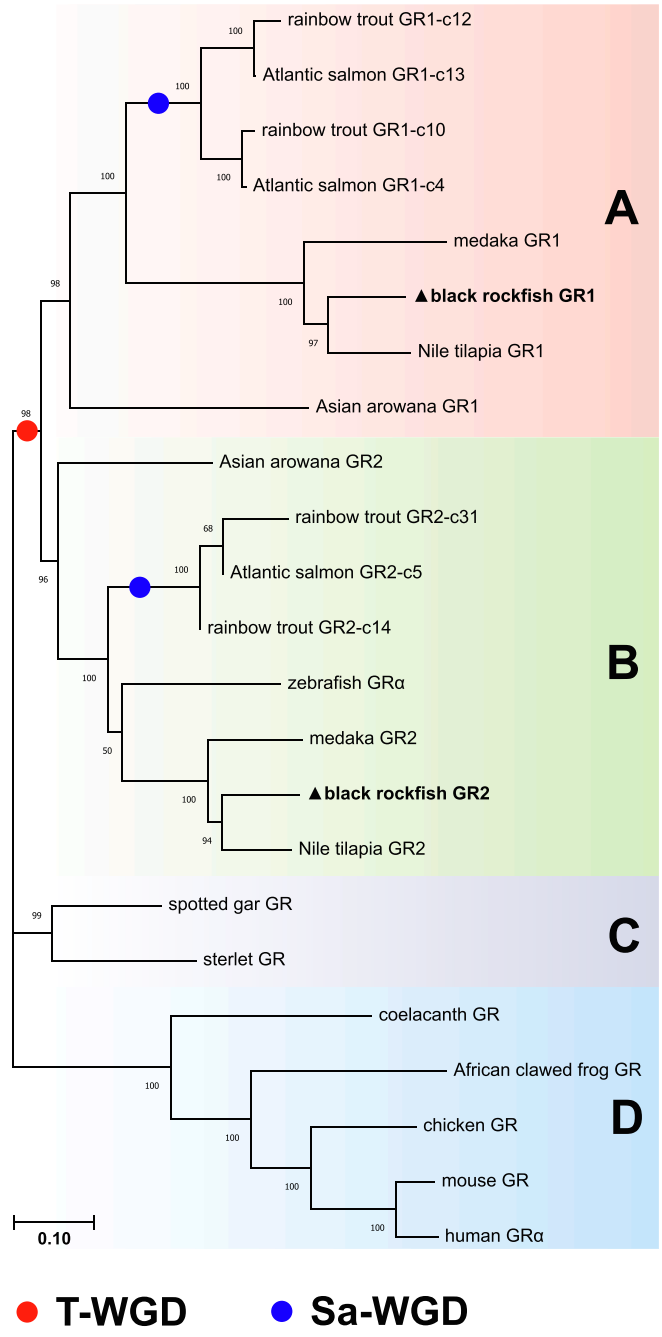

Fig. 2. Phylogenetic tree of vertebrate GRs. A: teleost GR1; B: teleost GR2; C: basal ray-finned fish GR; D: GR of lobe-finned fish and tetrapods. GR1 and GR2 of black rockfish are marked by black triangles (▲). The bar represents a sequence divergence of 0.1%. WGD: whole-genome duplication; T-WGD: teleost-specific WGD; Sa-WGD: Salmoniformes-specific WGD. The GenBank accession numbers for the sequences are African clawed frog *Xenopus laevis* GR (NP001081531.1); coelacanth *Latimeria chalumnae* GR (XP005996163.1); spotted gar *Lepisosteus oculatus* GR (XP015204944.1); sterlet *Acipenser ruthenus* GR (AFK14015.1); Asian arowana *Scleropages formosus* GR1 (XP018607685.1), GR2 (XP018611937.1); rainbow trout *Oncorhynchus mykiss* GR1-c12 (XP036793674.1), GR1-c10 (NP001118202.1), GR2-c31 (XP036826413.1), GR2-c14 (NP001117954.1); Atlantic salmon GR1-c13 (XP013992257.1), GR1-c4 (XP014053534.1), GR2-c5 (XP014054152.1). Rainbow trout and Atlantic salmon have four GR genes; each gene is named by its subtype and the chromosome on which it is located in this study, e.g., Atlantic salmon GR1-c13 means GR1 type located on chromosome 13. However, salmon GR2-c9 (XM_014213196.1) is a truncated nonfunctional protein that cannot be classified with any type of Salmoniformes GR, so only three salmon GRs are listed in this tree.

