Thesis for the Degree of Master of Fisheries Science

Molecular Cloning and mRNA Expression Analysis of Interleukin-1β, Cathepsin B and Cathepsin D from Siberian Sturgeon (*Acipenser baerii*)

by

S M Ashikur Rahman

KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

Pukyong National University

February 2016

Molecular Cloning and mRNA Expression Analysis of Interleukin-1β, Cathepsin B and Cathepsin D from Siberian Sturgeon (*Acipenser baerii*)

(시베리아 철갑상어 (*Acipenser baerii*) interleukin-1β, cathepsin B 및 cathepsin D 유전자의 클로닝 및 mRNA 발현 분석)

Advisor: Professor NAM Yoon Kwon

by

S M Ashikur Rahman

A thesis submitted in partial fulfillment of the requirement

for the degree of Master of Fisheries Science

in KOICA-PKNU International Graduate Program of Fisheries Science

Pukyong National University

February 2016

Molecular Cloning and mRNA Expression Analysis of Interleukin-1β, Cathepsin B and Cathepsin D from Siberian Sturgeon (*Acipenser baerii*)

A dissertation

by

S M Ashikur Rahman

Approved by:

(Chairman) Professor KIM Hyun-Woo

(Member) Professor GONG Seung Pyo

(Member) Professor NAM Yoon Kwon

February 26, 2016

Table of Contents

List of Figures

List of Tables

Molecular Cloning and mRNA Expression Analysis of Interleukin-1β, Cathepsin B and Cathepsin D from Siberian Sturgeon (*Acipenser baerii*)

S M Ashikur Rahman

KOICA-PKNU International Graduate Program of Fisheries Science

Pukyong National University

Abstract

The innate immunity provides the first line and fundamental defense against infection by pathogen in fish. Sturgeons, as an extant primitive fish group might possess immune functions largely different from more advanced bony fishes and regarded as a useful model for evolutionary genomics of immunity in vertebrates. Siberian sturgeon (*Acipenser baerii*) is a threaten species and one of the most important species for sturgeon farming. The aim of this study was isolation, cloning, sequencing and analyzing of interleukin-1β (IL-1β), Cathepsin B (CTSB) and Cathepsin D (CTSD) gene from Siberian sturgeon and evaluation of the expression level in the embryonic developmental stages and in different tissues of juvenile specimen. The cDNA of the genes, obtained by RT-PCR were cloned, sequenced and analyzed. The Siberian sturgeon IL-1β transcript consisted of 849 bp open reading frame (ORF) which translated into a 282 amino acids (aa) peptide. The amino acid sequence lacked a signal peptide but contained interleukin-1 propeptide site and a clearly identifiable IL-1 family signature. The sequence showed low level of similarity

with other known species IL-1β and the highest identity was 45% to Atlantic salmon IL-1β. The CTSB sequence consisted of 999 bp open reading frame (ORF) which encoded 332 aa. The amino acid sequence possessed a signal peptide, propeptide, mature peptide regions; active sites of cysteine, histidine and asparagine residues and also contain the papain family cysteine protease domain. The sequence showed the highest level of identity to orange-spotted grouper CTSB (81%). The CTSD sequence consisted of 1191 bp open reading frame (ORF) which encoded 396 aa. The amino acid sequence contained a signal peptide, propeptide domain, aspartic peptidase domain and two aspartic peptidase active sites. The sequence showed the highest level of identity to zebrafish CTSD (85%). The expression level of IL-1β gene mRNA was at a low level in early embryonic development stages and was up regulated from formation of large yolk plug stage up to first occurrence of advanced hatch stage but CTSB and CTSD gene mRNA showed up regulated expression for all the development stages. In the tissue-specific expression, CTSB and CTSD transcripts were relatively high in all examined tissue but IL-1β gene mRNA transcript was expressed relatively high in brain, fin, liver, and spleen than other tested tissue. The ubiquitously expression of IL-1β, CTSB and CTSD in every embryonic developmental stage and in all tested tissues revealed that these three genes may play a vital role in the innate immune functions of Siberian sturgeon. The information from this study could be a fundamental baseline data for detailed studying of innate immune functions and regulations in the development of this primitive fish species.

1. Introduction

The innate immune system is the only defense weapon in invertebrates and in higher vertebrates it plays an instructive role for the acquired immune system. In teleost, innate immunity occupies a more important position for the initial protection against pathogen invasion, due to the constraint on adaptive immunity in suboptimal environments (Ullal AJ et al., 2008). It has been considered as an essential component in combating disease incidents due to the constraints placed on the adaptive immune response by their poikilothermic nature plus the limited antibody repertoires, affinity maturation and memory and relatively slow lymphocyte proliferation (Magnadottir B., 2006). The main role of the innate immune system is believed to be the first line of host defense in opposing pathogenic organisms and to deal with any foreign material until the adaptive immune system was able and potent enough to take over. Teleost fish serve a key role as the bridge between innate and adaptive immune responses in that they are the earliest class of vertebrates possessing the elements of both innate and adaptive immunity (Whyte SK., 2007).

Innate immunity is generally subdivided into two parts, the cellular and the humoral defense responses. Cellular responses include the physical barrier such as mucus and epithelial tissues lining the skin, gills and stomach, which keeps infectious microorganisms from entering the body, and specialized cells (like monocytes/macrophages, granulocytes and nonspecific cytotoxic cells) capable of killing and digesting pathogens if the latter breaches the physical barriers. These cells are recruited in the infection site primarily by inflammatory cytokines. On the other hand, humoral responses employ a variety of proteins and glycoproteins capable of destroying or inhibiting growth of infectious microorganisms, which include Cytokines, anti-bacterial peptides, complement, transferrins and the antiviral myxovirus resistance-1 protein (Mx1)(Takashi Aoki et al., 2008).

Cytokines, the most important family in the innate immune system, are of low molecular weight proteins that are often glycosylated and are secreted by activated immune-related cells upon induction by various pathogens such as parasitic, bacterial, or viral components (Salazar-Mather TP and Hokeness KL., 2006). They are involved in several steps of the

immune response, from induction of the innate response to the generation of cytotoxic T cells and the production of antibodies. They can modulate immune responses through an autocrine or paracrine manner upon binding to their corresponding receptors. In higher vertebrates, the combination of cytokines that are secreted in response to an immune stimulation induces the expression of immune-related genes through multiple signaling pathways, which contributes to the initiation of the immune response. Cytokines are derived from macrophages, lymphocytes, granulocytes, DCs, mast cells, and epithelial cells, and can be divided into interleukins (ILs), tumor necrosis factors (TNFs), interferons (IFNs), colony stimulating factors, and chemokines (Savan R and Sakai M., 2006).

IL-1β is a pro-inflammatory cytokine involved in the initiation and/or increase of a wide variety of non-structural, function-associated genes expressed during inflammation. It is a member of the b-trefoil cytokines as it contains 12 β-sheets within the mature protein, and folds to create a trefoil-like structure (Nicola NA., 1994). IL-1β is produced by many cell types, but predominantly blood monocytes and tissue macrophages. It is

important in the host's response to microbial invasion, tissue injury and immunological reactions including autoimmune diseases via its ability to enhance phagocytic activity, lysozyme synthesis, macrophage proliferation and leucocyte migration (Dinarello CA., 1997). In addition to humans (Bensi G et al., 1987), mice (Telford J et al., 1986) and chicken (Weining KC et al., 1998), IL-1β has been characterized in cartilaginous fish (Bird S et al., 2002) and bony fish such as haddock (Corripio-Miyar Y et al., 2007), common carp (Fujiki K et al., 2000), rainbow trout (Hong S et al., 2001), yellow fin sea bream (Jiang SG et al., 2008), Nile tilapia (Lee D-S et al., 2007), orange-spotted grouper (Lu DQ et al., 2008), European sea bass (Scapigliati G et al., 2001), and channel catfish (Wang YP et al., 2006).

At present, cathepsins have gained extensive attention of researchers studying for their significant physiological roles in protein degradation/turnover (cathepsins B, L and H), bone resorption (cathepsin K), proenzyme activation (cathepsins B and C), antigen presentation and processing (cathepsins F, H, L, S and V), epidermal homeostasis (cathepsin L) and hormone maturation (cathepsins B and L) (Lecaille F et al., 2002). Cathepsins are a family of lysosomal proteases (enzymes that degrades

proteins) present in almost all animals as well as other organisms. According to the amino acid residues in their active sites, cathepsins can be divided into three groups: cysteine proteases (cathepsins B, C, F, H, K, L, O, S, W and X or Z), aspartic proteases (cathepsins D and E) and serine proteases (cathepsins A and G) (Conus S and Simon HU., 2010).

Majority of cysteine proteases belong to the C1 peptidase family, which are also known as member of the papain family (Colbert JD et al., 2009). Cathepsin B (CTSB), a unique member of this papain superfamily, plays important roles in pathological and physiological processes, such as apoptosis (Blomgran R et al., 2007), cancer, TLR9 signaling pathway (Matsumoto F et al., 2008) and TNF-a post translational processing in macrophages (Ha SD et al., 2008). To date, cathepsin B has been identified in teleosts such as *Paralichthys olivaceus* (Cha IS et al., 2012), *Oplegnathus fasciatus* (Whang I et al., 2011), *Cynoglossus semilaevis* (Chen L and Sun L., 2012) and orange-spotted grouper (Wei SN et al., 2014).

Cathepsin D (CTSD) is the major aspartic protease of the pepsin family that plays an important role in the degradation of intracellular and

endocytosed proteins in lysosomes (Lee DC et al., 2007; Jia A and Zhang XH., 2009). It is involved in immune recognition and in pathological changes, including inflammatory states, apoptosis, and mutagenesis (Park EM et al., 2009), extracellular proteolysis and processing, secretion and activation of enzymes and hormones (Baldocchi RA et al., 1993). CTSD is also known to be involved in oogenesis and embryogenesis in rainbow trout (Kwon JY et al., 2001). It has been cloned and sequenced in a number of mammalian species (Redecker B et al., 1991 and Hetman M et al., 1994), and in several species of fish such as tilapia (*Tilapia nilotica, Tilapia aurea*) (Jiang ST et al., 1991), rainbow trout (*Oncorhynchus mykiss*) (Brooks S et al., 1997), grass carp (Dong ZD et al., 2012). Antarctic ice fish (*Chionodraco hamatus*) (Capasso C et al., 1999), sea bream (*Sparus aurata*) (Carnevali O et al., 1999), zebrafish (*Danio rerio*) (Riggio M et al., 2000), common carp (*Cyprinus carpio*) (Goldmann T et al., 2001), herring (*Clupea harengus*) (Nielsen LB and Nielsen HH., 2001), pufferfish (*Takifugu rubripes*) (Kurokawa T et al., 2005), Atlantic cod (*Gadus morhua*) (Wang PA et al., 2007), and turbot (*Scophthalmus maximus*) (Jia A and Zhang XH., 2009).

Sturgeons are the most economically important phylogenetically primitive fish (Bronzi P et al., 2011) and are considered to be a transition between major taxa, thus useful in the understanding of vertebrate evolution. All living representatives of sturgeons are listed as threatened, vulnerable, and endangered in CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) (Bemis WE et al., 1997 and Ludwig A., 2008). Sturgeons are excessively harvested for their meat and eggs, sold worldwide as caviar and sturgeon caviar have the highest economical value. As a result, sturgeon aquaculture has increased considerably worldwide in the last two decades (Bronzi et al., 2011 and Wei Q et al., 2011). Siberian sturgeon (*Acipenser baerii*) is a threatened species (IUCN, 2011) and one of the most important sturgeon species whose farming has risen (Williot P et al., 2001).

