Thesis for the Degree of Master of Fisheries Science

Physiological and cellular responses of marine medaka fish (*Oryzias dancena*) to ammonia stress treatment

by

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KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

Pukyong National University

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바다송사리에서 암모니아 스트레스에 대한 세포생리학적 분석

Advisor: Prof. NAM Yoon Kwon

by

Ali Rashid Hamad

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Abstract

Ammonia is regarded as one of the most widely distributed chemical in aquatic environment as it enters to the aquatic environment by sewage effluents, also is produced by some aquatic organisms and can adversely pose physiological effects to the fish when is in high concentration or allowed to get in to contact with fish a long period of time. In order to understand those effects to fish, marine medaka fish (*Oryzias dancena*) which is model fish was exposed to ammonia in different concentrations. Adult medaka fish was exposed to 1.5 ppm for 24, 48 and 96 hours and juvenile medaka fish was treated with 0, 4.5, 9.0, 18 and 36 ppm for 48 hours and expression of gill and kidney anti-oxidant enzymes like catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and

superoxide dismutase (SOD) were measured using semi quantitative PCR and the result observed showed that the fish (*O. dancena*) responded by exhibiting expression of target genes as physiological mechanism to protect the body.

On the other hand, juvenile medaka fish also responded by showing differences in expression patterns between control group (0ppm) and experiment groups (4.5, 9, 18 and 36 ppm) signifying that ammonia can cause stress to the studied fish.

Keywords: Ammonia, physiological effects, anti-oxidant enzymes and *Oryzias dancena*.

1. Introduction

1.1. General introduction

Living systems encounter a variety of stresses during their continuous interaction with environment. (Lushchak and Stoliar, 2012). All organisms are strongly affected by their surrounding environment, and the environmental factors play an important part in shaping ecology and evolution of biological systems. Environmental stress is especially important at many levels of biological organization (Hoffmann and Parsons 1997; Hoffmann and Hercus 2000). The interactions between fish and their environment, specifically how the physiology of fishes is affected by and regulated in response to environmental factors (fish ecophysiology), provides the key to understanding the survival and maintenance of fish populations in changing aquatic environments (Rankin & Jensen, 1993).

Fish are the most diverse marine vertebrates. Therefore, diverse fish species are necessary for evaluating pollutant toxicity and understanding the mechanisms of action underlying this toxicity. Fish can also be useful as model species to study early warning signals for the effects of anthropogenic substances (Kim, et al, 2016).

Fish are particularly threatened by aquatic pollution, and the environmental stress they face may help shape their ecology, evolution, or biological systems (Sorensen and Loeschcke 2007). Fish are exposed to stressors both in the wild and in artificial conditions such as in the laboratory or in aquaculture.

The intensity and duration of exposure to the stressor, to a large extent, may determine whether the animal is capable of coping with the stressor. The categories of environmental, physical and biological stressors help to group the diverse possible stressors into a few themes (Iwama et al., 1999). Environmental stressors mainly include adverse chemical conditions of the water. Although pollutants are common environmental stressors, extreme conditions or changes in water quality parameters such as dissolved oxygen, ammonia, hardness, pH, gas content and partial pressures, and temperature can stress fish. High water concentrations of metals such as copper, cadmium, zinc and iron can also cause stress and death in fish. Contaminants such as arsenic, chlorine, cyanide, various phenols and polychlorinated biphenyls are potent stressors to all fish. (Barton, 2002).

