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Research Article

Cyclooxygenases of ovoviviparous black rockfish (*Sebastes schlegelii*): Cloning, tissue distribution and potential role in mating and parturition

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ABSTRACT

Prostaglandins are a series of unsaturated fatty acids that play critical roles in regulating reproductive events. The prostaglandins endoperoxide H synthases-1/2 (PGHS-1/2; also named cyclooxygenases-1/2, COX-1/2) catalyse the commitment step in prostaglandin synthesis. However, the of the *cox* genes in teleosts, especially ovoviviparous teleosts, is still unclear. The aim of the present study was to determine the potential role of *cox* genes in mating and parturition behaviour using black rockfish (*Sebastes schlegelii*) as a model species. Two transcripts, *cox1* and *cox2*, were cloned. The phylogenetic analysis results revealed that both *cox* genes were closely related to mammalian *coxs*. qPCR analyses of their tissue distribution showed that *cox1* was mainly expressed in the heart in both sexes, while *cox2* was mainly expressed in the testis and ovary. Detection of *cox* expression in samples from reproductive-related stages further showed that both *cox* genes may play important roles in mating and parturition processes. *In situ* hybridization further detected positive *cox* mRNA signals in the testis and ovary, where they are known to be involved in mating and parturition behaviour. These data suggest that *cox1* and *cox2* are crucial in inducing mating, gonad regeneration and parturition behaviour.

1. Introduction

Prostaglandins (PGs) are a group of lipid mediators generated in response to diverse stimuli, and they play important roles in normal physiological events. They oxygenate eicosanoids through the cyclooxygenase (COX), lipoxygenase (LOX) or P-450 epoxygenase pathways (Smith et al., 2000). PGs, which play roles in various processes of female reproduction including ovulation, fertilization, luteolysis and parturition, are synthesized through the COX pathway (Sugimoto et al., 2015). COX, also known as prostaglandin H2 synthase (PGHS), which was first purified in 1976 and then cloned in 1988 (DeWitt and Smith, 1988), is the key enzyme in the first two steps of prostaglandin biosynthesis from the substrate arachidonic acid (AA). Furthermore, a product functionally similar to COX was identified and named COX2 (Hla et al., 1999; Marnett et al., 1999; Smith and Dewitt, 1996; Smith et al., 1996).

Both isoforms of COX are identical in structure but show differences in intracellular location as well as substrate or inhibitor selectivity (Vane et al., 1998). COX1 is a constitutively active enzyme with various tissue distributions in most organs in the body and generally controls basal prostanoids with physiological functions including gastric cytoprotection, inflammation, platelet aggregation (Morita, 2002; Teeling et al., 2010). While COX2 is unexpressed in most tissues but inducible in response to some factors including proinflammatory factors such as cytokines (Di Costanzo et al., 2019). It was generally believed that the synthesis of arachidonic acid was the rate-limiting step of prostaglandin synthesis in early studies of prostaglandin synthesis (Herschman and Hall, 1994; Miyamoto et al., 2000). However, subsequent studies of COX2 indicated that cyclooxygenase is the rate-limiting enzyme that catalyses the synthesis of prostaglandins because the expression of COX2 is regulated by a series of factors including luteinizing hormone, interleukin-1β and tumor necrosis factor α (Chandrasekharan and Simmons, 2004; Feng et al., 1995; Liu et al., 2003; Sirois et al., 2004; Wolff et al., 1998; Zhou et al., 1999).

Sexual selection by females is believed to play a crucial role in the evolution of male sexual traits (displays and ornaments) and the maintenance of reproductive isolation among closely related species(Andersson, 1994; Kraaijeveld et al., 2011). In the process of mate selection, females evaluate potential mates based on male courtship displays, which is a means for potential mates to demonstrate their qualities (Byers et al., 2010; Candolin, 2003). Prostaglandins have been proven to

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play a crucial role in sexual behaviour in many animals, from insects to mammals, such as cricket (*Gryllus texensis*) (Worthington et al., 2015), zebrafish (*Danio rerio*) (Yabuki et al., 2016), African cichlid (*Astatotilapia burtoni*) (Juntti et al., 2016), snake (*Thamnophis sirtalis*) (Whitter and Crews, 1986), ferret (*Mustela putorius fur*) (Villars et al., 1990), boar (Estienne, 2014) and rat (Rodriguez-Sierra and Komisaruk, 1977). In recent years, an increasing number of studies have focused on the function of PGs in mating behaviour in oviparous teleosts. Previous studies have shown that the regulatory mechanisms of female sexual behaviours are responsive to either sex steroids or the fatty acid derivative prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Forlano and Bass, 2011; Kobayashi et al., 2002; Stacey, 2003). After that, it was proven that $PGF_{2\alpha}$ mediates male courtship behaviour *via* a specific olfactory receptor in zebrafish (Yabuki et al., 2016). Similar behavioural responses triggered by $PGF_{2\alpha}$ in both males and females have also been found in African cichlid (Juntti et al., 2016), lake trout (*Salvelinus namaycush*) (Buchinger et al., 2020) and goldfish (*Carassius auratus*) (Sorensen et al., 2018).

In addition to mating behaviour, PGs can also induce ovulation and spawning behaviour in teleost species. In the 1970s, studies in goldfish and rainbow trout (*Oncorhynchus mykiss*) found that $PGF_{2\alpha}$ can induce ovulation (Jalabert and Szöllösi, 1975; Stacey and Pandey, 1975). Subsequently, PGs have been shown to stimulate ovulation and spawning behaviour in various teleosts, including brown trout (*Salmo trutta*) (Crespo et al., 2015), Asian stinging catfish (*Heteropneustes fossilis*) (Joy and Singh, 2013), medaka (*Oryzias latipes*) (Hagiwara et al., 2014) and zebrafish (Knight and Van Der Kraak, 2015). Exogenous prostaglandin E_2 (PGE_2) or $PGF_{2\alpha}$ can trigger ovulation in mature oocytes of European seabass (*Dicentrarchus labrax*) (Ann Sorbera et al., 2001). Inhibition of PGs with indomethacin can suppress ovulation in Atlantic croaker (*Micropogonias undulatus*) and zebrafish (Lister and Van Der Kraak, 2008; Patiño et al., 2003).Furthermore, PGs also have a significant effect on parturition in mammals. Increasing evidence supports the idea that prostaglandins trigger foetal membrane rupture by stimulating matrix metalloproteinase (MMP) activity (McLaren et al., 2000), leading to extracellular matrix protein remodeling and the induction of cell apoptosis (Keelan et al., 2001). At the onset of labour, PGE_2 and $PGF_{2\alpha}$, which have been confirmed as inducers of spontaneous uterine contractility, are increased significantly in the principal site of the amnion (Crankshaw and Dyal, 1994; Olson et al., 1983). However, in ovoviviparous teleosts, which are rarely used as research models, research on PG function in parturition is still lacking. In the 1990s, a study of guppies showed that PGE and $PGF_{2\alpha}$ levels increased in late gestation, and cortisol injection suppressed parturition by inhibiting PG synthesis (Venkatesh et al., 1992a). Administration of PGE_2 and $PGF_{2\alpha}$ to pregnant guppies during late gestation induced premature parturition (Venkatesh et al., 1992b).