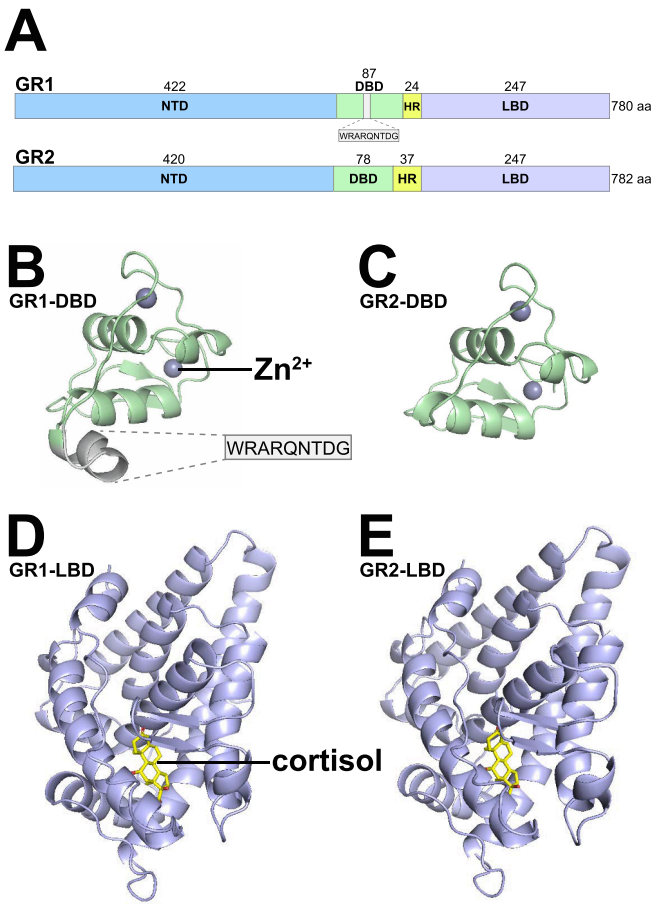

Fig. 3. Structures of black rockfish GR proteins. A: Linear structures. B, C: Predicted 3D structures of GR DBD using tetrapod GR DBD as a template (Protein Data Bank ID code: 1glu.1). D, E: Predicted 3D structures of GR LBD using tetrapod GR LBD as a template (Protein Data Bank ID code: 4p6x.5.A).

It has been reported that teleost GR1 and GR2 differ in ligand sensitivity, ligand specificity and transcriptional activity. Teleost GR2 has lower EC_{50} values (concentration for 50% of maximal effect) and greater fold induction; in other words, GR2 is more sensitive and tends to be activated at a basal ligand level; GR1 plays its role at a higher ligand level under stress conditions (Arterbery et al., 2011; Bury, 2017; Li et al., 2012). Furthermore, the 9 unique amino acids inserted in teleost GR1 DBD affect the interactions between DBD and GRE (glucocorticoid response element) of the target gene (Bury, 2017; Li et al., 2012). In brief, teleost GR1 and GR2 have distinct functional divergence after T-WGD. According to our research, the ligand cavity-related amino acids of teleost GR1 and GR2 were slightly different, which might have influenced ligand sensitivity. Black rockfish GR1/GR2 possess four modularized domains as well as the structural features of teleost GR1/GR2.

In humans, two subtypes of CYP11B have been identified, while teleosts have only one (Nebert et al., 2013; Tokarz et al., 2015). Teleost 11β-hydroxylase (teleost CYP11B or zebrafish CYP11C1) is the critical enzyme that catalyzes the following conversion: (1) 11-deoxycortisol → cortisol; (2) androgens → 11β-hydroxylated androgens (Rajakumar and Senthilkumaran, 2020; Tokarz et al., 2015). 11β-Hydroxylated androgens are precursors of 11-ketotestosterone (11-KT), an important androgen of teleosts. The highest expression level of cyp11b was detected in mature testes, indicating its role in androgen synthesis. Zebrafish *cyp11c1*$^{-/-}$ knockout results in a severe lack of serum cortisol and 11-KT (James et al., 2020; Zhang et al., 2020). In black rockfish, *cyp11b* transcripts were mainly expressed in the testis and the head