In the rearing environments, sturgeons are usually faced with numerous acute and chronic stress agents including poor water quality, high density, and handling, among others (Rafatnezhad et al., 2008; Rafatnezhad and Falahatkar., 2011). Similar to other teleosts rearing, the culture of larvae

is considered to be one of the most critical and difficult stages in intensive sturgeon farming, often associated with a high mortality rate during and after yolk sac absorption and onset of feeding (Conte et al., 1988 and Mohler et al., 2000). During this period, the larvae have to rely solely on innate mechanisms and possibly maternal antibody until they develop a functional adaptive immune system, which may take several months (Saurabh S and Sahoo PK., 2008).

Better understanding of the immune system during the embryonic and early larvae development stages is very important to manage or prevent mortality in developmental stages. The immune competence that the fish has acquired during their early life stages could be a significant factor for better survival in later stages or when they are reared in aquaculture facilities. In fish, maternal factors such as mRNAs, proteins, vitamins and minerals are passed from the mother to the unfertilized eggs and these factors are thought to influence fish egg quality and embryonic health (Bobe & Labbe, 2010). These factors are essential for the developing embryo until such time when its own transcriptional machinery becomes functional including its immune

system. During the embryonic developmental stages IL-1β molecules has been identified in common carp (Huttenhuis HBT et al., 2006), rainbow trout (Chettri JK et al., 2012) and brown trout (Cecchini et al., 2013). Cathepsin has been described in rainbow trout (Brooks S et al., 1997), sea bass (Carnevali O et al., 2001) and cod (Magnadottir B et al., 2004). Siberian sturgeon is a cold water fish species that produces large eggs and has a long developmental phase from fertilization to hatching. Park et al., 2013 described the embryonic developmental stages for the Siberian sturgeon and was the derivation of developmental gene expression assays.

Thus, this species is greatly invaluable for research on sturgeon physiology. A large number of studies have been performed on Siberian sturgeon concentrating on growth performance, toxicology, normal rearing conditions, reproduction, hematology, stress responses, and immunostimulation (Hamlin HJ et al., 2008; Kolman H., 2002; Ruchin AB., 2007; Sadati M A Y et al., 2011; Wlasow T and Fopp-Bayat D., 2011). But the innate immune functions and the expression of innate immune related genes of Siberian sturgeon in the embryonic developmental stages has been little explored. A better understanding of innate immune functions of

Siberian sturgeon is important to optimize aquaculture rearing conditions and potentially aid wildlife conservation programs. Therefore, the aim of the study was isolation, cloning, sequencing and analyzing of interleukin-1β (IL-1β), Cathepsin B (CTSB) and Cathepsin D (CTSD) gene from Siberian sturgeon; evaluate their expression level in the embryonic developmental stages and in different tissues of juvenile specimens.

2. Materials and methods

2.1 Brood fish rearing and induce breeding

Brood fish were originated in 2003 and reared in Dinoville sturgeon farm, Hamyang in South Korea by using well oxygenated ground water; water temperature was maintained between 12 to 20 ºC in RAS system. Stocking density was 1-2 fish in M^3 and fed by commercial feed. The fishes were sexed and tagged at six years ages by external gonad biopsy and sexually tagged fish were reared in RAS system.

In February 2015 the sexed mature 10 females (average body weight 14±7.2 kg) were checked by using metal biopsy method to determine the development stage of eggs. According to Van Eenennaam et al., 1996, polarization index of oozytes if (P1) score <0.1 was the reference point for oocytes development stages. Primary selection was performed, females were tagged and introduced to the spawning tanks and reared until artificial induce breeding. The temperature was maintained at 10±0.5 ºC until induce by hormone in the spawning tanks. Matured males were selected by

presence of milt with catherization using silicon tube connected syringe into the genital duct, tagged and introduced to the spawning tanks. The selected female fish were performed secondary selection to confirm the development stage of oocytes (germinal vesicle break down stage). The fully egg matured three female fish were selected and introduced to the spawning tanks individually. The water temperature was maintained at 14±0.5 ºC with proper aeration in spawning tank. Consequently, four males (average body weight 8±4.2 kg) were selected by presence of milt, using silicon tube connected syringe introduced to the genital duct or palpitation of abdomen. After three weeks from primary selection, selected males and females were stimulated by hormone to induce ovulation and spermiation. Female were injected luteinizing hormone- releasing hormone analog des-Gly^{10}, (D-Ala^{6}) LH-RH ethylamide (LHRHa; Syndel Laboratories ltd.), Qualicum beach, BC, Canada) 10-15 µg per kg body weight. After 12 hours LHRHa 90-135 µg per kg body weight were injected as secondary injection. The male were injected only one dose of LHRHa 100 µg per kg body weight. During injection, water temperature was maintained at 14±0.5 ºC with proper aeration in spawning tank. The first occurrence of eggs ovulation at

very low amount was observed after 32 hours of injection. Finally, eggs were collected after 34 hours of injection.

2.2 Artificial insemination and hatching

Spawning was recognized after first observed of ovulation. After confirmation of ovulation, milt were collected from injected males using a silicon tube connected syringe and stored at 2-4 ºC until used. The eggs were collected from spawned female by hand stripping and caesarean section. Consequently, collected eggs were mixed with milt (1:200 diluted with water) and for 150 seconds rinsed with fresh water to remove adhesiveness from the egg. After two minutes of insemination, fertilized eggs were washed with fuller's earth solution at 30 minutes until removed adhesiveness. Then eggs were incubated in McDonald hatching jar at 14.5 ºC until transferred to the lab. After reached to Genetic Engineering and Laboratory of Pukyong National University, incubation was continued at 18.5±0.5 ºC until hatched out.

2.3 Embryonic and larval samples collection

Eggs were collected from immediate after fertilization to mass hatch out for the samples of development stages. According to Park et al., 2013, the developmental stages of Siberian sturgeon were assigned 30 stages starting just after fertilized. Immediate after insemination to beginning of gastrulating samples were collected at every one hour, every three hours and every 4 hours period until the first occurrence of hatching. From each stage 20 embryos were collected and fixed with cold 4% paraformaldehyde and 20 embryos of each stage stored with RNAlater solution and stored at -80 ºC until RNA isolation. Every sample was examined under the microscope to identify the development stages of embryos and stages states were recorded.

2.4 Experimental fish rearing and samples collection

One year old healthy Siberian sturgeon fish was collected from the RAS system of Pukyong National University, Busan, South Korea for RNA isolation. Fish were reared by using well oxygenated ground water, fed with Woosung (Korea) commercial feed and water temperature was maintained at 12-20 ºC. Consequently, fish was anesthetized by using clove oil

(0.30 mg/L) and rapidly killed. Tissue samples were collected and stored at -80 ºC until total RNA was extracted.

2.5 Isolation of IL-1β, CTSB and CTSD cDNA

2.5.1 RNA extraction and isolation

Total RNA was extracted with RNeasy plus mini kit (Quiagen, Hilden, Germany) according to manufacturer's protocol. The collected tissues were added into 600 μl buffer RLT, homogenized by rotor-stator homogenizer. The lysate was centrifuged at 13,000 rpm for 3 min. supernatant was transferred to 1.5 ml tube and added 1 volume of 70 % ethanol. The mixture was transferred to RNeasy spin column, centrifuged at 13,000 rpm for 15 sec. and discarded flow-through. Subsequently, buffer RW1 700 μl added to spin column and centrifuged at 13,000 rpm for 5 sec. to wash the spin column the membrane. The buffer RPE 500 μl was added to spin column and centrifuged at 13,000 rpm for 15 sec. to wash the spin column the membrane. This step was repeated to spin column and centrifuged at 13,000 rpm for 2 min. RNeasy spin column was transferred to 1.5 ml new tube and 30 μl RNase free water was added directly to the membrane and centrifuged at 13,000 rpm for 1 min. to elute the RNA.

2.5.2 cDNA synthesis

Purified RNA was reverse-transcribed using Omniscript^{R} Reverse Transcription kit (Quiagen) as described. The first cDNA was synthesized with Omniscript Reverse Transcriptase in a reaction mixture of 20 μl containing 2 μg of total RNA and 2 μl oligo-DT primers.

2.5.3 RT-PCR

For amplification of cDNA template, RT-PCR reaction performed using forward and reverse primers. Primers were designed by using multiple sequence analysis of IL-11β, CTSB and CTSD cDNA from NGS database. RT-PCR reaction was performed using 1 μl of cDNA template, 2 μl each forward and reverse primer, 15 μl distilled water added to AccuPower^{R} PCR Premix (dNTP, Taq DNA polymerase, $Mgcl_2$) (Bioneer Corporation). The reactions were completed in a thermo cycler with following thermo-profile: the samples were amplified for 30 cycles at 95 ºC for 2 min., 55 ºC for 30 sec. and extension at 72 ºC for 1 min., 72 ºC for 5 min. For confirmation, 1μl RT-PCR products (1μl RT-PCR product, 5 μl PCR loading dye mixture) were used fo electrophoresis by 1% agarose gel and stained using Et-Br (10 μg/ml).

2.5.4 Purification of PCR product

PCR product purification was performed by GeneAll^{R} Expin^{TM} spin kit (GeneAll biotechnology, Seoul, South Korea) as following protocol. The PB buffer 5 volumes was added into 1 volume of PCR sample, mixed and transferred to a SV column. Mixture was centrifuged for 30 sec, the pass through was discarded and reinserted the SV column into the same tube. The NW buffer 700 µl was added and centrifuged for 30 sec. and discarded the pass-through. Sv column was reinserted into the collection tube. To remove the residual wash buffer, centrifuged for additional 1 min. and SV column transferred to new 1.5 ml tube. The EB buffer 23 µl was added into the center of membrane SV column, was stand at 1 min. and centrifuged for 1 min, discarded the SV mini column and stored eluted DNA at -20 ºC until used.

2.6 Cloning of IL-1β, CTSB and CTSD gene

2.6.1 Transformation into *E. coli*

The 5 µl of 2X Ligation buffer was added into purified 3 µl PCR product and ligated into 1 µl of pGEM-Teasy Vector (Pormega, USA). For this mixture, 1 µl T4 DNA ligase was added and mixed well by micro

pipette and incubated at 12.5 ºC for 12 hour in cool incubator. The 200 µl competent *E. coli* cells were thawed on ice for 5 min. and added into 10 µl ligated product, incubated on ice for 30 min, heat shock at 42 ºC for 90 sec. and mixture was incubated on ice for 3 min. the SOC (Super Optimal Broth) solution 800 µl was added into reaction mixture. The reaction mixture was transferred to 14 ml round bottom tube and incubated at 37 ºC for 45 min. in shake incubator. Then, the mixture was transferred into 1.5 ml tube and centrifuged for 10 sec. and partially removed supernatant and mix well. The transformed competent cells were spread into the LB media plate contained ampicillin (100 µg/ml), X-gal (40 µg/ml) and IPTG (Isopropylthio-β-Galactoside (Invitrogen)) (100 µg/ml), incubated at 37 ºC for overnight.