Black rockfish (*Sebastes schlegelii*) exhibits an ovoviviparous reproductive pattern and long-term sperm storage(Gao et al., 2018). In black rockfish, the process of spermatogenesis starts in late July and the mature sperms were found in December or January with individual differences, when mating also occurs through its modified urogenital papillae, which emits spermatozoa into the female ovary. The sperm are stored in the ovary cavity during the vitellogenesis period (Mori et al., 2003; Wang et al., 2021). In females, oocytes start vitellogenesis in November and mature in late March. After the activation of the sperm and fertilization in April, the females become pregnant and the fertilized eggs develop into larvae in the ovary, until parturition occurs in May (Mori et al., 2003; Wang et al., 2021).

These previous studies have focused on COXs functions in mating, ovulation, and spawning behaviour in oviparous teleosts, however, little is known about in ovoviviparous teleosts. To provide more endocrinology information about ovoviviparous teleost reproductive physiology, the present study first characterized COX (*cox1* and *cox2*) genes in black rockfish, followed by the expression pattern and mRNA localization in testis and ovary. This is the first to identify and characterize *coxs* in ovoviviparous black rockfish. Our results will provide novel information for the understanding of reproduction aspect in ovoviviparous teleost.

2. Materials and methods

2.1. Animals and ethics statement

Black rockfish (1.0 ± 0.2 kg) were obtained from marine cages located offshore of Penglai (37.6°N, 120.8°E) in the northern Yellow Sea, Shandong Province, China. All animal experiments in this research were approved by the Animal Research and Ethics Committees of Ocean University of China prior to the initiation of the study. The studies did not involve endangered or protected species. All experiments were performed in accordance with the relevant guidelines and regulations.

In total, 3 female and 3 male individuals were sampled in September 2019 and June 2020 for tissue distribution analysis. According to the reproductive cycle of black rockfish (Mori et al., 2003; Wang et al., 2021), 3 testis were sampled in each stage including the spermatogenesis stage (ES, early September 2019), mature stage (M, December 2019) and regressed stage (R. January 2020). Similarly, 3 ovaries were sampled in each stage including the previtellogenesis stage (Pv, October 2019), vitellogenesis stage (V, December 2019) and mature stage (M, March 2020). Nine female individuals were sampled (including ovarian substrate, ovary wall and cloaca), before parturition, during and 24 h after parturition. The ovarian substrate includes oocyte and other ovarian somatic cell except the muscle in ovary wall. And the ovary wall indicates the surrounding muscle layers separated from the whole ovary.

2.2. RNA extraction and cDNA preparation

Total RNA was extracted from different tissues (heart, liver, spleen, stomach, kidney, head kidney, intestine, gill, muscle, ovary, and testis) in December and gonads in different developmental stages in black rockfish using TRIzol reagent (Invitrogen, USA). The quality and quantity of the RNA were estimated by agarose gel electrophoresis and a biophotometer (OSTC, China), respectively. Total RNA was reverse-transcribed into complementary DNA (cDNA) using 1 μg of DNA per 20 μL reaction *via* the Prime ScriptTM RT reagent kit (TaKaRa, Japan) according to the manufacturer's instructions.

2.3. Sequence analysis of cox1 and cox2

According to the genome (CNA0000824) and transcriptome data (PRJNA573572), the open reading frames (ORFs) of *cox1* and *cox2* in black rockfish were predicted, and the cloning primers of both genes were designed by Primer 5 software (Premier Biosoft International) (Table 1). The PCR protocol was performed as previously reported (Zhang et al., 2019) in a study using cDNA samples from ovaries. Briefly, initial denaturation was performed at 94 °C for 3 min followed by 35 cycles at 94 °C for 30 s, 55–60 °C for 30 s and 72 °C for 1 min. The reaction was terminated with an extension for 5 min at 72 °C. The products were purified using a TIANgel Midi purification kit (TIANGEN, Beijing, China), subcloned into the pEASY-T1 cloning vector (TransGen Biotech, Beijing, China) and transformed into DH5α cells. Positive clones were selected for subsequent sequencing. The 3D structures were predicted by Swiss-Model (http://swissmodel.expasy.org/). Multiple sequences were aligned by Clustal X software, and a phylogenetic tree was constructed by MEGA 6 software using the neighbour-joining method with 1000 bootstrapping replicates. The signal peptides of the COXs were predicted using Signal P 4.1 software.

2.4. Expression patterns in tissues and reproductive periods

The mRNA levels of both *cox1* and *cox2* in different tissues and the gonadal development stage were evaluated by quantitative real-time PCR (qPCR), which was performed in triplicate as previously

Table 1
Primers sequences used for ORF cloning, ISH and qPCR.

Primers	Sequence (5′-3′)
Primers for ORFs clone	
cox1-orf-F	AGGCTGCTGTAATGAGATCCTC
cox1-orf-R	TCACAGCTCATCAGTCCTTACT
cox2-orf-F	AGTCTTTGGAGTATGAACAG
cox2-orf-R	TTAGAACTCAGTAGTCCTTTCT
Primers for ISH prober preparation	
cox1-ish-F	CGCATTTAGGTGACACTATAGAAGCGTCCTCTGTCCTAGGATCAGT
cox1-ish-R	CCGTAATACGACTCACTATAGGGAGACATCCCGTAGGAAGGTGTTGTT
cox2-ish-F	CGCATTTAGGTGACACTATAGAAGCGAGTGCTGGAGGAGTTCTATG
cox2-ish-R	CCGTAATACGACTCACTATAGGGAGACAGACTGTGGGGTTGACATCAT
Primer for qPCR	
cox1-F	TTCTTCGCACAGCACTTC
cox1-R	TCTCGCCGTCAACTAACT
cox2-F	CCAGGGAACAGATGATTACG
cox2-R	CTTGAAGTGGGTGAGCAG
18s-F	CCTGAGAAACGGCTACCACAT
18s-R	CCAATTACAGGGCCTCGAAAG

described. qPCR was performed on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, USA) using a SYBR Green I Kit (TaKaRa, Japan) according to the manufacturer's instructions. The expression of black rockfish *cox1* and *cox2* was detected by primers that generated a specific fragment (Table 1). After initial denaturation at 95 °C for 30 s, each template was amplified with 40 cycles of denaturation for 5 s at 95 °C and annealing for 30 s at 60 °C. The expression level of the target gene was calculated with the $2^{-\Delta\Delta ct}$ method. The *cox1* and *cox2* transcript levels were normalized against the 18S rRNA transcript levels.