At the cellular level, stress could be regarded as a disturbance to normal development, which may affect structure, function, stability, growth and survival. Stress of aquatic animals occurs due to any physical and physiological disturbances in the aquatic environment or system when transportation, crowding, handling and physical and chemical factor changes transpire. Stress has negative economic impact as it impairs aquatic and aquaculture systems by affecting fish health, productivity and final product quality. There is a general concept that stress in fish is any external stimuli (so-called stressor) that threats

homeostasis of the fish and results in neuroendocrine, physiological and behavioral alterations. The stress response is an assortment of changes in different physiological sections by stressor and an output reaction to neutralize the effect of the stressor and recover the homeostatic equilibrium. The environmental and micro environmental stimuli that act as potential stressors are numerous. Typically, they include hyper- or hypothermia, hypoxia, reactive oxygen species (ROS), radiation, starvation, hypo or hyperosmotic conditions, mutations, factors underlying metabolic deficiencies and other pathologic conditions, heavy metals, toxic agents and drugs (Tiligada *et al.* 2002).

Fish exhibit several responses to have the compensatory or adaptive mechanism that allow the fish to mitigate the stressors, maintain homeostasis and survive. These responses are so important to fish in a short-term stress; however, during the prolonged stressors with severe intensity, the stress response may be unable to maintain its homeostasis and fish health could be in danger (Barton 2002; Prunet et al. 2008; Tort & Teles 2012).

In living organisms like fish, a number of waste products are produced from excretion, microbial breaking down of organic matters and remaining of uneaten food. Nitrogenous waste products are a result of the breakdown of the amino acids in the proteins we digest for nutrition, and are universal to all higher vertebrates. Animals excrete this waste in the form of three main nitrogen products: ammonia, urea, and uric acid. These three products have a direct relationship between water conservation and energy requirements, meaning the more energy spent the more water conserved. Ammonia, the least water conserving form and therefore the least energy requiring, is highly toxic. Due to its toxicity it must

either be excreted or converted into the less toxic variants, such as urea or uric acid. Uric acid, which is mainly excreted by birds, reptiles, and many terrestrial invertebrates, requires the least amount of water loss along with the highest energy cost and does not appear to be excreted in significant quantities by fishes (Wood & Wright, 2009).

Generally, ammonia in aquatic environment exists in two forms (ionized form, NH_4+ and un-ionized form, NH_3). Given that it is able to easily diffuse across the epithelial membranes of aquatic animals due to easily dissolve in lipids, the form of NH_3 is considered much more toxic to aquatic animals than NH_4+ (Armstrong et al., 2012).

Harmful effects of ammonia on aquatic organisms have been extensively studied. In fish, for instance, ammonia can disrupt the normal functions of internal organs such as damage to the gill epithelium and disruption of normal metabolic functioning of the liver and kidneys (Wang & Leung, 2015). The increased concentrations of ammonia in aquatic environment can induce the deleterious effects in aquatic animals, due to its toxicity on the central nervous system of aquatic animals. In addition, the acute ammonia exposure causes the increased gill ventilation, loss of equilibrium, convulsions, ionic balance failure, and hyper excitability in aquatic animals (Kim, 2015).

One method of bio-monitoring is to use biomarkers. Biochemical biomarkers are molecules that are present in the body fluids, cells and tissues of organisms and whose activity is altered by the presence of toxic agents in the environment. One important set of biomarkers is the antioxidant defense enzymes that decompose the Reactive Oxygen Species (ROS).

These enzymes have an important role in the control, production and elimination of ROS, which in excess can alter the normal functions of the cell and lead to oxidation of the cell membranes as well as lesions in mitochondria, proteins, DNA and other components of the cell. Natural or anthropic changes in the environment introduce oxidative stress that results in disequilibrium in the cell by increasing the production of ROS (Batista, 2014).

In response to the production of ROS by the ammonia exposure, superoxide dismutase (SOD) is an antioxidant defense mechanism in aquatic animals that catalyzes the conversion of reactive superoxide anions to hydrogen peroxide (H_2O_2), and catalase (CAT) play a critical role in detoxification mechanism of hydrogen peroxide (H_2O_2) into oxygen (O_2) and water (H_2O_2) . Glutathione S-transferase (GST) is also one of antioxidant enzymes, which is associated to the conjugation and elimination of xenobiotics (Atli and Canli, 2010).Glutathione (GSH), a non-enzymatic antioxidant, decreases both H_2O_2 and lipid hydroperoxides involved in glutathione peroxidases (GPx) and glutathione reductase (GR) through the reaction of oxidation–reduction. Overproduction of ROS can damage important biomolecules, such as DNA, proteins and lipids, and initiate a cascade of events, bringing about impaired cellular function. These enzymes and non-enzyme play an important role in the antioxidant defense in vertebrates, preventing cells from adverse effects of oxidative stress. (Pinto et al., 2016).