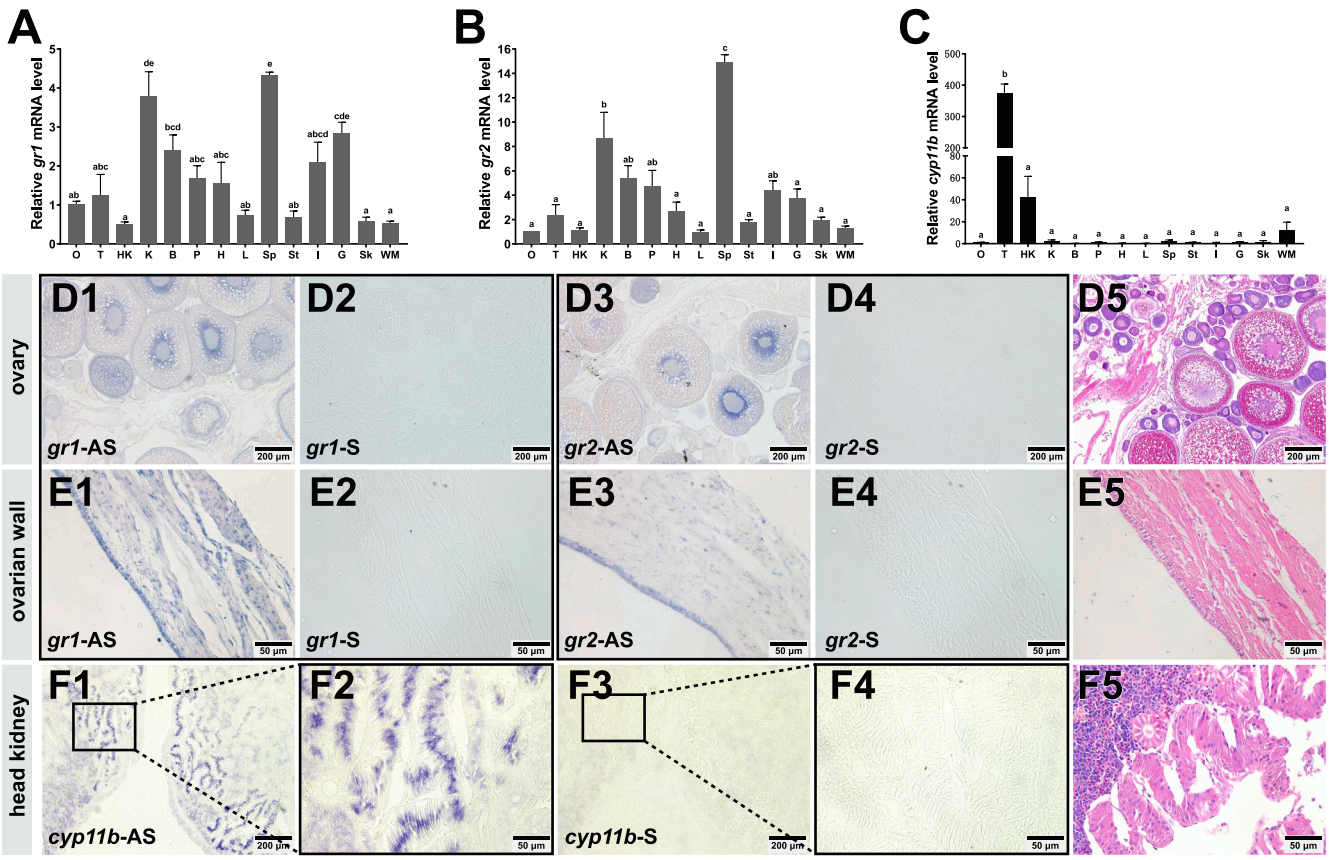

Fig. 4. mRNA tissue distribution of *gr1* (A), *gr2* (B) and *cyp11b* (C) and *in situ* hybridization staining of *gr1* (D), *gr2* (E) and *cyp11b* (F). D1-D4: Localization of *gr1* and *gr2* mRNA in ovary (at vitellogenesis phase); D5: ovary with HE staining. E1-E4: Localization of *gr1* and *gr2* mRNA in the ovarian wall; D5: ovarian wall with HE staining. F1-F4: Localization of *cyp11b* mRNA in interrenal glands of the head kidney. F5: interrenal glands with HE staining. O: ovary at the vitellogenesis stage; T: mature testis; HK: head kidney; K: kidney; B: brain; P: pituitary; H: heart; L: liver; Sp: spleen; St: stomach; I: intestine; G: gill; Sk: skin; WM: white muscle. Bars with different letters indicate significant differences ($P < 0.05$). AS: tissue section treated with antisense probes; S: section treated with sense probes as a negative control.