2.6.2 Purification of recombinant plasmid

Randomly selected white color single colonies were inoculated into 2 ml LB medium which contained ampicillin (100 µg/ml) and incubated for overnight at 37 ºC in shaker incubator. The grown cell culture 1.2 ml was used to isolate for recombinant plasmid by using GeneAll^{R} Exprep^{TM} plasmid SV mini kit (GeneAll biotechnology, Seoul, South Korea) as following protocol. Pellet bacterial cells were collected by centrifuged at

13,000 rpm for 30 sec. and discarded the supernatant. Re suspended pellet bacterial cells thoroughly in 250 µl of buffer S1 by vortex and transferred to a new 1.5 ml tube. Added 250 µl of S2 buffer and mixed by inverting the tube 4 times, incubated until the cell suspension becomes clear and viscous not more than 5 min. The S3 buffer 350 µl was added and imidiately mixed by inverting the tube 4-6 times and centrifuged for 10 min. The supernatant was transferred to SV column and centrifuged at 13,000 rpm for 30 sec., pass through was discarded. Consequently, 700 µl of buffer PW was added to SV column and centrifuged for 30 sec. and discarded pass through. The SV column was centrifuged additional for 2 min. to remove residual washed buffer and SV column was transferred to new 1.5 ml tube. 50 µl of EB buffer was added into the tube and stand for 2 min. and centrifuged for 1 min. and stored at -20 ºC. For confirmation, 1 µl plasmid product and 5 µl 1X dye were used for 0.70% gel electrophoresis. Plasmid products were used for sequencing at Cosmo Genetech Co. Ltd., South Korea.

2.7 Synthesis of IL-1β, CTSB and CTSD genomic DNA

The Siberian sturgeon IL-1β, CTSB and CTSD cDNA was used to amplify the IL-1β, CTSB and CTSD genomic DNA. Subsequently, the following protocol was performed. 30 μl of distilled water added into 40 μl of PCR premix and mixed well by pipette, 4 μl each forward and reverse primer were added and finally 2 μl cDNA sample was added and mixed well. For amplification, following protocols were performed with two groups of samples.

A set of primers, specific for each intron was prepared from the Siberian IL-1β, CTSB and CTSD cDNA sequence. The reactions were completed in a thermo cycler with following thermo-profile: the samples were amplified for 35 cycles at 95 ºC for 2 min., 95 ºC for 30 sec., 55 ºC for 30 sec., 68 ºC for 4 min. and extension at 68 ºC for 5 min. with combination of the gene-specific primer listed in Table 1. Consequently, PCR products were confirmed through 0.7% agarose gel electrophoresis.

Table 1. Primers used to amplify IL-1β, CTSB and CTSD cDNA and for expression studies

Primer name	Primer sequences (5' - 3')
AB_cIL-1β_1F	ATACCGCCAGCAAAGCAAAG
AB_cIL-1β_1R	AATTCGGATGACCGACCAGT
qAB_IL-1β_2F	TATGTGCGCTCCATGAGACA
qAB_IL-1β_2R	CGTCTCAGCTCGGTCTCAAA
AB_cCATHEPSIN B_1F	ATGTGGTGCCTAGTTGCCTT
AB_cCATHEPSIN-B_1R	CTGGTCCTGTTAACAGCTCA
qAB_CATHEPSIN B_2F	GGAGTACACTGGTGATGTCA
qAB_CATHEPSIN B_2R	GATCCTCTGCAGAGATCTCA
AB_cCATHEPSIN-D_1F	ACCATTCAAGGAACCAACGT
AB_cCATHEPSIN-D_1R	CCTGTTGCATCATCATCTCA
qAB_CATHEPSIN-D_2F	AAGAACTCTGTCCGATGCTG
qAB_CATHEPSIN-D_2R	CGGTATCAAAGACCACAGTG

2.8 Gel purification of genomic DNA

Gel purification was done with GeneAll^{R} Exprep^{TM} Gel spin method. The DNA bands of interest were excised using an ethanol cleaned razor blade and weighted the gel slices in a micro centrifuge tube separately. The

GB buffer 3 volume added to 1 volume of (mg) gel. Mixture was incubated at 50 ºC for 5-10 min. until the agarose gel completely melted; during the melting vortex the tube every 2-3 min. then 1 gel volume of isopropanol was added into the sample and mixed well with the vortex. The mixture was transferred to SV column, centrifuged for 1 min. and pass-through was discarded and reinserted the SV column into collection tube. Added 500 µl of GB buffer to the SV column and centrifuged for 30 sec. and discarded the pass-through. Added 700 µl of NW buffer to the reinserted SV column and centrifuged for 30 sec. and discarded the pass-through. The SV column was reinserted into the collection tube and centrifuged for additional 1 min. to remove residual wash buffer. The SV column was transferred to new 1.5 ml tube. Consequently, added 23 µl of EB buffer to the centre of the membrane in the SV colum, stand for 1 min. and centrifuged for 1 min. An eluted PCR product was stored at 4 ºC until used. The purified genomic DNA was confirmed by 0.7% agarose gel. Purified 3 µl products were ligated into 1 µl of pGEM-teasy Vector (Pormega, USA).

2.9 Distribution of IL-1β, CTSB and CTSD transcripts in embryonic development stages and tissues

Total RNA was extracted with RNeasy plus mini kit (Quiagen, Hilden, Germany) according to manufacturer's protocol from development stages (Stage 1, Stage 2, Stage 3, Stage 6, Stage 7, Stage 9, Stage 11, Stage 13, Stage 14, Stage 17, Stage 23, Stage 25, Stage 26, Stage 27, Stage 29, D4 and D9) and tissues such as, Brain (B), Eye (E), Fin (F), Gill (G), Heart (H), Intestine (I), Kidney (K), Liver (L), Muscle (M), Spleen (S), Gonad (Go).

Purified RNA was reverse-transcribed using Omniscript^{R} Reverse Transcription kit (Quiagen) as described. The first cDNA was synthesized with Omniscript Reverse Transcriptase in a reaction mixture of 20 µl containing 2 µg of total RNA and 2 µl oligo-DT primers. Both forward and reverse primers (Table 1) were used to RT-PCR. The reactions were completed in a thermo cycler with following thermo-profile: the samples were amplified for 20 and 28 cycles at 95 ºC for 2 min., 95 ºC for 20 sec., 58 ºC for 20 sec. and extension at 72 ºC for 20 sec. PCR products were confirmed using 5 µl pcr product through 1.5% agarose gel electrophoresis.

Table 2. Development stages of Siberian sturgeon embryos and early larvae (Park et al., 2013)

Stages	Development state
Stage 1	Fertilization
Stage 2	First cleavage (two cells)
Stage 3	Second cleavage (four cells)
Stage 6	Fifth cleavage; blastomeres
Stage 7	Irregular pattern of cleavages continues
Stage 9	Blastula-late phase of blastula
Stage 11	Gastrulation-dorsal blastopore lip formed
Stage 13	Formation of large yolk plug
Stage 14	Formation of small yolk plug
Stage 17	Wide neural plate formed
Stage 23	Rudimentary heart visible and tail rod-shaped
Stage 25	S-shaped heart
Stage 26	Tail straightened; rudimentary fin bud in caudal region
Stage 27	Tail approaches s-heart and eye pigmentation evident
Stage 29	First occurrence of advanced hatch
D4	4 days post hatch

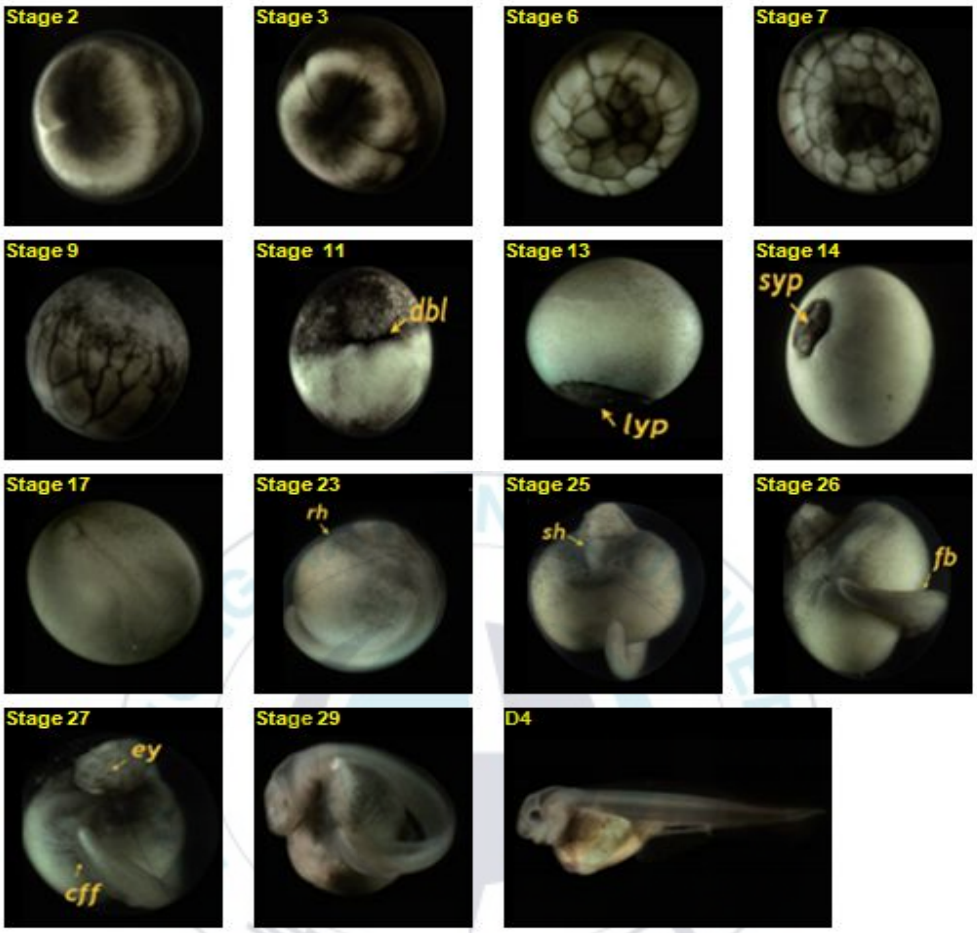

Figure 1. Embryonic developmental stages (Stage 2, Stage 3, Stage 6, Stage 7, Stage 9, Stage 11, Stage 13, Stage 14, Stage 17, Stage 23, Stage 25, Stage 26, Stage 27, Stage 29 and D4) of Siberian sturgeon (Park et al., 2013).

3. Results

3.1 cDNA nucleotide and amino acid sequence analysis

3.1.1 IL-1β gene

ataccgccagcaaagcaaagcgaagcgacgcagctgcactgcaatatacttcataacgac
acacagcgccacatcagcttgtgaaa**ATG**AGCTACAACACACGCGTATCTTCTGAAATGG
CAAACGAAATCGCCAGCGAGATCTTTGACTTTGAAGCACAACACTGCCCCCTGGAGGAGA
AGAGAATGCAAGATGAGCGGCCGCGGAAGGTCGGCAGGATGGGAGACAGCGGTCTCGAGT
TGCGGGTGTCCGGCTACGTGCGCTCCATGAGACAGGCAGTGACGCTGGTCCTGGCCGTGG
AGAAGATGAAGAGACCGCACAGGAAAGCGATCGGGTCCGAGTTTTCCGACTTAGACCTGC
TGAACATGTTGATGGAGCACGTGAATGAAGAGCACGTGATGCTGGAGGTGAATCCCGACT
TGGAGCGCTTTGAGACCGGGCTGAGACAGCGCCGCTCCGACGAGCCCCACTCGATAAGGG
ACTCCGAGAAGAAGTGCTGGGTGATGAACGAGCTGGATGGACGCCCCGCGGAGCTGACAG
CCAGCATGCTGCAGGGCCCCAACATCTCACAGCAAGTGAATCTGCGCCTCTCTACCTACA
TACCGCAGACCCAACCCGGATTGGGGCAGCCCGTGGTTCTGGGCATCGGGGGGAGTAATT
ACTATCTGTCCTGCAACGGCTCGCCCGACTCACCCGTCCTGGAGCTTGAGAAGGTGGAGA
ATCGGGATGGTTTGAAGACGATCAGCCTGAGCAGCGACGCGACCCGGTTCCTGTTTTACA
AGAGAGACAGCGGCAGCACCTCCACTTTCGAGTCGGCTCGGTTCCCCGGCTGGTTTATCA
GCACCGCCAGGAACGTGGACCGCGTGAGAGTGGCCATGTGTAACGAGAGAGCGTCCGCAG
GGCGCGTCACCGACTTCCAGGTGTCGCGCATT**tga**cgggagggacgcaacacggagcgac
ccacacaccaaaccaagcgttatttggtttaaatctatatataatgtatgtaccaagtac
acctctttataaaagaaaacggtaaacacttcgatgttcggttaccaatttcaggtagaa
gggacatacgtgtcctacaaaaacaaaacaacttttattattattattattattattatt
attattattattattatttgtttgtttaaaggaggctttgcacgggttcaggggttt
caaatttagtgacacgggtggggaactggtcggtcatccgaat

Figure 2. cDNA nucleotide sequence of Siberian sturgeon IL-1β gene. Start codon (ATG) and stop codon (tga) are bold and ORF is underlined. A mRNA instability motif is grey shaded.