2.5. In situ hybridization (ISH)

ISH was performed as previously reported (Zhang et al., 2019). The gonads of black rockfish ($n = 6$, 3 males and 3 females) were collected and fixed in buffered 4% paraformaldehyde over 24 h and then embedded in paraffin. Seven-micrometre sections were cut, placed onto aminopropylsilane-treated glass slides, and dried at 37 °C. Sense and antisense digoxigenin (DIG)-labelled riboprobes were synthesized according to the *cox1* and *cox2* genes using a DIG RNA Labelling Kit (Roche Diagnostics, Mannheim, Germany). The sections were dewaxed in xylene and rehydrated by a graded series of ethanol solutions (100%–70%), permeabilized with 0.1 M HCl for 10 min followed by proteinase K (10 μg/mL) digestion for 2 min, prehybridized at 42 °C for 1 h, and then hybridized with DIG-labelled riboprobes diluted in hybridization buffer at 58 °C overnight in a wet box. After hybridization, the sections were washed in a graded series of saline sodium citrate (SSC) and then with PBS, followed by blocking reagent (Roche Diagnostics, Germany). DIG was detected with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics; diluted 1:400), and chromogenic development was conducted with an NBT/BCIP stock solution (Roche Diagnostics).

2.6. Haematoxylin-eosin (H&E) staining

The 4% paraformaldehyde-fixed tissues were cut into seven-micrometre sections by a microtome (Leica, Wetzler, Germany) and stained with haematoxylin-eosin. The magnified photographs of the sections were taken by an Olympus bright field light microscope (Olympus, Tokyo, Japan).

2.7. Statistical analysis

All data are expressed as the mean ± S.E.M. Data analyses were performed by one-way ANOVA followed by LSD and the Dunnett T3 multiple range test, and significance was considered at $P < 0.05$. Statistical analysis method was chosen according to the previous reports (Björnsson et al., 2018; Davis et al., 2010; Du Toit et al., 2018). All statistical processes were performed using SPSS 19.0 software (SPSS, Chicago, IL, USA).

3. Results

3.1. Gene cloning and in silico sequence analysis of COX1 and COX2 of black rockfish

Cox1 and *cox2* were identified through genomic data (CNA0000824) and RNA-seq data (PRJNA573572) mining. The gene cloning results showed that the ORFs of *cox1* (accession number: MT862757) and *cox2* (accession number: MT862758) were 1803 bp and 1827 bp, coding for a 600 -amino acid (AA) peptide and a 608-amino acid (AA) peptide, respectively (Supplementary 1). Sequence comparison analysis and conserved domain prediction of both COX1 and COX2 among teleosts, mammals and avians showed one signal peptide and two highly conserved domains including an epidermal growth factor-like domain (EGF-like domain) and a prostaglandin endoperoxide synthase domain (Fig. 1A, B). *In silico* protein structural prediction results showed that both COX1 (Fig. 2A) and COX2 homodimers (Fig. 2B) were highly conserved (Fig. 2C). In COX1, Ile^{432} and Ile^{521} were replaced with Val^{433} and Val^{512} in COX2, respectively, and Ile^{521} in COX1 and Val^{512} in COX2 were superimposed completely (Fig. 2D). Phylogenetic analyses (Fig. 3) revealed that black rockfish was highly conserved with other vertebrates and clustered into two clades: COX1 and COX2. In general, *cox* genes were relatively conserved during evolution.

3.2. Expression profiles of cox1 and cox2 in different tissues of black rockfish

Three male and three female black rockfish were used to investigate the expression patterns of both *cox1* and *cox2* in different tissues *via* qPCR. As shown in Fig. 4, *cox1* was highly expressed in spleen and stomach in male, as well as in heart in both sexes (Fig. 4A, C). The mRNA of *cox2* was mainly detected in reproduction-related tissues: ovary, testis, cloaca, and urinogenital papillae, as well as other tissues including heart, spleen, kidney and skin (Fig. 4B, D).

3.3. Expression patterns of cox1 and cox2 in different gonad developmental stages and delivery periods in black rockfish

Testis and ovary in different stages were collected to test *cox1* and *cox2* expression pattern. In testis, both *cox1* and *cox2* showed similar expression patterns, with significant expression in the regressed stage (Fig. 5A). In ovary, *cox1* showed varied expression in different