1.2. Target genes

1.2.1 Catalase (CAT)

Catalase, which protects tissues against damage by hydrogen peroxide, was one of the first enzymes proposed to be an effective marker of oxidative stress (Vasylkiv et al., 2011). Catalase is an important member of the enzymatic antioxidant system and exists in all oxygen respiring organisms. CAT can effectively catalyze the decomposition of hydrogen peroxide (H_2O_2) to keep the balance between de novo H_2O_2 generation and efficient elimination, which is essential for innate immunity. Additionally, the up- or down-regulation of catalase can influence diverse biological processes, including cell proliferation, differentiation, migration and apoptosis, as the H_2O_2 involves in sensing and signaling of these biological events (Wang et al., 2013**).** The enzyme CAT is widely distributed in biological tissues and is involved in the decomposition of hydrogen peroxide into oxygen and water. It is one of the most prominent enzymes involved in defense against oxidative stress in both vertebrates and invertebrates (Batista et al. 2014).

1.2.2 Glutathione peroxidase (GPx)

Glutathione peroxidase (EC 1.11.1.9 and EC 1.11.1.12) is the general name for a family of multiple isozymes that catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH) as an electron donor (H_2O_2 + 2GSH → GS-SG + $2H_2O$) (Margis et al., 2008). Glutathione peroxidase (GPx, EC1.11.1.9)

reduces hydroperoxides, including hydrogen peroxides, in the presence of reduced glutathione as a means of protecting organisms from oxidative damage (Yamashita, 2012). There are at least five GPX isoenzymes found in mammals. Among them, GPX1 is considered as the major enzyme responsible for removing H_2O_2. Overexpression of this enzyme was observed to protect cells against oxidative damage. Elevation of GPX1 activity in both FL5 (Hockenbery, et al., 1997) and MDBK cells (Kayanoki, et al., 1996) suppressed apoptosis induced by H_2O_2. This evidence indicates that GPX1 is a major antioxidant enzyme that protects cells against lethal oxidative stress (Li, et al., 2000).

1.2.3 **Glutathione-s-transferase (GST)**

Glutathione-S-Transferases (GSTs) are a multigene family of dimeric, mainly cytosolic enzymes, which play an important role in the biotransformation and detoxification of a number of electropholic compounds (Rao, 2006). The alterations in the GST activities directly reflect the metabolic disturbances and cell damage in specific organs of fish (Livingstone et al., 2001). Glutathione-S-transferases constitute a complex family of proteins that play roles in both normal cellular metabolism and in the detoxification of a wide variety of xenobiotic compounds. They are predominantly cytosolic defense systems responsible for protecting cellular components against various toxic effects and oxidative stress (Sen& Semiz, 2007). It has also been demonstrated that the role of GST in oxidative stress is of conjugating endogenously-produced electrophiles such as membrane lipid peroxides in animals (Halliwell & Gutteridge, 1999) as well as in plants (Cummins *et al*., 1999).

1.2.4 **Superoxide dismutase (SOD)**

SOD generally exists in two forms inside the eukaryotic cell, Cu/ZnSOD in the cytoplasm and outer mitochondrial space (Sturtz et al., 2001), and MnSOD exclusively in the inner mitochondrial space. In addition, many species have an extracellular Cu/ ZnSOD. Superoxide generated by the mitochondria and other sources is converted to H_2O_2 and O2 by SOD. Abundant catalase enzyme then converts H_2O_2 to H_2O and O_2. (Landis and Tower, 2005). MnSOD (EC 1.15.1.1) is an essential primary antioxidant enzyme that converts $O\cdot_2$ to H_2O_2 and O_2 within the mitochondrial matrix. A variety of cancer cells have reduced levels of antioxidant enzymes, especially MnSOD, when compared with their normal counterpart (Li, et al., 2000).