The cDNA nucleotide sequence of Siberian sturgeon IL-1β contained 1243 bp with an open reading frame (ORF) of 849 bp. The 5' un translated region (UTR) and 3' un translated region (UTR) consisted of 86 bp and 308 bp, respectively. The 3' UTR contained one region of ATT repeats and one instability motif (ATTTA) (**Figure 2**).

MSYNTRVSSEMANEIASEIFD**FEAQHCPLEEKRMQDERPRKVGRMGDSGLE**
LRVSGYVRSMRQAVTLVLAVEKMKRPHRKAIGSEFSDLDLLNMLMEHVNEE
HVMLEVNPDLERFETGLRQRRSDEPHSIRDSEKKCWVMNELDGRPAELTAS
MLQGPNISQQVNLRLSTYIPQTQPGLGQPVVLGIGGSNYYLSCNGSPDSPV
LELEKVENRDGLKTISLSSDATRFLFYKRDSGSTSTFESARFPGWFISTAR
NVDRVRVAMCNERASAGRVTDFQVSRI*

Figure 3. The deduced amino acid sequences of Siberian sturgeon IL-1β cDNA. Interleukin-1 propeptides are bold. The interleukin-1 family signature is underlined. N-glycosylation sites are grey shaded.

The ORF of IL-1β cDNA nucleotide sequence translated into a 282 amino acids peptide with an estimated molecular weight (MW) of 31925.09 Da and a theoretical isoelectric point (p*I*) of 6.04**.** The amino acid sequences contained predicted interleukin-1 propeptide at 22-102 aa, two predicted N-

glycosylation sites at 159-161 aa and 197-199 aa. A clearly identifiable interleukin-1 family signature was conserved at 155-278 aa (**Figure 3**).

CLUSTAL 2.1 Multiple sequence alignment

```
A.baerii          MSYNTRVSSEMANEIASEIFDFEAQHCPLEEKRMQDERPRKVGRMGDSGLELRVSGYVRS 60
I.punctatus       ---MDDKDLLTLERSFDSDCGFDSDAMDFDELDCSDPLAMSGRCDLHEGLRIEVTKEPLS 57
T.rubripes        --------------------MESQMKSNESKMLQSQMP--------EGLELEITHHPLT 31
A.schlegelii      ------------------------------------MP--------EGLDLEIAHHPLT 15
D.rerio           MACGQYEVTIAPKNLWETDGAVYSD---SDEMDCSDPLAMSYRCDMHEGIRLGMWTSQHK 57
C.carpio          MAYHKYVHPLDLSEAFETDSAIYSDSADSDELDCPDPQSMSCQCDMHD-IKLELSSHPHS 59
S.salar           ---------------MEFESNYSLIKNTSESKAWSSKLP--------QGLDLEVSHHPIT 37
O.mykiss          ---------------MDFESNYSLIKNTSESAAWSSKLP--------QGLDLEVSHHPIT 37
E.coioides        --------------------MESQMTCNVSEMWSSRMP--------EGLELEISHHPLT 31
G.morhua          ---------------MAFQCEYSSMKQTTSSEHWSDRMP--------QGMDLEITNHPLT 37
P.olivaceus       --------------------MESKMECNVSQMWSAKMP--------QGLNLEISHHPMT 31
H.sapiens         MAEVPELASEMMAYYSGNEDDLFFEADGPKQMKCSFQDLDLCPLDGGIQLRISDHHYSKG 60
                                                                 : :

A.baerii          MRQAVTLVLAVEKMKR-PHRKAIGSEFSDLDLLNMLMEHVNEEHVMLEVNPDLER-FETG 118
I.punctatus       MRQVANVVIALQRLKL--TQNIQSTEFTDQELFNVFIENVIEESMVINLKCTESK----S 111
T.rubripes        MKSVVNLVIAMERLKGNRSESLLSTEFRDENLLSMMMDTIVEEQIVFERYSAPPV--QKY 89
A.schlegelii      MKRVVNLIIAMDRLKGKVSESPRGTEFTDDNLLNMLLESAVEERTVFERTAKP----AQY 71
D.rerio           MKQLVNVIIALNRMKH--IKPQST-EFGEKEVLDMLMANVIQEREVNVVDSVP------S 108
C.carpio          MRQVVNIIIAVERLKH--IKNMSSGKFCDEELLGFILENVIEERLVKPLNETP------I 111
S.salar           MRHIANLIIAMERLKGGEGVT-MGTEFKDKDLLNFLLESAVEEHIVLELESAPPTSRREA 96
O.mykiss          MRHIANLIIAMERLKGGEGVT-MGTEFKDKDLLNFLLESAVEEHIVLELESAPPASRRAA 96
E.coioides        MKHVANLIIATQRFKGIISESVLGTEFRDEHLLSIMLESIVEERNVFGCEATPPT--DED 89
G.morhua          MRQVVNLVIAMDRLKGSQSEKVQSSEFRDEDLLNLLLENALDEQLVLELTEAAPP--RGF 95
P.olivaceus       MRSVVNLIIAMERLKGSHSESVLSTSFTDENLLNIMMENIVEEHIVCERSSSPP-----D 86
H.sapiens         FRQAASVVVAMDKLRK--MLVPCPQTFQENDLSTFFPFIFEEEPIFFDTWDNEAY----V 114
                  ::  ..::::* ::::          * : .:  .:     :*  .

A.baerii          LRQRRSDEPHSIRDSEKKCWVMNE-LDGRPAELTASMLQGPNISQQVNLRLSTYIP-QTQ 176
I.punctatus       YSLQDKVVRCTICDKSKRALVQRE----KLPILLAFTLKGGNKDNKAWFNLSAYTPPNCT 167
T.rubripes        MRMSMQEY--SVSDSEKRSLIR----VPDSMELNAVMLQGGSECRKVNLNLSTYVH-LES 142
A.schlegelii      TYNFQSPY--SVMDSEQRHLVR----VPNSMEIHAVMLQGGTGNCQVQLNMATYLP-PTP 124
D.rerio           YTKTKNVLQCTICDQYKKSLVRSG----GSPHLQAVTLRAGSSDLKVRFSMSTYASPSAP 164
C.carpio          YNKTSLTLQCTICDKYKKTMVQSNKLSDEPLHLKAVTLSAGAMQYKVQFSMSTFVS-SAT 170
S.salar           GFSSTSQYECSVTDSENKCWVL----MSEAMELHAMMLQGGSGYHKVHLNLSTYVT-PVP 151
O.mykiss          GFSSTSQYECSVTDSENKCWVL----MNEAMELHAMMLQGGSSYHKVHLNLSSYVT-PVP 151
E.coioides        MITRTREYDCTVEDEEKRSLVR----VNNSLVLHAVMLQGGTDLKQVKLNMSMYLH-PAP 144
G.morhua          TAIEPSQQ-CMLRDHQKRSMVL----VKEAMGLHAIRLQGGTTDHEVSLNMSTYLD-PRP 149
P.olivaceus       QFSRRGVYTCNITDSQKRNFIL----VQNSMELHAVMLQGGSSNRKVLLNMSTYVH-PSP 141
H.sapiens         HDAPVRSLNCTLRDSQQKSLVMSG-----PYELKALHLQGQDMEQQVVFSMSFVQG---E 166
                            : *  ::  :           : *  * .     :. : ::
```

Figure 4. Multiple alignment of Siberian sturgeon IL-1β amino acid sequence with others known IL-1β sequence. Identical amino acids to the Siberian sturgeon sequence are indicated by asterisks (*), similar amino acid residues are indicated by dots (.).

```
A.baerii          -PGLGQPVVLGIGGSNYYLSCNGSPDS--PVLELEKVENRDGLKTISLSSDATRFLFYKR 233
I.punctatus       ENTKGQPVCLGIVKTNLFLSCTLENET--PFLGLEEVKDKERLKSIQENDGMERFLFFRN 225
T.rubripes        -SINAQTVALGIRGTNFFLSCHKDGEE--PTLHLEAVEDKEQLSSISSDSELVRFLFNKH 199
A.schlegelii      -STEAVTVTLCIKDTNLYLSCHKEGNE--PSLHLEAVNDKDSLLRITPGSDMVRFLFHKH 181
D.rerio           ATS-AQPVCLGISKSNLYLACCPAEGSTSPHLVLKEISGSLETIKAGDPNGYDQLLFFRK 223
C.carpio          QKE-AQPVCLGISNSNLYLACTQLDGS-SPVLILKEASGSVNTIKAGDPN--DSLLFFRK 226
S.salar           IETKARPVALGIKGSNLYLSCSKSGGR--PTLHLEEVANKEQLKSISQQSDMVRFLFYRR 209
O.mykiss          IETEARPVALGIKGSNLYLSCSKSGGR--PTLHLEEVADKDQLKSISQQSDMVRFLFYRR 209
E.coioides        -SVEGRTVALGIKGTQYYLTCRKDGTQ--PTLHLETIT-KDSLASIDPNSDMVRFLFYKQ 200
G.morhua          -SASAQPVALGIRGTKLYLSCTQKADR--PTLHLEEVENTDDLKSISKDSDMVRFLFYRT 206
P.olivaceus       -TIEARPVVLGIKDTDFFLSCQKNGAE--PTLHLERVENKCDLEAFSRDSEMVRFLFYKQ 198
H.sapiens         ESNDKIPVALGLKEKNLYLSCVLKDDK--PTLQLESVDPKNYPK-----KKMEKRFVFNK 219
                        .* * :  .. :*:*       * * *:             .     :. .

A.baerii          DSGS-TSTFESARFPGWFISTARNVDRVRVAMCNERASAGRVTDFQVSRI---------- 282
I.punctatus       GTGDSLNTFESVKYPGWFITTSKEDY--KPVQMCKQQS-SHLQLFTLHDETVVSQNEI-- 280
T.rubripes        ITGINLCTLVSVPYSDWYISTAVEEN--KPVEMCLESA-QRNTIFTLLALKKTPTCEGEM 256
A.schlegelii      VTGLNNSTLVSVPFSNWYISTAEENN--KPVDMCQESA-RRHRIFKFLPPK-PAVSNGEC 237
D.rerio           ETGSSINTFESVKCPGWFISTAYEDS--QMVEMDRKDT-ERIINFELQDKVRI------- 273
C.carpio          ETGTRYNTFESVKYPGWFISTAFDDW--EKVEMNQMPT-TRTTNFTLEDQKRI------- 276
S.salar           NTGVDISTLESAGFRNWFISTDMQQDNTKPVDMCQKAAPNRLTTFTIQRHN--------- 260
O.mykiss          NTGVDISTLESASFRNWFISTDMQQDYTKPVDMCQKAAPNRLTTFTIQRHN--------- 260
E.coioides        ISGVNVSTLMSVAHPNWYISTAEADN--MPVEMCQEST-SRYRAFTFSAIKEETPTA--- 254
G.morhua          DIGVSASSLVSALHSGWYISTATEDN--LPVEVCLQSE-SRYRSFTILQG---------- 253
P.olivaceus       DSGVSISTLMSARFPNWYISTSEQDN--RPVMVCQKNA-RCYQTFNIQHQS--------- 246
H.sapiens         IEINNKLEFESAQFPNWYISTSQAEN--MPVFLGGTKGGQDITDFTMQFVSS-------- 269
                        : *.   .*:*:*        .           * .
```