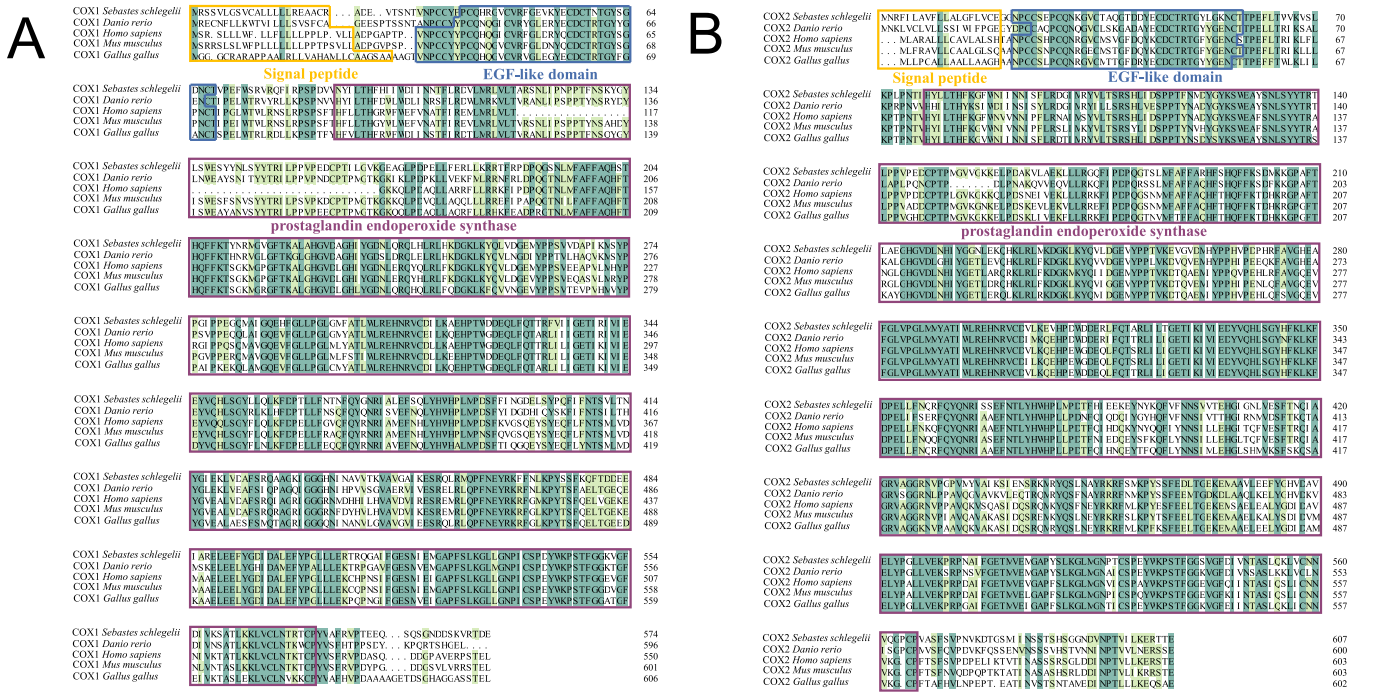

Fig. 1. Multiple amino acid sequence comparisons of the amino acid sequences of COX1 (A) and COX2 (B). The boxed letters indicate the sequences of different domains among black rockfish, zebrafish, human, mice, and chickens.

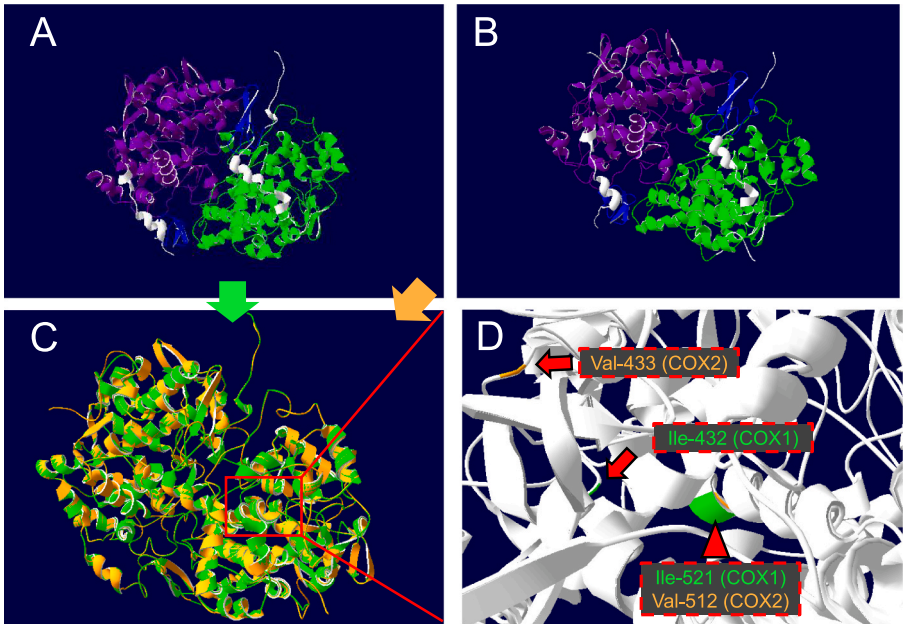

Fig. 2. 3D structure prediction analysis of the COX1 homodimer (A) and COX2 homodimer (B) of black rockfish showed high conservation (C) between the COX1 homodimer (green) and COX2 homodimer (orange); red arrows indicate the differences between the two homodimers. Ile^{432} and Ile^{521} (green) in COX1 were replaced by Val^{433} and Val^{512} in COX2, respectively. Ile^{521} in COX1 and Val^{512} in COX2 were superimposed completely (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

developmental stages with the highest expression in the vitellogenesis stage. Likely, *cox2* also showed a dramatic variation in stage vitellogenesis ovaries, which is an important period for mating behaviour in black rockfish (Fig. 5B).

As an ovoviviparous teleost, female black rockfish undergo a one-month pregnancy period, followed by delivery. In the late stage of pregnancy, the cloaca undergo expansion by the larval fish, which could be visualized through the cloaca (Supplementary 2). During the perinatal period, no variation in *cox1* was observed in either the ovary or cloaca. In the ovary wall, a significant increase was detected 24 h after delivery (Fig. 5C). Unlike *cox1*, *cox2* expression decreased significantly after delivery in the ovary and cloaca, while it decreased after parturition in the ovary wall (Fig. 5D).

3.4. mRNA localization of cox1 and cox2 in the testis and ovary

Based on the qPCR results, mRNA localization of *cox1* and *cox2* was performed in testis and ovaries *via* ISH. As shown in Fig. 6, the *cox1* mRNA signal in the regressed testis was mainly detected in Sertoli cells of black rockfish compared to the negative controls (Fig. 6A–C). Positive