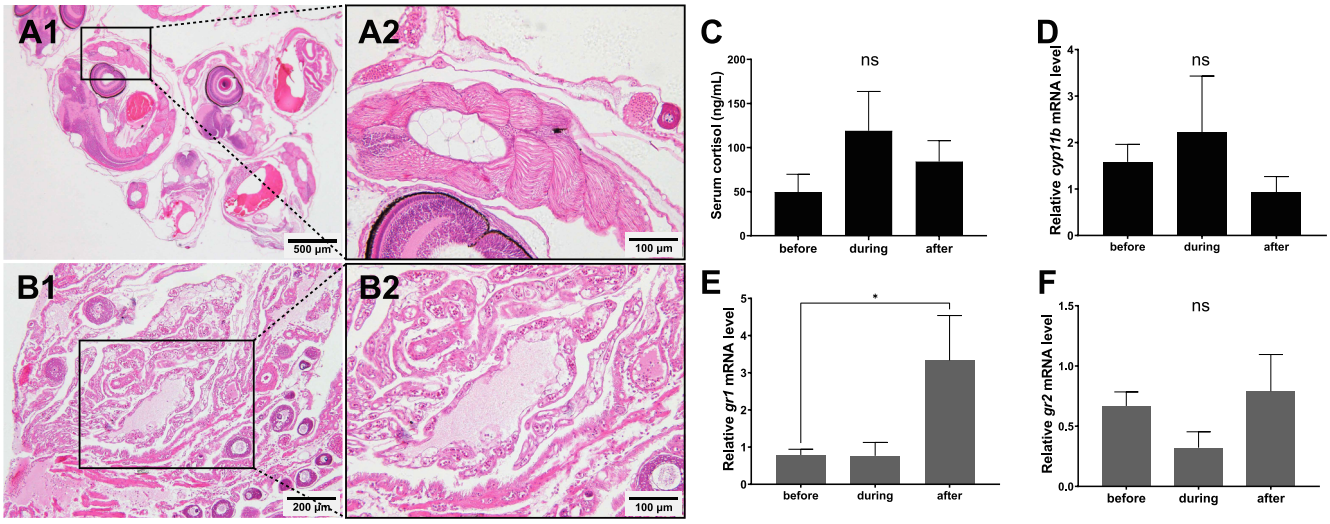

Fig. 5. Ovarian histology, serum cortisol level and gene expression patterns before/during/after the delivery process. A: Ovary at the gestation stage. B: Ovary immediately after parturition. C: Serum cortisol levels. D-E: Relative mRNA levels of *gr1*, *gr2* and *cyp11b*. Significant differences are indicated (*$P < 0.05$).

kidney. In addition, ISH revealed that *cyp11b* transcripts were localized in the interrenal glands of the head kidney, where CYP11A (cholesterol side-chain cleavage enzyme) proteins were located according to our previous research (Wang et al., 2021). These results indicate that interrenal glands are likely to be homologous to the mammalian adrenal cortex and a major source of serum cortisol in black rockfish. In humans, local cortisol synthesis can be detected in injured tissue, which acts as negative feedback to prevent excessive inflammation (Vukelic et al., 2011). In this study, neither serum cortisol nor ovarian local *cyp11b* expression levels changed significantly during parturition. The expression level of black rockfish *gr1* transcripts was upregulated significantly after parturition, which might indicate the unique functions of GR1