Figure 4. (Continued)

Multiple alignment of Siberian sturgeon IL-1β with other known teleost IL-1β, as well as human sequences revealed low level of identities. The highest identy showed to *Salmo salar* (45%), followed by *Oncorhynchus mykiss* (44%), *Gadus morhua* (39%), *Homo sapiens* (32%) and the lowest identy to *Ictalurus punctatus* (31%). As with all other known teleost IL-1β sequences, there was an absence of both a signal peptide and an IL-1β converting enzyme (ICE) cleavage site. The Siberian sturgeon IL-1β amino acid shared areas of good amino acid conservation, particularly within the predicted 12 β-sheets (**Figure 4**).

3.1.2 CTSB gene

agaggaagaatcaaaccagcaagagaaggcgaacgagctagctagcaaaaactgcaagacg
gcgttgggttcagtttactcacaaagc**ATG**TGGTGCCTAGTTGCCTTGCTCTGCCTCCTCT
CGGTGCTCGCCAGAGCACGTACGCTGCCTCATCTCCCACCTCTGTCAAATGAGCTCATCAA
CTACATCAACAAGGCCAACACCACCTGGAAGGCCGGACACAATTTCCCTAATGCTGACCCG
AGCTATGTTAAGAAGCTCTGTGGGACCTTCCTCAATGGCCCCAAGCTCCCTGTAATGGTGG
AGTACACTGGTGATGTCAAACTACCTGATAGCTTTGACCCCAGGGAGCAGTGGCCCAACTG
CCCGACAATCCAGGAGATCCGAGACCAGGGCTCTTGTGGCTCTTGCTGGGCTTTTGGTGCT
GCCGAGGCAATCTCTGACCGGTTCTGCGTTCAGTCTAATGGGAAGGTCAGCGTTGAGATCT
CTGCAGAGGATCTGCTCTCCTGCTGTGACTCGTGTGGCATGGGGTGTAATGGGGGGTATCC
CTCTGCAGCCTGGAACTTCTGGACCGAGAAAGGACTGGTATCTGGTGGATTATACGACTCG
CATGTGGGTTGCCGCCCATACTCAATCCCTCCCTGTGAGCACCATGTAAATGGCTCCCGAC
CCCCTTGCACTGGTGAAAAAGGAGACACTCCTAAGTGTGTGCTACAGTGTGAGGATGGATA
CAGCCCCTCCTACAAGCAAGACAAGCGCTATGGGGTGACGTCCTATGCAGTGCCCTCTTCG
GAGAAGCAGATCATGAGAGAGCTCTACAAGAATGGGCCAGTCGAGGGCGCTTTTACAGTCT
ATGAGGATTTCCTCATGTACAAGACCGGAGTGTACCAGCATGTGAGTGGTGAGGCTGTTGG
TGGTCACGCTATCAAGATCCTAGGCTGGGGAGTGGAGGGAAGCACCCCGTACTGGCTGGCA
GCTAACTCCTGGAACACTGACTGGGGTGAAAATGGGTTCTTTAAGATTCTGCGTGGACAGG
ATCACTGTGGAATCGAATCTGAGATTGTTGCTGGCAAGCCTAAAAGT**taa**tcctcttccaa
caggctttaaccaggaagtcaacagaacatcaccctctgtatcttcaccaggggtgtttct
gtagcaacacaaaattgacaaagcaccacttactgggggggcaggggaatagattggaccc
aatcctgctgtgagcgttgtcctaatatatggcttggagagacgcttgtttagctgcaaat
aataatgaaacagtgtataattcacatttagcatttttttttttttttttaaatcagtatt
tttattttaaaaatcacaagtatgatttacaactgtaatgtaaatagacagctcctaaaat
tgataggttgcagtgacttttcagcaaagattaaaatcttgaaactaacactgcatcgtat
aagttgagctgttaacaggaccag

Figure 5. cDNA nucleotide sequence of Siberian sturgeon CTSB gene. Start codon (ATG) and stop codon (taa) are bold and ORF is underlined. Two mRNA instability motifs are grey shaded.

The cDNA nucleotide sequence of Siberian sturgeon CTSB consisted of 1488 bp with an open reading frame (ORF) of 999 bp. It contained 88 bp length 5' UTR and 401 bp length 3' UTR. Two mRNA instability motifs (ATTTA) were harbored in 3' UTR (**Figure 5**).

MWCLVALLCLLSVLARARTLPHLPP**LSNELINYINKANTTWKAGHNFPNAD**
PSYVKKLCGTFLNGPKLPVMVEYTGDVKLPDSFDPREQWPNCPTIQEIRDQ
GSCGSCWAFGAAEAISDRFCVQSNGKVSVEISAEDLLSCCDSCGMGCNGGY
PSAAWNFWTEKGLVSGGLYDSHVGCRPYSIPPCEHHVNGSRPPCTGEKGDT
PKCVLQCEDGYSPSYKQDKRYGVTSYAVPSSEKQIMRELYKNGPVEGAFTV
YEDFLMYKTGVYQHVSGEAVGGHAIKILGWGVEGSTPYWLAANSWNTDWGE
NGFFKILRGQDHCGIESEIVAGKPKS*

Figure 6. The deduced amino acid sequences of Siberian sturgeon CTSB cDNA. signal peptides are bold, peptidase C1A propeptide domain is bold and underlined. Papain C-terminal domain is underlined. Active sites of cysteine, histidine and asparagines are grey shaded. Two N-glycosylation sites are boxed.

The ORF of CTSB cDNA nucleotide sequence translated into a putative peptide of 332 amino acid residues with a putative molecular mass of 36377.14 Da and a 5.78 isoelectric point (p*I*). The deduced amino acid sequence contained putative signal peptides (1-17 aa) and C1 peptidase

propeptide (26-66 aa). The papain family cysteine protease (80-329 aa), eukaryotic thiol protease cysteine (102-113 aa), thiol protease histidine (276-286 aa), thiol protease asparagine (293-312 aa) and two N-glycosylation sites (38-40 aa and 191-193 aa) were identified within the amino acid sequence of Siberian sturgeon CTSB (**Figure 6)**.

Multiple alignment of Siberian sturgeon CTSB sequence with those from other species showed the highest level of identity to the *Epinephelus coioides* (81%), followed by *Sparus aurata* (80%), *Ophichthus fasciatus* and *Lutjanus argentimaculatus* (78%), and the lowest level to *Cynoglossus semilaevis* (74%) whereas to *Homo sapiens* (76%). Alignment analyses also revealed that the cysteine active site (102-113 aa), the histidine active site (276-286 aa), the asparagine active site (293-312 aa), the N-glycosylation sites (38-40 aa and 191-193 aa) and the cysteine residues for the disulphide bridges of the deduced protein were well conserved in all CTSB sequences (**Figure 7**).

CLUSTAL 2.1 Multiple sequence alignment

Figure 7. Multiple alignment of the Siberian sturgeon CTSB amino acid sequence with other known CTSB sequences. The predicted N-glycosylation site is indicated by the box. Active site residues of cysteine, histidine, and asparagine are indicated by grey shadow. The conserved cysteine residues are indicated by down arrows (↓).

Figure 7. (Continued)

3.1.3 CTSD gene

The cDNA nucleotide sequence of Siberian sturgeon CTSD was 1686 bp and contained an open reading frame (ORF) of 1191 bp. The 5' un translated region (UTR) and 3' un translated region (UTR) contained 52 bp

accattcaaggaaccaacgtttagcattaacatccagaagaacaacaacata**ATG**AAATTCT
CGTATATAATCCTGTTTGCTGCCTTTATCGCGATCACCAATGCTATTGTCAGAATTCCTCTT
AAAAAGTTCCGATCTCTCAGAAGAACTCTGTCCGATGCTGGTAGAAGTGTTGAAGGGCTGGT
CTCCGAAGAACATAATTTAAAATACAATCTGGGCTTCCCTTCCAGTGATGGGCCTACCCCAG
AGACTCTCAAAAACTATCTTGATGCTCAGTACTATGGAGAGATTGGGATCGGGACTCCTCCT
CAGCTTTTCACTGTGGTCTTTGATACCGGCTCTTCCAACCTGTGGGTGCCTTCTATCCACTG
CTCGCTGACTGATATTGCTTGCTTGCTTCACCACAAGTATAACTCTGCAAAATCAAGCACCT
ACGTGAAGAATGGGGCAGCATTTGCAATCCAGTATGGCTCGGGTAGTCTTTCTGGGTATCTT
AGCCAAGATACGTGCAAGATTGGGGACATTTCTGTAGACGGTCAGGTGTTTGGGGAAGCCAT
CAAACAACCTGGGGTGGCGTTCATTGCTGCCAAGTTTGATGGAATCCTGGGCATGGCTTACC
CAAAGATCGCTGTGGATGAAGTGACTCCAGTCTTTGATAATATCATGAGTCAGAAAAAAGTC
GAAAAGAATGTCTTCTCTTTTTACCTAAACAGGAACCCAGCCACTCAACCAGGGGGTGAACT
GCTGCTGGGGGGAACTGATCCCAAATATTACACTGGAGAGTTCAACTACCTGAACATCACAC
GCCAGGCCTACTGGCAGATCCATATGGATGAGATGGGTGTTGGAAGCCAGCTGACTCTGTGC
AAGGGGGGTTGTGAAGCCATCGTGGACACTGGAACCTCGCTCATCACTGGACCGTCGGCAGA
GGTGAAAGCACTGCAGAAAGCCATCGGAGCTGTCCCACTCATCCAGGGGGAGTACATGGTTG
ATTGTAAGAAAGTGGCCACTCTTCCTGCCATCTCTTTCAAACTTGGAGGTCAGACCTACACC
CTGACAGGAGATCAGTACATCTTGAAGGAAAGTCAAGCCGGCCAGACTATCTGTCTGAGTGG
CTTCATGGGGCTTGACATCCCAGCCCCGGCTGGGCCTCTGTGGATCCTGGGAGATGTGTTTA
TTGGCCAGTATTACACTGAGTTTGATCGGGAAAACAACAGGGTGGGTTTTGCAAAATCGAAA
taaacgtgaccattttcacaatctgtgtgggaggggaaaaaaatactaaaattgcacacgc
attgtttccgttactcctacacactttagaaccaagtagcaatatgtttgcattaaaggtag
accccagtgactcatttattaatagatgagtgctttttctgttactaaatgcagaaaaaaaa
ataaaaagttcaattcaatgtgagagttgtaattcagggctccatgctgttctgtttttgtt
taggtttttggcaaaatgtttgttttatctgagatgatgatgcaacaggtttgtttgtttgg
ttttttaacaggaatgttagcaatgtctagtttaatgtaactgctattacgtatcagaaat
cgtacctcttgatgttgcagccttttgtgtaataattggatctaaataaattaacaagctg
caaaaaaaaaaa

Figure 8. cDNA nucleotide sequence of Siberian sturgeon CTSD gene. Start codon (ATG) and stop codon (taa) are bold and ORF is underlined. The polyadenylation signal is grey shaded.

and 443 bp respectively. The putative polyadenylation signal sequence (aataaa) was located 23 bp upstream of the poly-A tail in 3' UTR (**Figure 8**).