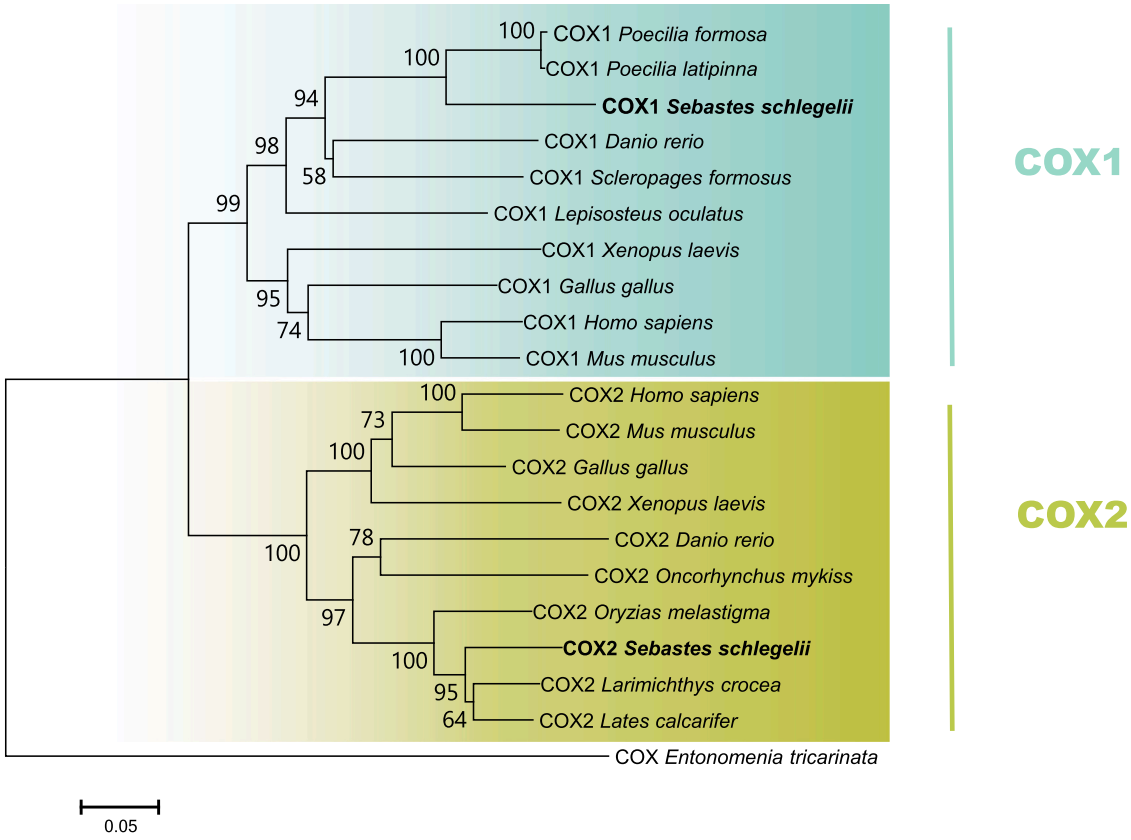

Fig. 3. A phylogenetic tree was constructed by MEGA 6 software using the neighbour-joining method. Data were resampled with 1000 bootstrap replicates. The accession numbers of each sequence are *Poecilia formosa* cox1 (XP_007571574.1), *Poecilia latipinna* cox1 (XP_014913654.1), *Danio rerio* cox1 (NP_705942.1), *Scleropages formosus* cox1 (XP_018610694.2), *Lepisosteus oculatus* cox1 (XP_015222331.1), *Xenopus laevis* cox1 (NP_001091389.1), *Gallus gallus* cox1 (XP_425326.4), *Homo sapiens* cox1 (NP_001258093.1), *Mus musculus* cox1 (NP_032995.1), *H. sapiens* cox2 (NP_000954.1), *M. musculus* cox2 (NP_035328.2), *G. gallus* cox2 (NP_001161191.1), *X. laevis* cox2 (NP_001086946.1), *D. rerio* cox2 (NP_705943.1), *Oncorhynchus mykiss* cox2 (NP_001117820.1), *Oryzias melastigma* cox2 (XP_024135124.1), *Larimichthys crocea* cox2 (XP_010732791.3), *Lates calcarifer* cox2 (XP_018551532.1), *Entonomenia tricarinata* cox (ALG96661.1).

signals of *cox1* mRNA were also observed in the inner layer of the ovary wall after delivery, which is composed of two layers of smooth muscle and may play important roles in parturition by muscle contraction (Fig. 6D–F). However, no significant signal was detected in ovary (data not shown), which may be due to the low basal expression level.

On the other hand, positive signals of *cox2* mRNA in the testis were mainly detected in Leydig cells after ejaculation, which was consistent with our qPCR result (Fig. 7A–C). Interestingly, strong positive signals of *cox2* in the ovary after delivery were detected in the follicular cell layer distributed around the lumen that had surrounded an embryo (Fig. 7D–F). In contrast, no signal was observed in the follicular epithelium around the early-stage oocytes (Fig. 7D–F).

4. Discussion

Mating choice is an important evolutionary process that imposes sexual selection on the other sex and accounts for spectacular ornaments that would otherwise remain unexplained by natural selection (Andersson, 1994; Darwin, 1871). In zebrafish (Yabuki et al., 2016) and African cichlid (Juntti et al., 2016), $PGF_{2\alpha}$ was reported to activate male or female mating behaviours. In goldfish, high circulating $PGF_{2\alpha}$ is associated with ovulation (Sorensen et al., 2018). PGs are used to clinically induce labour since prostaglandins of the E and F series induce uterine activity (O'Brien, 1995). In teleosts, PGs were reported to induce premature parturition in guppies (Venkatesh et al., 1992b). Accordingly, PGs play a conservative role in inducing teleost reproductive behaviours including both mating and delivery. However, COXs, the rate-limited enzymes of PG biosynthesis remain largely unexplored in teleosts.

In the evolutionary study of COXs, it has been suggested that teleosts sometimes possess additional copies of *cox1* or *cox2* likely due to a whole-genome duplication event (Kawamura et al., 2014). However, not all teleosts possess the same forms of COX. It has been reported that Atlantic croaker (*M. undulatus*), Atlantic cod (*Gadus morhua*) and brook trout (*Salvelinus fontinalis*) have one COX1 and one COX2 form (Kawamura et al., 2014; Roberts et al., 2000). In the present study, we have identified two isoforms of *cox* genes in black rockfish. This result might be due to differential loss of the *cox* gene after a whole-genome duplication event.

By sequence and structure analysis, COX2 has been shown to be very similar in structure to constitutive COX1. The three-dimensional X-ray crystal structure of human or murine COX2 can be superimposed on that of COX1. The residues that form the substrate binding channel, the catalytic sites, and the residues immediately adjacent are all identical except for two small variations(Vane et al., 1998). In these two residues, Ile in COX1 is substituted for Val in COX2 at residues 434 and 523. In black rockfish, COX1 and COX2 were also superimposed with small variations in the residues: Ile^{432} and Ile^{521} in COX1 were replaced with Val^{433} and Val^{512}, respectively. The structural differences between the two isoforms indicate functional differences. Supporting evidence has shown that mutation of Ile^{523} in COX1 allows the binding of selective COX2 inhibitors (Wong et al., 1997), and a mutant of COX2 in which Val^{523} is exchanged for Ile shows similar inhibitor binding selectivity in human (Gierse et al., 1996).