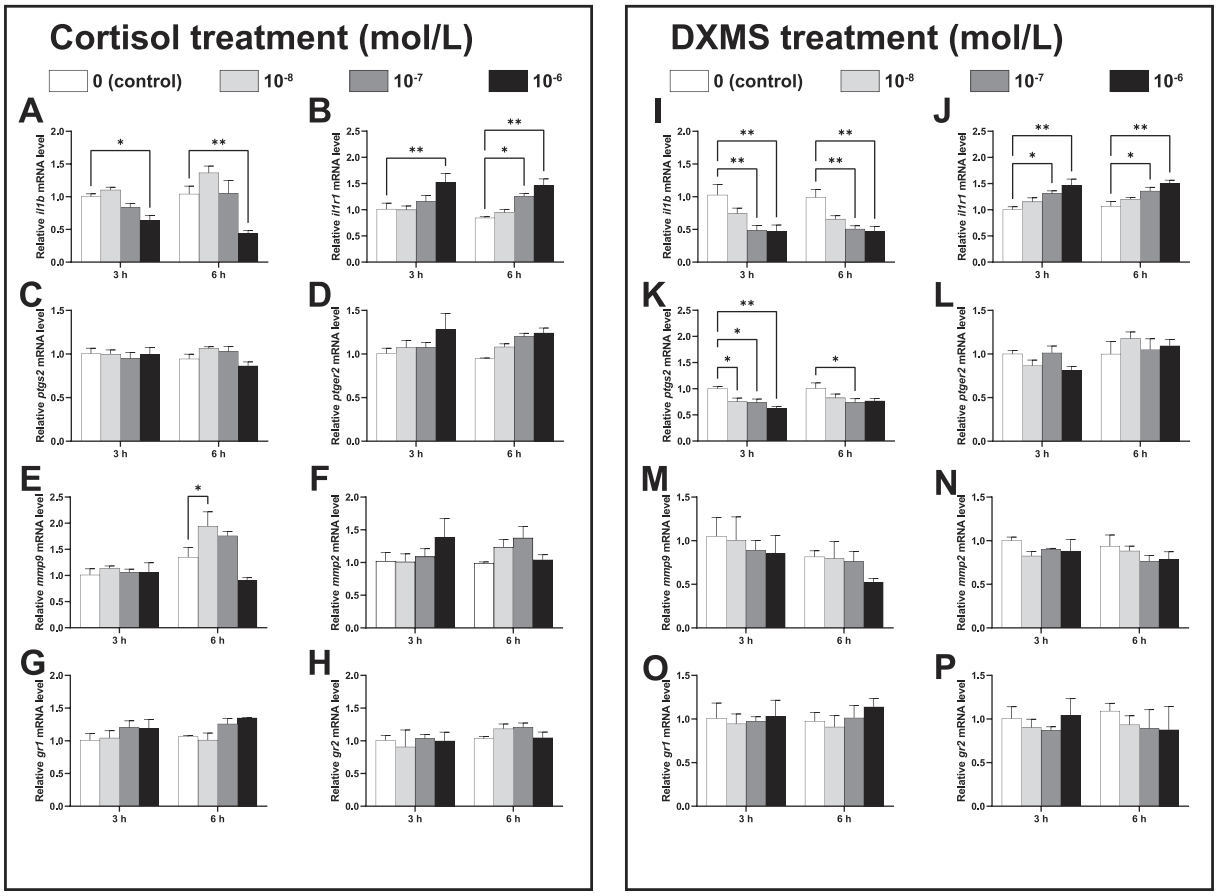

Fig. 6. Effects of cortisol/DXMS stimulation on the gene expression of ovarian primary cells. A-F: Relative mRNA levels of *il1b*, *il1r1*, *cox2*, *ptger2*, *mmp2* and *mmp9* after cortisol stimulation. G-L: Relative mRNA expression levels of *il1b*, *il1r1*, *cox2*, *ptger2*, *mmp2* and *mmp9* after DXMS stimulation. Significant differences between the control and treatment groups are indicated (*P < 0.05, **P < 0.01).

during the delivery process.

In humans, it is well recognized that many physiological reproductive events, such as ovulation, menstruation, implantation and onset of parturition, are accompanied by a pronounced inflammatory response (Jabbour et al., 2009; Reinl and England, 2015). In addition to mammals, proinflammatory cytokines (e.g., IL-1α and IL-1β) are identified at the fetomaternal interface of viviparous lizards and shark near parturition (Cateni et al., 2003; Paulesu et al., 2005). Nevertheless, the inflammatory response of viviparous teleosts at parturition has not yet been reported. Based on our research, fetuses of black rockfish continue to develop in ovaries after fertilization. The fetomaternal interface is formed by a layer of connective tissue originating from the follicle layer. Moreover, significant blood vessel dilation was observed in the connective tissue layers of ovaries immediately after parturition, which was considered to be a typical feature of the inflammatory response (Kvietys and Granger, 2012).

As an anti-inflammatory hormone, cortisol plays a role in negative feedback regulation and downregulates the expression of proinflammatory factors, such as interleukin-1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α), limiting inflammation to avoid toxicity and tissue damage (Castro et al., 2011; Su et al., 2020). These processes have been verified by this research. After *in vitro* cortisol or DXMS treatment, *il1b* expression in ovarian cells was downregulated significantly, indicating the anti-inflammatory effects of GCs; in contrast, the levels of *il1r1* transcripts were upregulated significantly after GC stimulation. The activity of IL-1β is mediated by its membrane receptors, the complex of IL1R1 and IL1RAP (interleukin-1 receptor accessory protein) (Boraschi and Tagliabue, 2013). In addition to the membrane form, a soluble form of IL1R1 (sIL1R1) has been identified, which is capable of binding IL-1 competitively and blocking the activity of ligands (Francis et al., 2001; Klasing and Peng, 2001). In black rockfish, GCs can upregulate the expression of IL1R1, and sIL1R1 might be generated by extracellular matrix (ECM) hydrolysis in parturition.