MKFSYIILFAAFIAITNAIVRIPLKKFRSLRRTLSDAGRSVEGLVSEEHNL
KYNLGFPSSDGPTPETLKNYLDAQYYGEIGIGTPPQLFTVVFDTGSSNLWV
PSIHCSLTDIACLLHHKYNSAKSSTYVKNGAAFAIQYGSGSLSGYLSQDTC
KIGDISVDGQVFGEAIKQPGVAFIAAKFDGILGMAYPKIAVDEVTPVFDNI
MSQKKVEKNVFSFYLNRNPATQPGGELLLGGTDPKYYTGEFNYLNITRQAY
WQIHMDEMGVGSQLTLCKGGCEAIVDTGTSLITGPSAEVKALQKAIGAVPL
IQGEYMVDCKKVATLPAISFKLGGQTYTLTGDQYILKESQAGQTICLSGFM
GLDIPAPAGPLWILGDVFIGQYYTEFDRENNRVGFAKSK*

Figure 9. The deduced amino acid sequences of Siberian sturgeon CTSD cDNA. The predicted signal peptides are bold and propeptides are bold and grey shaded. N-glycosylation site is boxed. Aspartic peptidase domain is underlined and aspartic peptidase active sites are grey shaded.

The ORF of Siberian sturgeon CTSD encodes a putative protein of 396 residues, predicted to have a molecular mass of 43037.27 Da and an isoelectric point (pI) of 6.24. The amino acid sequence contained a predicted signal peptide (1-18 aa), a propeptide domain (19-42 aa), a aspartic peptidase domain (7-395 aa), two aspartic peptidase active sites (91-102 aa and 278-289 aa) and one N-glycosylation site (249-251 aa) (**Figure 9**).

Multiple alignment of Siberian sturgeon CTSD amino acid sequence with those from other species showed the highest level of identity to the *Danio rerio* (85%), followed by *Ctenopharyngodon idella, Paralichthys olivaceus* and *Cynoglossus semilaevis* (83%), and the lowest level of identity to *Sparus aurata* (56%) whereas to *Homo sapiens* (69%). Alignment analyses revealed that the aspartic peptidase active sites (91-102 aa and 278-289 aa) were well conserved in all of the CTSD sequences. The deduced protein in all of the CTSD sequences showed well conserved two N-glycosylation sites (131-133 aa) except Siberian sturgeon and (249-251 aa) except silver carp, rainbow trout and zebrafish. The CTSD amino acid sequences of Siberian sturgeon (249-251 aa) and rainbow trout (131-133 aa) showed only one N-glycosylation site while all other species showed two sites **(Figure 10)**.

CLUSTAL 2.1 Multiple sequence alignment

Figure 10. Multiple alignment of Siberian sturgeon CTSD amino acid sequence with others known CTSD sequence. Identical amino acids to the Siberian sturgeon sequence are indicated by asterisks (*), similar amino acid residues are indicated by dots (.). The active sites and N-glycosylation sites are indicated by grey shade and the box, respectively.

Figure 10. (Continued)

3.2 Expression of IL-1β, CTSB and CTSD transcripts in embryonic developmental stages and early larvae stages

The RT-PCR data showed different mRNA expression level of development stages for the selected gene. IL-1β, CTSB and CTSD gene mRNA expression was found at every developmental stage but the relative expression levels of different development stages were different for the genes.

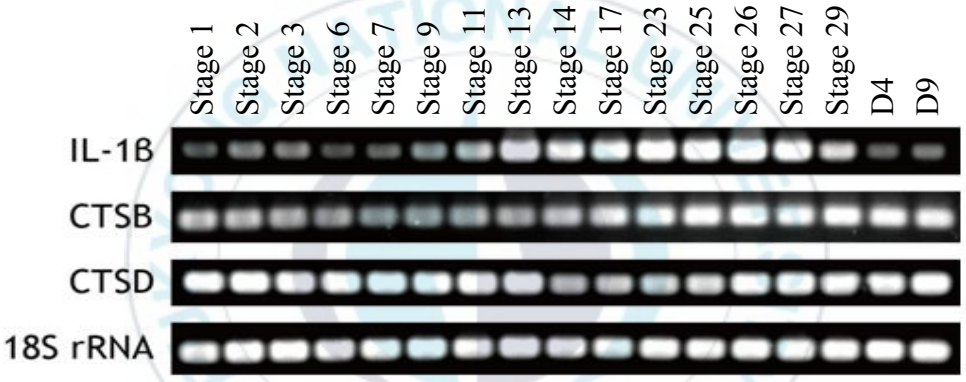

Figure 11. RT-PCR analysis of Siberian sturgeon IL-1β, CTSB and CTSD gene mRNA expression in embryonic development stages. Such as Stage 1, Stage 2, Stage 3, Stage 6, Stage 7, Stage 9, Stage 11, Stage 13, Stage 14, Stage 17, Stage 23, Stage 25, Stage 26, Stage 27, Stage 29, D4 and D9 where 18S rRNA served as internal reference. Confirmed by 1.5% agarose gel electrophoresis.

The expression level of IL-1β gene was at a low level in early embryonic developmental stages and was up regulated from stage 13 (formation of large yolk plug) to stage 29 (first occurrence of advanced hatch), and CTSB gene mRNA expression level was up regulated for all the development stages. High level of expressions were found for CTSD gene of all development stages except from stage 14 (formation of small yolk plug) to stage 25 (S-shaped heart) (**Figure 11**).

3.3 Tissue-specific expression of IL-1β, CTSB and CTSD transcripts

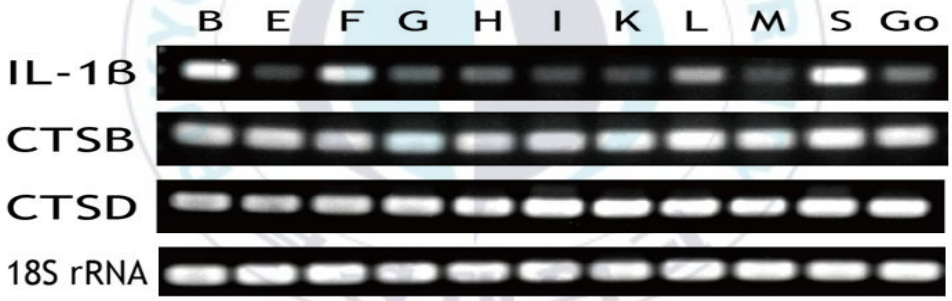

Figure 12. RT-PCR analysis of Siberian sturgeon IL-1β, CTSB and CTSD gene mRNA expression in tissues. Such as Brain (B), Eye (E), Fin (F), Gill (G), Heart (H), Intestine (I), Kidney (K), Liver (L), Muscle (M), Spleen (S) and Gonad (Go). Confirmed by 1.5% agarose gel electrophoresis.

In the tissue-specific expression study, constitutive expressions of CTSB, CTSD and IL-1β transcripts were detected in all the examined tissues. The expression of CTSB and CTSD transcripts were relatively high in all examined tissue but IL-1β gene mRNA transcript was expressed relatively high in brain, fin, liver, and spleen than other tissue (**Figure 12)**.

4. Discussion

The innate immune system involves a large number of molecules dissolved in body fluids that provides an array of protective mechanisms that are inherently available, and offer immediate and permanent protection against a wide variety of pathogens. In this study, cDNA nucleotide sequence of three innate immune related genes from Siberian sturgeon, IL-1β (a pro-inflamatory cytokines), cathepsin B (a cysteine cathepsin), and cathepsin D (an aspartic cathepsin) were isolated, cloned, sequenced and analyzed.

The identified cDNA nucleotide sequence of Siberian sturgeon IL-1β encoded 282 amino acids that showed a low degree of identity with known fish and mamal IL-1β cDNAs. The highest identity was with *Salmo salar* IL-1β (45%). The deduced amino acid well conserved interleukin-1 propeptide, clearly identifiable IL-1 family signature, indicating the deduced amino acid belongs to pro inflammatory interleukin-1 family. The nucleotide sequence contained consensus sequence (ATTTA) in the 3'UTR

which was known to be important for mRNA degradation (Sachs AB, 1993) but no discernable polyadenylation signal (AATAAA) was identified, that indicated the sequence may not be complete. The Siberian sturgeon IL-1β, like other teleosts molecules did not appear to have a signal peptide and an IL-1β converting enzyme (ICE) cleavage site. This is a common feature of IL-1β molecules which are synthesized as precursors and go through a non-classical secretion pathway (Dinarello, 1997).

The deduced amino acids of Siberian sturgeon CTSB consisted of 332 aa residues and possessed a signal peptide, propeptide, mature peptide regions and papain family cysteine protease domain like other all known CTSB profiles. The multiple alignments results revealed that Siberian sturgeon CTSB with other known CTSB contained most of the common features, including N-glycosylation sites, cysteine residues for the disulphide bridges of the deduced protein and high level conservation of the cysteine, histidine and asparagine active sites which play important roles in the formation and stabilization of the catalytic site of the activated enzyme. Lecaille et al., 2002 reported that all papain-like cysteine proteases contain conserved active sites of cysteine, histidine and asparagine residues which

play crucial roles in the formation and stabilization of the catalytic site of the activated enzyme.

The full-length CTSD cDNA of Siberian sturgeon encoded a peptide of 396 aa which were very similar to most of the teleosts. The deduced amino acids of CTSD possessed the signal peptide, propeptide domain, aspartic peptidase domain, and polyadenylation signal, the same as all known CTSD profiles. The deduced amino acid sequences consisted of two aspartic peptidase active sites, which were estimated to generate angiotensin I from angiotensinogen in the plasma (Balakrishnan S et al., 2010). The N-glycosylation site may be important for cathepsin transportation into lysosomes (Shi GP et al., 1992) but Siberian sturgeon CTSD amino acid contains only one N-glycosylation site like rainbow trout whereas, other known CTSD had two or more N-glycosylation sites.

Fish eggs, hatched embryos and larvae need to have defensive factors to live in a pathogenically hostile environment. For that, the developmental stages needs to be well-protected by several innate and adaptive immune substances. RT-PCR was performed to investigate the

expression pattern of IL-1β, CTSB and CTSD at the embryonic development stages and early larvae stages. The result showed that all three transcripts were expressed at every embryonic and larval developmental stage. According to S Cecchini et al., 2013, IL-1β gene expressions appeared about 7 days before hatching, probably indicating a preparation of the brown trout immune system for the post-hatching period. In *Cyprinus carpio,* IL-1β expression in embryos implied a functional embryonic innate defense system (Huttenhuis HBT et al., 2006). In rainbow trout at the yolk-sac larval stage the expression of IL-1β was up regulated by the infection in 3 month-old fry (Chettri JK et al., 2012). He also mentioned that at the yolk-sac stage larvae might be covered by protective shield of different immune factors which protect against a broad range of pathogens. Cathepsin activity has been described in eggs and larvae of different fish species like sea bass (Carnevali O et al., 2001), cod (Magnadottir B et al., 2004), and rainbow trout (Brooks S et al., 1997). Its primary function is believed to be the digestion of the yolk proteins in oocytes, fertilized eggs and the yolk sac, contributing to the quality and viability of the eggs and the energy source of the embryo (Carnevali O et al., 1999). In fish, CTSD appears to be of major

importance for digestion of yolk proteins in developing eggs (Press CM and Evensen O., 1999; Romano M et al., 2004).