Cox2 was found to be mainly expressed in black rockfish gonads. In a study of male mice (*Mus musculus*) and Syrian hamsters (*Mesocricetus auratus*), *cox2* was found in Leydig cells and was involved in the

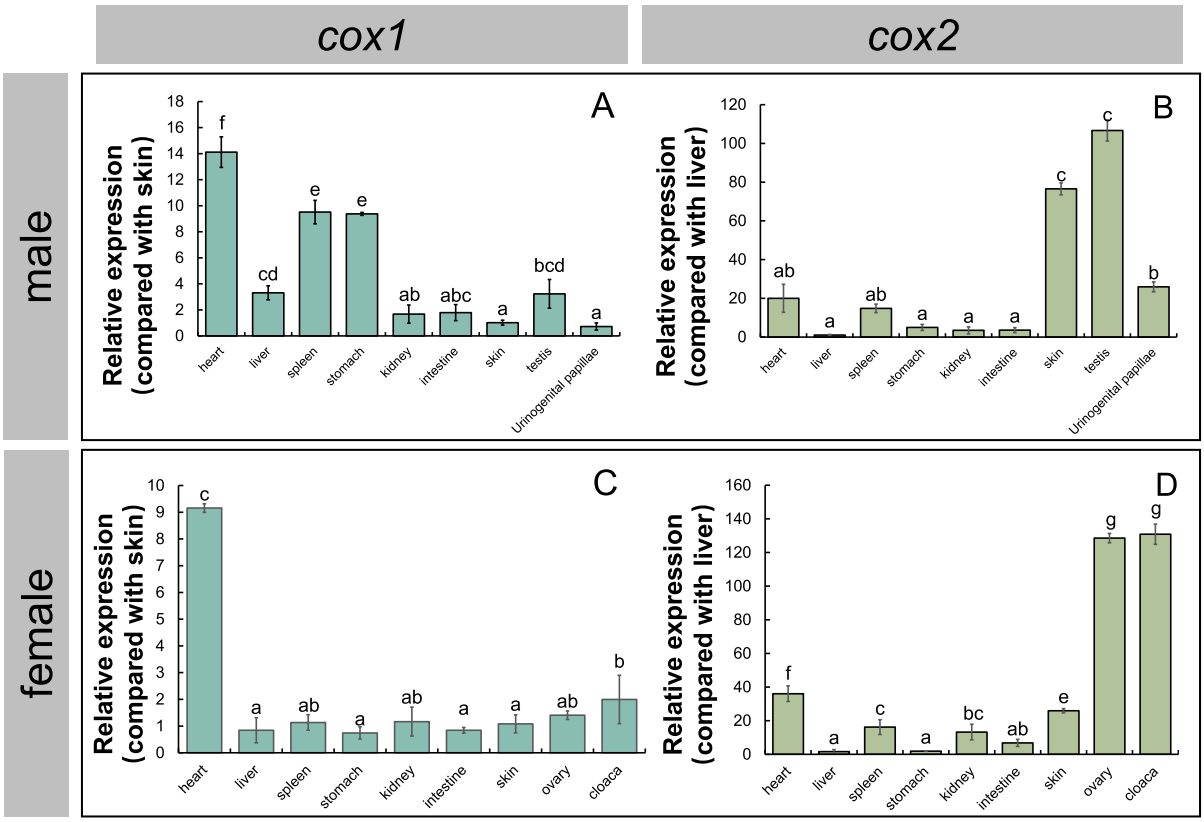

Fig. 4. Gene expression patterns of *cox1* and *cox2* in different tissues in black rockfish. Relative expression levels of *cox1* (A, C) and *cox2* (B, D) in different organs (heart, liver, spleen, stomach, kidney, intestine, skin, testis, ovary, cloaca, and urinogenital papillae) in both sexes in December. The X axis indicates different tissues. The Y axis indicates the relative expression normalized by 18S RNA. Data analyses were performed by one-way ANOVA followed by LSD (A, C, D) and the Dunnett T3 (B) multiple range test. The data are shown as the mean ± S.E.M. ($n = 3$), and different letters indicate significant differences ($P < 0.05$). The F statistic of Fig. 4A, B, C, D are 88.686, 240.745, 79.624, and 494.729, respectively. The degrees of freedom of Fig. 4A, B, C, D are 8 (between groups) and 18 (within groups).

synthesis of PG which regulated factors involved in steroidogenesis such as StAR (steroidogenic acute regulatory protein) and 17β-hydroxysteroid dehydrogenase (Frungieri et al., 2015; Frungieri et al., 2006). Meanwhile, COXs also play an important role in the ovary. *Cox2* deficient mice were incapable of PGE_2 synthesis in the ovary under GtH stimulation(Davis et al., 1999). The natural ovulation, fertilization, and implantation of *cox2* deficient mice were compromised (Lim et al., 1997).

Since both *cox1* and *cox2* showed high expression levels in the testis and ovary, we examined the expression profile of both genes at three different stages and delivery periods of black rockfish. In male black rockfish, *cox1* and *cox2* were both increased significantly in the regressed stage. It is clear that inflammatory factors present in the normal human testis participate in the regulation of spermatogenesis, including the proliferation of premeiotic germ cells (GCs) and spermatocyte apoptosis (Loveland et al., 2017). Several studies also indicated the positive regulation of prostaglandins and apoptosis in mammalian testis (Indrei et al., 2001; Matzkin et al., 2016), which supported the upregulation of *cox2* in regressed testis in the present study. Similarly, in teleosts, inflammatory factors including PGs, IL-1 and TNFα are present in the testis and play an important role in androgen biosynthesis and adaptation to the testicular microenvironment (Chaves-Pozo et al., 2008; Chaves-Pozo et al., 2005; Lister and Van Der Kraak, 2002; Wade and Van Der Kraak, 1993). In seasonal breeding teleosts, the testis experiences a testicular degeneration stage, followed by a resting stage. During the regression stage, phagocytosis of spermatozoa by Sertoli cells is evident. In addition, apoptosis is also involved in the testicular remodeling stimulated by hormones (Huettenbrenner et al., 2003; Kaptaner and Kankaya, 2013; Takle and Andersen, 2007). Accordingly, the increase in *cox1* and *cox2* in degeneration stage testis could provide suitable conditions for the initiation of testis development by synthesizing prostaglandins.

PG synthesized by COXs in ovary is crucial for ovulation during mating in ovulated teleosts (Baker and Van der Kraak, 2019). During this period, overdoses of PG are release into environment with urine by female, which can trigger the male mating response (Appelt, 1995). In zebrafish, $PGF_{2\alpha}$ was able to activate olfactory receptors in the olfactory sac to trigger male courtship behaviour (Yabuki et al., 2016). $PGF_{2\alpha}$ injection into African cichlid could also induce a mating response (Juntti et al., 2016). In the present study, the *cox2* expression level increased significantly during mating periods in female black rockfish. Black rockfish normally store sperm from several different males to produce diverse offspring (Gao et al., 2016). However, the amount of sperm from each male is limited (approximately 500 μL with a concentration of 1×10^8/mL on average). Accordingly, females need to attract several males to obtain enough sperm for future fertilization. Enhanced *cox2* expression in the ovary during the mating period could provide prostaglandins for important reproductive behaviour.