In human parturition, the preweakening and rupture of fetal membranes occur due to reduced collagen content and ECM remodeling (Babwah and Bhattacharya, 2017). Matrix metalloproteinases (MMPs) are a large family of calcium-dependent zinc-containing endopeptidases that play a role in ECM remodeling (Verma and Hansch, 2007). GRs downregulate MMPs indirectly by inhibiting the expression of IL-1β and IL-8 (Marinello et al., 2020). In addition, IL-1β stimulates prostaglandin (PG) production via transcriptional activation of PTGS2 (also known as cyclooxygenase-2/COX-2) (Newton et al., 1997). Prostaglandin E_2 (PGE_2) plays an important role in human parturition by softening the cervix and causing uterine contraction (Li et al., 2021). Intraperitoneal administration of prostaglandin $F_{2\alpha}$ ($PG_{2\alpha}$) significantly induced premature parturition of guppies (Lyu et al., 2021). According to ovarian cell stimulations in this study, the relative expression levels of *ptgs2*, *ptger2* and *mmp2/9* were not significant after cortisol stimulation; for DXMS treatment, only the expression levels of *ptgs2* and *mmp9* were inhibited, and with less significance. As a result, we speculate that GCs may play a role in black rockfish parturition by inhibiting the functions of proinflammatory factors (e.g., IL-1β) instead of directly regulating the functions of PG or MMP2/9. Moreover, cortisol or DXMS could not regulate the transcription of *gr1* or *gr2* directly.

Except for mammals, some other vertebrates, including cartilaginous fishes, ray-finned fish, lobe-finned fishes, amphibians and reptiles, contain viviparous species. For viviparous vertebrates, gestation is thought to be an immune tolerance process, and parturition is a result of

the inflammatory response (Hansen et al., 2017). In human cases, infection or inflammation are well-defined risk factors for preterm birth (PTB) (Cappelletti et al., 2016). DXMS treatment is used to prevent PTB and to accelerate fetal lung maturation (Roberts et al., 2017). The relationship among inflammation, GCs and parturition in viviparous teleosts is still not clear, even though various groups of viviparous teleosts have been identified to date. Our study provides evidence of the potential role of GRs in viviparous teleosts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the National Key R&D Program of China (2018YFD0901204) and the National Natural Science Foundation of China (41976089).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2021.113947.

References

Alberto, D., Giorgio, L., Camilla Maria, F., Natascia, T., Francesco, A., Luisa Dalla, V., 2020. Glucocorticoid receptor activities in the zebrafish model: a review. J. Endocrinol. 247 (3), R63–R82.

Alsop, D., Vijayan, M.M., 2009. Molecular programming of the corticosteroid stress axis during zebrafish development. Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 153 (1), 49–54.

Arterbery, A.S., Fergus, D.J., Fogarty, E.A., Mayberry, J., Deitcher, D.L., Lee Kraus, W., Bass, A.H., 2011. Evolution of ligand specificity in vertebrate corticosteroid receptors. BMC Evol. Biol. 11 (1), 14.

Babwah, A.V., Bhattacharya, M., 2017. A Birth Story: Cortisol-Stimulated Autophagy in Parturition. Endocrinology 158 (4), 693–695.

Boraschi, D., Tagliabue, A., 2013. The interleukin-1 receptor family. Semin. Immunol. 25 (6), 394–407.

Bury, N.R., 2017. The evolution, structure and function of the ray finned fish (Actinopterygii) glucocorticoid receptors. Gen. Comp. Endocrinol. 251, 4–11.

Cappelletti, M., Della Bella, S., Ferrazzi, E., Mavilio, D., Divanovic, S., 2016. Inflammation and preterm birth. J. Leukoc. Biol. 99 (1), 67–78.

Castro, R., Zou, J., Secombes, C.J., Martin, S.A.M., 2011. Cortisol modulates the induction of inflammatory gene expression in a rainbow trout macrophage cell line. Fish Shellfish Immunol. 30 (1), 215–223.

Cateni, C., Paulesu, L., Bigliardi, E., Hamlett, W.C., 2003. The interleukin 1 (IL-1) system in the uteroplacental complex of a cartilaginous fish, the smoothhound shark, *Mustelus canis*. Reprod. Biol. Endocrinol. 1 (1), 25.

Francis, J., MohanKumar, P.S., MohanKumar, S.M.J., 2001. Lipopolysaccharide stimulates norepinephrine efflux from the rat hypothalamus in vitro: blockade by soluble IL-1 receptor. Neurosci. Lett. 308 (2), 71–74.