In the tissue specific expression study, RT-PCR results demonstrated constitutive tissue-specific expression patterns for IL-1β in all tested eleven tissues. This indicates that expression of IL-1β genes does not necessarily need to be subject to triggering by pathogens. In common carp, IL-1β was constitutively expressed in several organs such as head kidney, spleen and brain (Engelsma MY et al., 2001), and constitutively expression was seen for the IL-1β gene in the RTS11 macrophage cell line from rainbow trout (Brubacher JL et al., 2000).

RT-PCR results showed ubiquitously expression of CTSB and CTSD in all tested eleven tissues. These results suggested that both CTSB and CTSD genes may be involved in protease function actively. The higher level of CTSB and CTSD expressions in liver, spleen, kidney and intestine should be related to the immune responses and digestive functions. In miiuy croaker, higher level in immune tissues and bacterial infection can increase the expression of the CTSB gene may suggest that CTSB play key roles in

innate immune response in miiuy croaker (R. Che et al., 2014). Similarly, ubiquitously expressed CTSB was identified in all examined tissues of flounder, rock bream, orange spotted grouper and up regulated by pathogen challenge (Zhang FT et al., 2008; Whang I et al., 2011; S. Wei et al., 2014).

It has been reported that the CTSD is involved in post mortem degradation of muscle proteins and thus contributes to textural changes in fish, because CTSD from fish muscle is capable of cleaving muscle myofibrillar proteins (Cheret R et al 2007). CTSD was detected higher expression levels in liver, head kidney, intestine, gill and spleen in turbot (Jia A and Zhang XH., 2009). In most fish species, CTSD was found to be mainly expressed in the spleen and liver (Wang PA et al., 2007), but it is also abundant in skin mucosa (Cho JH et al., 2002) and muscle based on protein levels (Nielsen LB and Nielsen HH., 2001). Because of major functions in protein degradation in the lysosomes and production of parasin I (an antimicrobial peptide), it is believed that cathepsins are more important for innate immunity (Cho JH et al., 2002). These observations indicated an involvement of CTSB and CTSD in host innate immune response.

5. Conclusion

The innate immune system is the fundamental defense weapon of fish at early stage of life and it is the only defense mechanism until the complete development of the acquired immune system. In this study, three innate immune related gene IL-1β, CTSB and CTSD cDNA nucleotide sequence and amino acid sequence were isolated, cloned and analyzed from Siberian sturgeon. The expression level of IL-1β, CTSB and CTSD transcript in embryonic developmental stages and different tissues were also described. The ubiquitously expression of IL-1β, CTSB and CTSD in every embryonic developmental stage and in all tested tissues revealed that these three genes may be played a vital role in the immune functions of Siberian sturgeon. The generated information of this study could contribute to better understand the embryonic and larval innate immune system of Siberian sturgeon. Despite this, further studies including expression analysis in unfertilized eggs, blood tissue and in response to a bacterial challenge need to be done for confirmation and analyze the detailed immune related functions of IL-1β, CTSB and CTSD genes in Siberian sturgeon.

6. Acknowledgements

I would like to express my cordial thanks and gratitude to my supervisor Prof. Nam Yoon Kwon, Department of Marine Bio-Materials and Aquaculture, Pukyong National University for his scholastic guidance, valuable advice and inspiration to complete this work.

I express deep sense of gratitude to Prof. Kim Hyun-woo and Prof. Gong Seung Pyo for managing their valuable times for reviewing this thesis, making valuable suggestions and criticisms as a chairman and member, respectively, of my thesis advisory committee.

I am grateful to KOICA-PKNU International Graduate program of Fisheries science from KOICA for providing me fund for this study. I also like to express gratitude to the authority of Department of Fisheries and Ministry of Fisheries and Livestock, Bangladesh to grant study leave for this program.

It is my pleasure to thank my laboratory colleagues including Mr. Lee Sang Yoon for his instruction and support in molecular works, Victor David Gultom, Lee Jung Ae and Kim Eun Jeong for their kind assistance in laboratory experiment.

I would like to thank Prof. KANG Kyoungmi, Ph.D. and Ms. KIM Sulki for facilitating necessary steps and generous assistance to complete my study.

Finally, I extend my heartfelt thanks to my wife, daughters and parents for encouraging and provide mental strength to complete this work.

7. References

Balakrishnan S, Pannerselvam S, Samuel S. 2010. Functional annotation of renin and angiotensin converting enzymes by sequence analysis. BIOMIRROR 3: 28-37.

Baldocchi RA, Tan L, King DS, Nicoll CS. 1993. Mass spectrometric analysis of the fragments produced by cleavage and reduction of rat prolactin: evidence that the cleaving enzyme is cathepsin D. Endocrinology 133: 935-938.

Bemis WE, Findeis EK, Grande L. 1997. An overview of Acipenseriformes. Environ Biol Fish 48: 25-71.

Bensi G, Raugei G, Palla E, Carinci V, Tornese Buonamassa D, Melli M. 1987. Human interleukin-1β gene. Gene 52: 95–101.

Bird S, Wang T, Zou J, Cunningham C, Secombes CJ. 2002. The first cytokine sequence within cartilaginous fish: IL-1β in the small spotted cat shark (*Scyliorhinus canicula*). Journal of Immunology 168: 3329–3340.

Blomgran R, Zheng L, Stendahl O. 2007. Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization. J Leukoc Biol 81: 1213-1223.

Bobe J. & Labbe C. 2010. Egg and sperm quality in fish. General and Comparative Endocrinology 165: 535–548.

Bronzi P, Rosenthal H, Gessner J. 2011. Global sturgeon aquaculture production: An overview. J. of Applied Ichthyology 27: 169–175.

Brooks S, Tyler CR, Carnevali O, Coward K, Sumpter JP. 1997. Molecular characterisation of ovarian cathepsin D in the rainbow trout, *Oncorhynchus mykiss*. Gene 201: 45-54.

Brubacher JL, Secombes CJ, Zou J, Bols NC. 2000. Constitutive and LPS-induced gene expression in a macrophage-like cell line from the rainbow trout (*Oncorhynchus mykiss*). Dev Comp Immunol 24: 565-574.

Capasso C, Lees WE, Capasso A, Scudiero R, Carginale V, Kille P, et al., 1999. Cathepsin D from the liver of the antarctic ice fish *Chionodraco hamatus* exhibits unusual activity and stability at high temperatures. Biochim Biophys Acta 1431: 64-73.

Carnevali O, Carletta R, Cambi A, Vita A, Bromage N. 1999. Yolk formation and degradation during oocyte maturation in seabream *Sparus aurata*: involvement of two lysosomal proteinases. Biol Reprod 60:140-146.

Carnevali O, Mosconi G, Cambi A, Ridolfi S, Zanuy S, Polzonetti-Magni AM. 2001. Changes of lysosomal enzyme activities in sea bass (*Dicentrarchus labrax*) eggs and developing embryos. Aquaculture 202: 249-256.

Carnevali O, Centonze F, Brooks S, Marota I, Sumpter JP. 1999. Molecular cloning and expression of ovarian cathepsin D in seabream, *Sparus aurata.* Biol Reprod 61: 785-791.

Cha IS, Kwon J, Mun JY, Park SB, Jang HB, Nho SW, et al., 2012. Cathepsins in the kidney of olive flounder, *Paralichthys olivaceus*, and their responses to bacterial infection. Dev Comp Immunol 38: 538-544.

Cheret R, Delbarre-Ladrat C, de Lamballerie-Anton M, Verrez-Bagnis V. 2007. Cal- pain and cathepsin activities in post mortem fish and meat muscles. Food Chem 101: 1474-1479.

Chettri JK, Raida MK, Kania PW, Buchmann K. 2012. Differential immune response of rainbow trout (*Oncorhynchus mykiss*) at early developmental stages (larvae and fry) against the bacterial pathogen Yersinia ruckeri. Dev Comp Immunol 36: 463-474.

Cho JH, Park IY, Kim HS, Lee WT, Kim MS, Kim SC. 2002. Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. FASEB J 16: 429-431.

Chulhong Park, Sang Yoon Lee, Dong Soo Kim and Yoon Kwon Nam. 2013. Embryonic Development of Siberian Sturgeon *Acipenser baerii* under Hatchery Conditions: An Image Guide with Embryological Descriptions. Fish Aquat Sci 16(1): 15-23.

Colbert JD, Matthews SP, Miller G, Watts C. 2009. Diverse regulatory roles for lysosomal proteases in the immune response. Eur J Immunol 39: 2955-2965.

Conte FS, Doroshov SI, Lutes PB, Strange EM. 1988. Hatchery manual for white sturgeon, *Acipenser transmontanus* R., with application to other North American Acipenseridae. Div. Agric. Nat. Res., University of California, Oakland p. 67.

Conus S, Simon HU. 2010. Cathepsins and their involvement in immune responses. Swiss Med Wkly 140: w13042.

Corripio-Miyar Y, Bird S, Tsamopoulos K, Secombes CJ. 2007. Cloning and expression analysis of two pro-inflammatory cytokines, IL-1β and IL-8, in haddock (*Melanogrammus aeglefinus*). Molecular Immunology 44: 1361–1373.

Dinarello CA. 1997. Interleukin-1. Cytokine & Growth Factor Reviews 8: 253–265.

Dong ZD, Zhang J, Ji XS, Zhou FN, Fu Y, Chen W, et al., 2012. Molecular cloning, characterization and expression of cathepsin D from grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol 33: 1207-1214.

Engelsma MY, Stet RJ, Schipper H, Verburg-van Kemenade BM. 2001. Regulation of interleukin 1β RNA expression in the common carp, *Cyprinus carpio* L. Dev Comp Immunol 25: 195-203.

Fujiki K, Shin D-H, Nakao M, Yano T. 2000. Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1β, high affinity immunoglobulin E Fc receptor gamma subunit and serum amyloid A. Fish & Shellfish Immunology 20: 229–242.

Goldmann T, Moorkamp A, Wiedorn KH, Suter L, Otto F. 2001. The prognostic value of the expression of collagenase IV, cathepsin D and metallothionein in squamous cell carcinomas of the skin determined by immunohistochemistry. Arch Dermatol Res 293: 115-120.

Ha SD, Martins A, Khazaie K, Han JH, Chan BMC, Kim SO. 2008. Cathepsin B is involved in the trafficking of TNF-alpha-containing vesicles to the plasma membrane in macrophages. J Immunol 181: 690-697.

Hamlin HJ, Moore BC, Edwards TM, Larkin IL V, Boggs A, High WJ B, Guillette LJ. 2008. Nitrate-induced elevations in circulating sex steroid concentrations in female Siberian sturgeon (*Acipenser baeri*) in commercial aquaculture. Aquaculture 281: 118–125.

Hetman M, Perschl A, Saftig P, Von Figura K, Peters C. 1994. Mouse cathepsin D gene: molecular organization, characterization of the promoter, and chromosomal localization. DNA Cell Biol 13: 419-427.