On the other hand, *cox1* expression in the ovary wall increased significantly after delivery. Inflammation has been implicated in the mechanisms responsible for preterm and term parturition in mammals (Romero et al., 2006). In black rockfish, the ovary wall acts as the uterus, with a smooth muscle layer to contain and then release the fries. After parturition, inflammation is obvious in the ovary wall, with a red and swollen phenotype. COX1 may participate in the anti-inflammatory process and thus initiate a new reproductive cycle. In the tested tissues, including the ovarian substrate, ovary wall and cloaca, *cox2* expression was significantly higher before and during delivery process

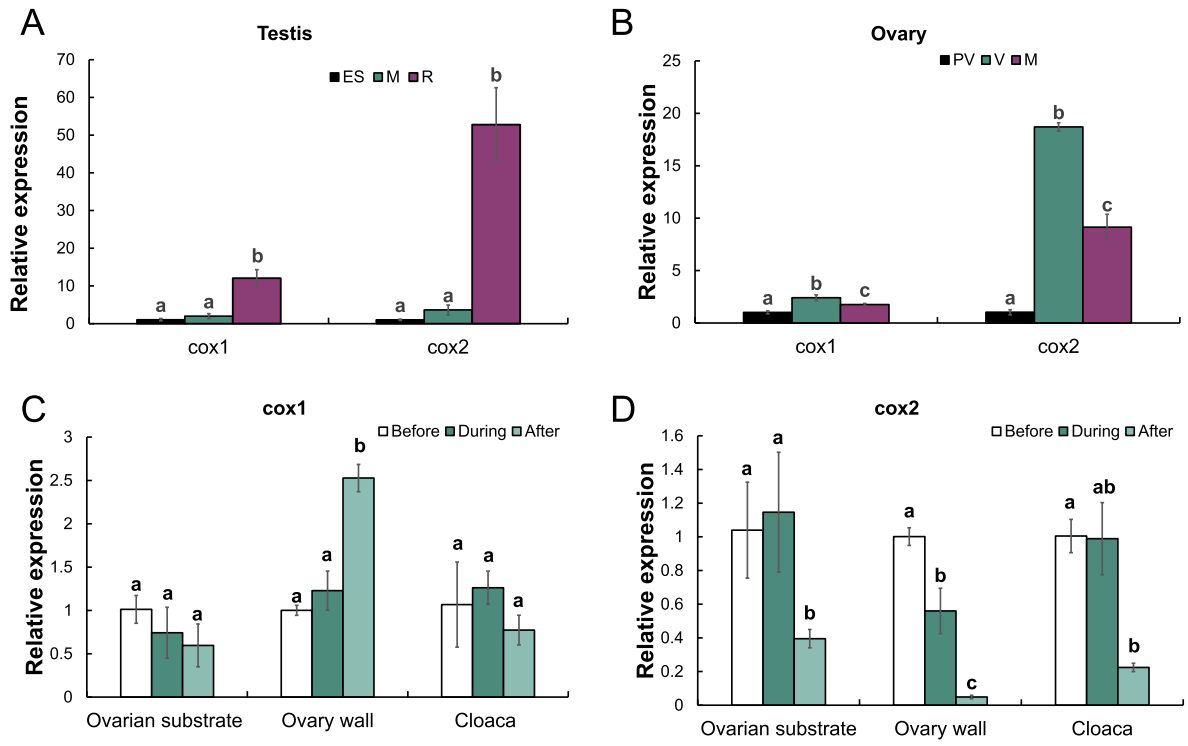

Fig. 5. Gene expression of *cox1* and *cox2* in different gonad development stages and delivery periods in black rockfish. Relative expression levels of *cox1* and *cox2* in testis (A) and ovaries (B) at different stages. The X axis indicates different genes in different stages. The Y axis indicates the relative expression normalized by 18S RNA. Relative expression levels of *cox1* (C) and *cox2* (D) before delivery, during delivery and 24 h after delivery. The X axis indicates different regions including ovarian substrate, ovary wall and cloaca. The Y axis indicates the relative expression normalized by 18S RNA. Data analyses were performed by one-way ANOVA followed by LSD (A, B, C, *ovarian substrate* and *ovary wall* in D) and the Dunnett T3 (*Cloaca* in D) multiple range test. The data are shown as the mean ± S.E.M. (n = 3), and different letters indicate significant differences ($P < 0.05$). The F statistics are 60.015 (cox1) and 78.153 (cox2) (Fig. 5A). The F statistics are 28.338 (cox1) and 407.001 (cox2) (Fig. 5B). The F statistics are 1.973 (ovarian substrate), 58.593 (ovary wall) and 1.699 (cloaca) (Fig. 5C). The F statistics are 6.997 (ovarian substrate), 96.693 (ovary wall) and 22.484 (cloaca) (Fig. 5D). The degrees of freedom of Fig. 5A, B, C, D are 2 (between groups) and 6 (within groups). ES: early spermatogenesis; M: mature; R: regressed; PV: previtellogenesis; V: vitellogenesis.