Glasauer, S.M.K., Neuhauss, S.C.F., 2014. Whole-genome duplication in teleost fishes and its evolutionary consequences. Mol. Genet. Genomics 289 (6), 1045–1060.

Hansen, V.L., Faber, L.S., Salehpoor, A.A., Miller, R.D., 2017. A pronounced uterine pro-inflammatory response at parturition is an ancient feature in mammals. Proc. Royal Soc. B: Biol. Sci. 284 (1865), 20171694. https://doi.org/10.1098/rspb.2017.1694.

He, Y., Chang, Y., Bao, L., Yu, M., Li, R., Niu, J., Fan, G., Song, W., Seim, I., Qin, Y., Li, X., Liu, J., Kong, X., Peng, M., Sun, M., Wang, M., Qu, J., Wang, X., Liu, X., Wu, X., Zhao, X.i., Wang, X., Zhang, Y., Guo, J., Liu, Y., Liu, K., Wang, Y., Zhang, H.e., Liu, L., Wang, M., Yu, H., Wang, X., Cheng, J., Wang, Z., Xu, X., Wang, J., Yang, H., Lee, S.-Y., Liu, X., Zhang, Q., Qi, J., 2019. A chromosome-level genome of black rockfish, *Sebastes schlegelii*, provides insights into the evolution of live birth. Mol. Ecol. Resour. 19 (5), 1309–1321.

Jabbour, H.N., Sales, K.J., Catalano, R.D., Norman, J.E., 2009. Inflammatory pathways in female reproductive health and disease. Reproduction 138 (6), 903–919.

James, A.O., Lise, B., Karl-Heinz, S., Vincent, T.C., Nils, P.K., 2020. 11β-Hydroxylase loss disrupts steroidogenesis and reproductive function in zebrafish. J. Endocrinol. 247 (2), 197–212.

Klasing, K.C., Peng, R.K., 2001. Soluble type-I interleukin-1 receptor blocks chicken IL-1 activity. Dev. Comp. Immunol. 25 (4), 345–352.

Kvietys, P.R., Granger, D.N., 2012. Role of reactive oxygen and nitrogen species in the vascular responses to inflammation. Free Radical Biol. Med. 52 (3), 556–592.

Li, W.-J., Lu, J.-W., Zhang, C.-Y., Wang, W.-S., Ying, H., Myatt, L., Sun, K., 2021. PGE2 vs PGF2α in human parturition. Placenta 104, 208–219.

Li, Y., Sturm, A., Cunningham, P., Bury, N.R., 2012. Evidence for a divergence in function between two glucocorticoid receptors from a basal teleost. BMC Evol. Biol. 12 (1), 137.

Liman, M.a., Wenji, W., Conghui, L., Haiyang, Y.u., Zhigang, W., Xubo, W., Jie, Q.i., Quanqi, Z., 2013. Selection of reference genes for reverse transcription quantitative real-time PCR normalization in black rockfish (*Sebastes schlegeli*). Mar. Geonomics 11, 67–73.

Lyu, L.K., Li, J.S., Wang, X.J., Yao, Y.J., Li, J.F., Li, Y., Wen, H.S., Qi, X., 2021. Arg-Vasotocin Directly Activates Isotocin Receptors and Induces COX2 Expression in Ovoviviparous Guppies. Front. Endocrinol. 12 (347).

Marinello, W., Feng, L., Allen, T.K., 2020. Progestins Inhibit Interleukin-1β-Induced Matrix Metalloproteinase 1 and Interleukin 8 Expression via the Glucocorticoid Receptor in Primary Human Amnion Mesenchymal Cells. Front. Physiol. 11, 900.

Milla, S., Wang, N., Mandiki, S.N.M., Kestemont, P., 2009. Corticosteroids: Friends or foes of teleost fish reproduction? Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 153 (3), 242–251.

Mori, H., Nakagawa, M., Soyano, K., Koya, Y., 2003. Annual reproductive cycle of black rockfish Sebastes schlegeli in captivity. Fish. Sci. 69 (5), 910–923.

Nebert, D.W., Wikvall, K., Miller, W.L., 2013. Human cytochromes P450 in health and disease. Philosophical Transactions of the Royal Society B: Biological Sciences 368 (1612), 20120431. https://doi.org/10.1098/rstb.2012.0431.

Newton, R., Kuitert, L.M.E., Bergmann, M., Adcock, I.M., Barnes, P.J., 1997. Evidence for Involvement of NF-κB in the Transcriptional Control of COX-2 Gene Expression by IL-1β. Biochem. Biophys. Res. Commun. 237 (1), 28–32.