Hong S, Zou J, Crampe M, Peddie S, Scapigliata G, Bols N, et al., 2001. The production and bioactivity of rainbow trout (*Oncorhynchus mykiss*) recombinant IL-1β. Vet Immu and Immu 81: 1–14.

Huttenhuis HBT, Taverne-Thiele AJ, Grou CPO, Bergsma J, Saeij JPJ, Nakayasu C,et al., 2006. Ontogeny of the common carp (*Cyprinus carpio* L.) innate immune system. Dev Comp Immunol 30: 557-574.

International Union for Conservation of Nature (IUCN). 2011. *Acipenser baerii.* Retrieved from http://www.iucnredlist.org/apps/redlist/details/244/0.

Jia A and Zhang XH. 2009. Molecular cloning, characterization and expression analysis of cathepsin D gene from turbot, *Scophthalmus maximus*. Fish Shellfish Immunol 26: 606-613.

Jiang SG, Zhang DC, Li JZ, Liu ZX. 2008. Molecular characterization, recombinant expression and bioactivity analysis of the interleukin-1β from the yellowfin sea bream, *Acanthopagrus latus* (Houttuyn). Fish & Shellfish Immunology 24: 323–336.

Jiang ST, Wang YT, Chen CS. 1991. Purification and characterization of a proteinase identified as cathepsin D from tilapia muscle (*Tilapia nilotica, Tilapia aurea*). J Agric Food Chem 39: 1597-1601.

Kolman H. 2002. Primary humoral response in Siberian sturgeon after exposure to anti-furunculosis bacterin. Czech Journal of Animal Science 47: 183–188.

Kurokawa T, Uji S, Suzuki T. 2005. Identification of pepsinogen gene in the genome of stomachless fish, *Takifugu rubripes*. Comp Biochem Physiol B Biochem Mol Biol 140: 133-140.

Kwon JY, Prat F, Randall C, Tyler CR. 2001. Molecular characterization of putative yolk processing enzymes and their expression during oogenesis and embryogenesis in rainbow trout (*Oncorhynchus mykiss*). Biol Reprod 65: 1701-1709.

Lecaille F, Kaleta J, Bromme D. 2002. Human and parasitic papain-like cysteine proteases: their role in physiology and pathology and recent developments in inhibitor design. Chem Rev 102: 4459-4488.

Lee DC, Womble TA, Mason CW, Jackson IM, Lamango NS, Severs WB, et al., 2007. 6-Hydroxydopamine induces cystatin C-mediated cysteine protease suppression and cathepsin D activation. Neurochem Int 50: 607-618.

Lee D-S, Hong SH, Lee H-J, Jun LJ, Chung J-K, Kim KH, et al., 2006. Molecular cDNA cloning and analysis of the organization and expression of the IL-1β gene in Nile tilapia, *Oreochromis niloticus*. Comparative Biochemistry and Physiology 143: 307–314.

Ludwig A. 2008. Identification of Acipenseriformes species in trade. J Appl Ichthyol 24: 2-19.

Lu DQ, Bei JX, Feng LN, Zhang Y, Liu XC, Wang L, et al., 2008. Interleukin-1β gene in orange-spotted grouper, *Epinephelus coioides:* molecular cloning, expression, biological activities and signal transduction. Molecular Immunology 45: 857–867.

Lv-yun Zhu, Li Nie, Guan Zhu, Li-xin Xiang, Jian-zhong Shao. 2013. Advances in research of fish immune-relevant genes: A comparative overview of innate and adaptive immunity in teleosts. Developmental and Comparative Immunology 39: 39–62.

Magnadottir B. 2006. Innate immunity of fish (overview). Fish & Shellfish Immunology 20: 137–151.

Magnadottir B, Lange S, Steinarsson A, Gudmundsdottir S. 2004. The ontogenic development of innate immune parameters of cod (*Gadus morhua* L.). Comp Biochem Biophys 139: 217-224.

Matsumoto F, Saitoh S, Fukui R, Kobayashi T, Tanimura N, Konno K, et al., 2008. Cathepsins are required for Toll-like receptor 9 responses. Biochem Biophys Res Commun 367: 693-699.

Mohler JW, King MK, Farrell PR. 2000. Growth and survival of first-feeding and fingerling Atlantic sturgeon under culture conditions. N. Am. J. Aquacult 62: 174–183.

Nicola NA. 1994. Introduction to cytokines and their receptors. Guidebook to cytokines. Oxford University Press.

Nielsen LB, Nielsen HH. 2001. Purification and characterization of cathepsin D from herring muscle (*Clupea harengus*). Comp Biochem Physiol B Biochem Mol Biol 128: 351-363.

Park EM, Kim YO, Nam BH, Kong HJ, Kim WJ, Lee SJ, et al., 2009. Cloning and expression analysis of cathepsin D in the oliver flounder, *Paralichthys olivaceus*. Biosci Biotechnol Biochem 73: 1856-1859.

Press CM, Evensen O. 1999. The morphology of the immune system in teleost fishes. Fish Shellfish Immunol 9:309–318.

Rafatnezhad S and Falahatkar B. 2011. Nitrogenous compounds and oxygen concentration as the key density dependent factors to optimize growth of beluga *Huso huso* (Actinopterygii: Acipenseriformes: Acipenseridae), in circular fiberglass tanks. Acta Ichthyologica Et Piscatoria 41: 285–291.

Rafatnezhad S, Falahatkar B, Tolouei Gilani MH. 2008. Effects of stocking density on haematological parameters, growth and fin erosion of great sturgeon (*Huso huso*) juveniles. Aquaculture Research 39: 1506–1513.

Redecker B, Heckendorf B, Grosch HW, Mersmann G, Hasilik A. 1991. Molecular organization of the human cathepsin D gene. DNA Cell Biol 10: 423-431.

Riggio M, Scudiero R, Filosa S, Parisi E. 2000. Sex- and tissue-specific expression of aspartic proteinases in *Danio rerio* (zebrafish). Gene 260: 67-75.

Romano M, Rosanova P, Anteo C, Limatola E. 2004. Vertebrate yolk proteins: a review. Mol Reprod Dev 69: 109–116.

R Che, Rixin Wang, Tianjun Xu. 2014. Comparative genomic of the teleost cathepsin B and H and involvement in bacterial induced immunity of miiuy croaker. Fish & Shellfish Immunology 41: 163-171.

Ruchin AB. 2007. Effect of photoperiod on growth, physiological and hematological indices of juvenile Siberian sturgeon *Acipenser baerii*. Biology Bulletin 34: 583–589.

Sachs AB. 1993. Messenger RNA degradation in eukaryotes. Cell 74: 413-421.

Sadati M A Y, Pourkazemi M, Shakurian M, Hasani MHS, Pourali HR, Pourasaadi M, Yousefi A. 2011. Effects of daily temperature fluctuations on growth and hematology of juvenile *Acipenser baerii*. Journal of Applied Ichthyology 27: 591–594.

Salazar-Mather TP, Hokeness KL. 2006. Cytokine and chemokine networks: pathways to antiviral defense. Curr. Top. Microbiol. Immunol 303: 29–46.

Saurabh S and Sahoo PK. 2008. Lysozyme: an important defence molecule of fish innate immune system. Aquaculture Research 39: 223-239.

Savan R and Sakai M. 2006. Genomics of fish cytokines. Comp Biochem Physiol Part D: Genom Proteom 1: 89–101.

Scapigliati G, Buonocore F, Bird S, Zou J, Pelegrin P, Falasca C, et al., 2001. Phylogeny of cytokines: molecular cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1β. Fish & Shellfish Immunology 11: 711–726.

Shina Wei, Youhua Huang, Xiaohong Huang, Jia Cai, Yang Yan, Chuanyu Guo, Qiwei Qin. 2014. Characterization of cathepsin B gene from orange-spotted grouper, *Epinephelus coioides* involved in SGIV infection. Fish & Shellfish Immunology 36: 194-205.

Shi GP, Munger JS, Meara JP, Rich DH, Chapman HA. 1992. Molecular cloning and expression of human alveolar macrophage cathepsin S, an elastinolytic cysteine protease. J Biol Chem 267: 7258-7262.

Stefano Cecchini, Mariateresa Paciolla, Elio Biffali, Marco Borra, Matilde V. Ursini, Maria B. Lioi. 2013. Ontogenetic profile of innate immune related genes and their tissue specific expression in brown trout, *Salmo trutta* (Linnaeus, 1758). Fish & Shellfish Immunology 35: 988-992.

Takashi Aoki, Tomokazu Takano, Mudjekeewis D. Santos, Hidehiro Kondo and Ikuo Hirono. 2008. Molecular Innate Immunity in Teleost Fish: Review and Future Perspectives. Fisheries for Global Welfare and Environment, 5th World Fisheries Congress 263–276.

Telford J, Macchia G, Massone A, Carinci V, Palla E, Melli M. 1986. The murine interleukin-1β gene: structure and evolution. Nucleic Acids Research 14: 9955–9963.

Ullal AJ, Litaker RW, Noga EJ. 2008. Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (*Ictalurus punctatus*, Rafinesque). Dev. Comp. Immuno 32: 1301–1312.

Wang PA, Stenvik J, Larsen R, Maehre H, Olsen RL. 2007. Cathepsin D from Atlantic cod (*Gadus morhua* L.) liver. Isolation and comparative studies. Comp Biochem Physiol B Biochem Mol Biol 147: 504-511.

Wang YP, Wang Q, Baoprasertkul P, Peatman E, Liu ZJ. 2006. Genomic organization, gene duplication, and expression analysis of interleukin-1β in channel catfish (*Ictalurus punctatus*). Molecular Immunology 43: 1653–1664.

Weining KC, Sick C, Kaspers B, Staeheli P. 1998. A chicken homolog of mammalian interleukin-1β: cDNA cloning and purification of active recombinant protein. European Journal of Biochemistry 258: 994-1000.

Wei Q, He J, Yang D, Zheng W, Li L. 2004. Status of sturgeon aquaculture and sturgeon trade in China: a review based on two recent nationwide surveys. J Appl Ichthyol 20: 321-332.

Wei SN, Huang YH, Huang XH, Cai J, Yan Y, Guo CY, et al., 2014. Characterization of cathepsin B gene from orange-spotted grouper, *Epinephelus coioides* involved in SGIV infection. Fish Shellfish Immunol 36: 194-205.

Wei QW, Zou Y, Li P, Li L. 2011. Sturgeon aquaculture in China: Progress, strategies and prospects assessed on the basis of nation-wide surveys (2007–2009). Journal of Applied Ichthyology 27: 162–168.

Whang I, De Zoysa M, Nikapitiya C, Lee Y, Kim Y, Lee S, et al., 2011. Molecular characterization and expression analysis of cathepsin B and L cysteine proteases from rock bream (*Oplegnathus fasciatus*). Fish Shellfish Immunol 30: 763-772.

Whyte SK. 2007. The innate immune response of finfish- A review of current knowledge. Fish & Shellfish Immunology 23: 1127-1151.

Williot P, Sabeau L, Gessner J, Arlati G, Bronzi P, Gulyas T, et al., 2001. Sturgeon farming in Western Europe: recent developments and perspectives. Aquat Living Resour 14: 367-374.

Wlasow T, Fopp-Bayat D. 2011. The effect of thermal shock on morphological characteristics of blood cells in Siberian sturgeon (*Acipenser baerii*) triploids. Acta Veterinaria 80: 215–218.

Zhang FT, Zhang YB, Chen YD, Zhu R, Dong CW, Li YY, et al., 2008. Expressional induction of *Paralichthys olivaceus* cathepsin B gene in response to virus, poly I:C and lipopolysaccharide. Fish Shellfish Immunol 25: 542-549.