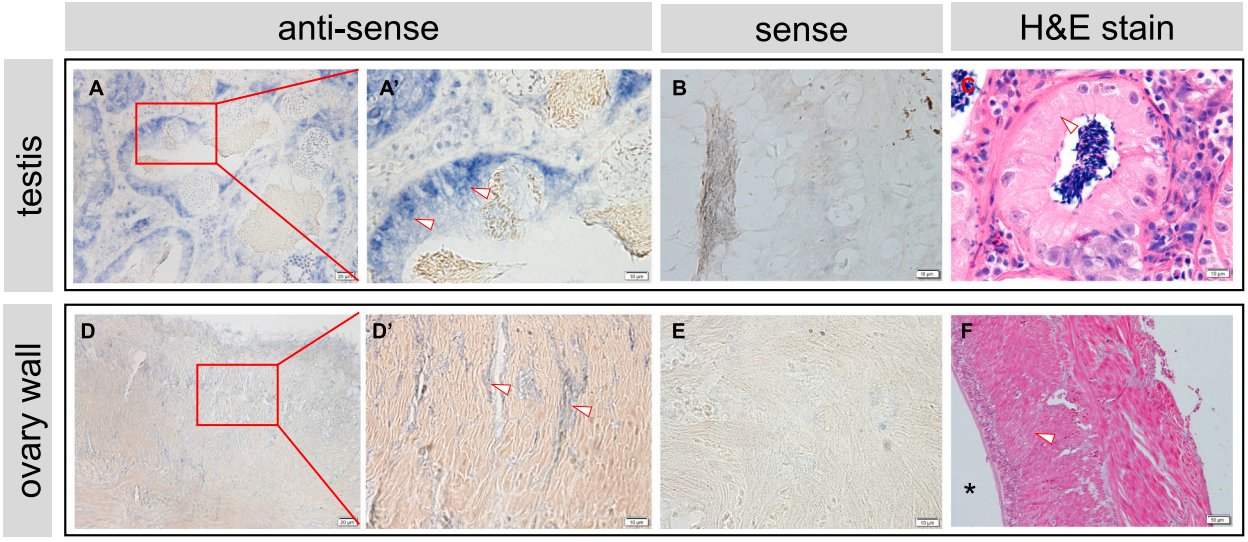

Fig. 6. Localization of *cox1* mRNA in the testis and ovaries of black rockfish *via in situ* hybridization. Antisense probe signal of *cox1* presented in Sertoli cells. The arrowhead indicates positive signals in the Sertoli cells (A, A'). H&E staining of the testis of male black rockfish in the regressed stage. The arrowhead indicates the Sertoli cells (C). Antisense probe signal of *cox1* in the inner layer of the smooth muscle in the ovary wall (D, D′). H&E staining of the ovary wall of female black rockfish after delivery. The asterisk indicates the inner cavity of the ovary. The arrowhead indicates the inner layer of the smooth muscle (F). Negative signal with sense probes at the same position (B, E). Scale bars (A, D) = 20 μm. Scale bars (A', B, C, D′, E) = 10 μm. Scale bars (F) = 50 μm.

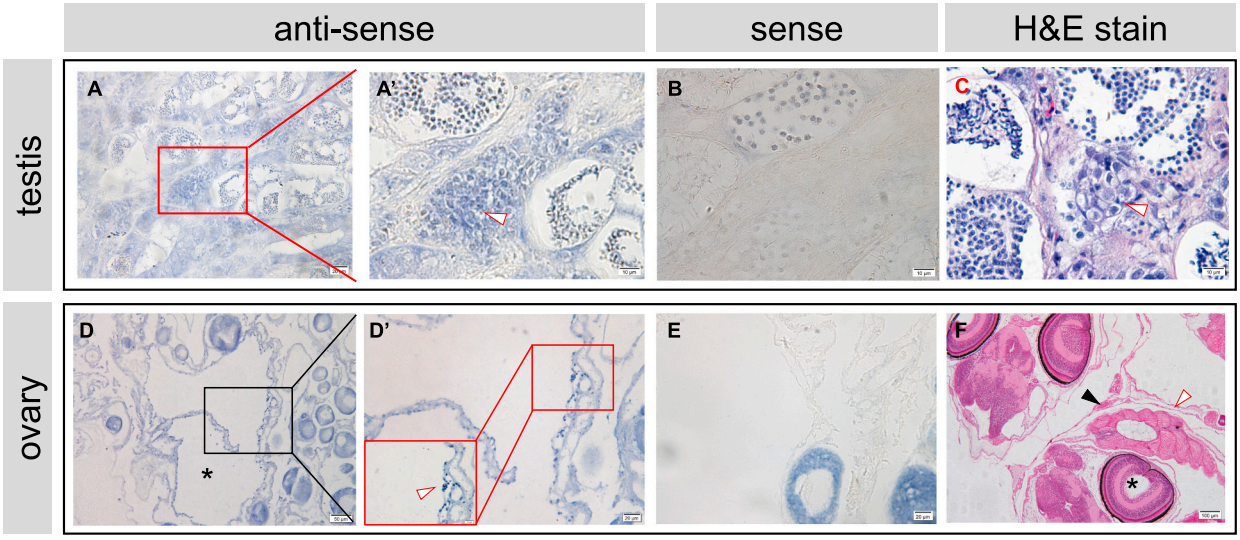

Fig. 7. Localization of *cox2* mRNA in the testis and ovaries of black rockfish *via in situ* hybridization. Antisense probe signal of *cox2* in the Leydig cells. The arrowhead indicates positive signals in the Leydig cells (A, A'). H&E staining of the testis of male black rockfish in the regressed stage. The arrowhead indicated the Leydig cells (C). The antisense probe signal of *cox2* in follicular cell layer in the ovary. The asterisk indicates the lumen that surrounded an embryo previously (D, D′). H&E staining of the ovary wall of female black rockfish before delivery. The asterisk indicates an embryo. The white arrowhead indicates the follicular cell layer. The black arrowhead indicated the blood capillary (F). Negative signal with sense probes at the same position (B, E). Scale bars (F) = 100 μm. Scale bars (D) = 50 μm. Scale bars (A, D′ E) = 20 μm. Scale bars (A', B, C) = 10 μm.

than after delivery. $PGF_{2\alpha}$ was elevated in the amniotic fluid of 40.2% of patients with preterm labour (Park et al., 2016). In oviparous teleosts, ovulation is associated with high circulating $PGF_{2\alpha}$ (Sorensen et al., 2018). Inhibition of COXs or cytosolic phospholipase A2 will lead to the failure of ovulation in zebrafish (Lister and Van Der Kraak, 2008; Tang et al., 2018). These results suggested that *cox2* plays an important role in the parturition of viviparity and oviparity vertebrates.

Finally, the localization of *cox1* and *cox2* was observed in the testis and ovary. The results showed that *cox1* and *cox2* signals in black rockfish testis were mainly located in Sertoli cells and Leydig cells, respectively. Similar results have been shown in rat and Syrian hamsters, in which COX2 was constitutively expressed in Leydig cells, indicating a role in testosterone synthesis (Balaji et al., 2007; Frungieri et al., 2006). *Cox2* was strongly expressed in follicular cell layer in the ovary during delivery, when follicular detached from the embryo and vascularized (Fig. 7F). It is coincident with the results in ostrich (*Struthio camelus*) (Rodler and Sinowatz, 2015b), quail (*Coturnix japonica*) (Rodler and Sinowatz, 2015a) and hen (*Gallus domesticus*) (Hales et al., 2008). These similar results indicate a conserved function of both *cox* genes.

Our present study provides important insights into the potential function of *cox* genes in ovoviviparous teleosts. In summary, we demonstrated that *cox1* and *cox2* are expressed differently in tissues and show different patterns during the mating and parturition periods. Furthermore, the localization of the products of both genes indicates that the *cox* genes are conserved throughout vertebrates. These results suggest that *cox* genes are important regulators of reproductive behaviours.

Summary sentence

Cyclooxygenase identification and potential roles in ovoviviparous teleost reproductive behaviours.

Declaration of interest statement

All authors declare that no conflict of interest exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpb.2021.110677.

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