Pasquier, J., Cabau, C., Nguyen, T., Jouanno, E., Severac, D., Braasch, I., Journot, L., Pontarotti, P., Klopp, C., Postlethwait, J.H., Guiguen, Y., Bobe, J., 2016. Gene evolution and gene expression after whole genome duplication in fish: the PhyloFish database. BMC Genomics 17 (1), 368.

Paulesu, L., Bigliardi, E., Paccagnini, E., Ietta, F., Cateni, C., Guillaume, C.P., Heulin, B., 2005. Cytokines in the oviparity/viviparity transition: evidence of the interleukin-1 system in a species with reproductive bimodality, the lizard *Lacerta vivipar*a. Evol. Dev. 7 (4), 282–288.

Rajakumar, A., Senthilkumaran, B., 2020. Steroidogenesis and its regulation in teleost-a review. Fish Physiol. Biochem. 46 (3), 803–818.

Reinl, E.L., England, S.K., 2015. Fetal-to-maternal signaling to initiate parturition. J. Clin. Investig. 125 (7), 2569–2571.

Roberts, D., Brown, J., Medley, N., Dalziel, S.R., 2017. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. Cochrane Database Syst. Rev. 3.

Romero, A., Vega, M., Santibáñez, N., Spies, J., Pérez, T., Enríquez, R., Kausel, G., Oliver, C., Oyarzún, R., Tort, L., Vargas-Chacoff, L., 2020. *Salmo salar* glucocorticoid receptors analyses of alternative splicing variants under stress conditions. Gen. Comp. Endocrinol. 293, 113466. https://doi.org/10.1016/j.ygcen.2020.113466.

Sanders, K., Mol, J.A., Kooistra, H.S., Slob, A., Galac, S., 2016. New Insights in the Functional Zonation of the Canine Adrenal Cortex. J. Vet. Intern. Med. 30 (3), 741–750.

Schiffer, L., Anderko, S., Hannemann, F., Eiden-Plach, A., Bernhardt, R., 2015. The CYP11B subfamily. J. Steroid Biochem. Mol. Biol. 151, 38–51.

Su, M., Zhang, R., Liu, N., Zhang, J., 2020. Modulation of inflammatory response by cortisol in the kidney of spotted scat (*Scatophagus argus*) in vitro under different osmotic stresses. Fish Shellfish Immunol. 104, 46–54.

Thornton, J.W., 2001. Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. Proc. Natl. Acad. Sci. 98 (10), 5671–5676.

Thornton, J.W., Need, E., Crews, D., 2003. Resurrecting the Ancestral Steroid Receptor: Ancient Origin of Estrogen Signaling. Science 301 (5640), 1714–1717.

Tokarz, J., Möller, G., Hrabě de Angelis, M., Adamski, J., 2015. Steroids in teleost fishes: A functional point of view. Steroids 103, 123–144.

Verma, R.P., Hansch, C., 2007. Matrix metalloproteinases (MMPs): Chemical–biological functions and (Q)SARs. Bioorg. Med. Chem. 15 (6), 2223–2268.

Vukelic, S., Stojadinovic, O., Pastar, I., Rabach, M., Krzyzanowska, A., Lebrun, E., Davis, S.C., Resnik, S., Brem, H., Tomic-Canic, M., 2011. Cortisol synthesis in epidermis is induced by IL-1 and tissue injury. J. Biol. Chem. 286 (12), 10265–10275.

Wang, W., Guo, C., Sun, K., 2020. Cortisol Regeneration in the Fetal Membranes, A Coincidental or Requisite Event in Human Parturition? Front. Physiol. 11, 462.

Wang, X., Wen, H., Li, Y., Lyu, L., Song, M., Zhang, Y., Li, J., Yao, Y., Li, J., Qi, X., 2021. Characterization of CYP11A1 and its potential role in sex asynchronous gonadal development of viviparous black rockfish *Sebastes schlegelii* (Sebastidae). Gen. Comp. Endocrinol. 302, 113689.

Weikum, E.R., Liu, X., Ortlund, E.A., 2018. The nuclear receptor superfamily: A structural perspective. Protein Sci. 27 (11), 1876–1892.

Zhang, Q., Ye, D., Wang, H., Wang, Y., Hu, W., Sun, Y., 2020. Zebrafish cyp11c1 Knockout Reveals the Roles of 11-ketotestosterone and Cortisol in Sexual Development and Reproduction. Endocrinology 161 (6).