The SODs convert superoxide radical into hydrogen peroxide. The CATs and GPXs convert hydrogen peroxide into water. In this way, two toxic species, superoxide radical and hydrogen peroxide, are converted into the harmless product water. GPX requires several secondary enzymes (GR and G-6-PDH) and cofactors (GSH, NADPH, and glucose 6-phosphate) to function. In this scheme, GR and G-6-PDH are considered secondary antioxidant enzymes, because they do not act on ROS directly but enable GPX to function. (Li, et al., 2000).

In order to explore stresses effect and how the fish respond to them is important aspect especially to fish farmers as they will make sure to keep their fish in optimum conditions that will in turn lead to maximization of high productivity and economic return. Different

approaches like bio-monitoring are used to measure stress problems to discover the response of fish to induced stressor. Therefore, ammonia chemical was used to induce stress and mRNA expression of our target genes which are catalase (CAT), glutathione peroxidase (GPx), glutathione-s-transferase (GST) and superoxide dismutase was analyzed by using semi quantitative PCR technique.

1.3. Objectives of the study

a) To measure effects of ammonia on mRNA expression of catalase (CAT), glutathione peroxidase (GPx), glutathione-s-transferase and superoxide dismutase (SOD) expression of marine medaka fish (*Oryzias dancena*).
b) To analyze expression pattern on different treatment conditions like ammonia concentration.

2. Materials and methods

2.1. Target genes sequences and RNA isolation.

To get DNA sequences of target genes used in this study, nucleotides were obtained from NGS database of our laboratory (unpublished data) and consensus sequences were constructed by using Sequencher 4.9 software.

Total RNA was extracted by using RNeasy plus mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The target tissues were added to 600 µl buffer RLT and homogenized by rotor-stator homogenizer. The lysate was centrifuged at 13,000 rpm for 3 minutes then supernatant was transferred to 1.5 ml tube and 1 volume of 70% ethanol was added. The mixture was then transferred to RNeasy spin column, centrifuged at 13,000 for 15 seconds and discarded flow-through. After that, 700 µl RW1 buffer was added to spin column and centrifuged at 13,000 rpm for 5 seconds to wash the spin column membrane. Then 500 µl of buffer RPE was added to spin column and centrifuged at 13,000 rpm for 15 seconds to wash the spin column membrane. The step was repeated for 2 minutes and RNeasy spin column was transferred to another 1.5 ml tube and 30 µl RNeasy free water was added to the membrane and centrifuged at 13,000 rpm for 1 minute to elute the RNA.

2.2. Bioinformatics sequence analysis and multiple sequence alignment

The structure and organization of OdCAT, OdGST, OdGPx, and OdSOD were compared with those of the corresponding orthologues available in GenBank (http://www.ncbi.nlm.nih.gov) by running BLAST x and first five orthologues were obtained to be compared with medaka fish.

Then multiple sequence alignment involving marine medaka amino acid sequence with that of other 5 selected species was done using http://www.clustalw.ddbj.nig.ac.jp/ and Jalview 2.10.1 software then phylogenetic trees were constructed by using MEGA7 software.

Other information of amino acids were like molecular weight in kilo Dalton (kDa) and theoretical p.I value were obtained from http://web.expasy.org/protparam/ searching tool.

2.3. Fish and laboratory management

Fish used in this study was laboratory stock propagated strain maintained in the Institute of Marine Living Organisms, Pukyong National University. 6 months adult marine medaka fish (*Oryzias dancena*) average total length of 2.75 cm and total weight of 0.32 grams and 2 months juvenile marine medaka fish (*O. dancena*) average total length of 1.4 cm and

average total weight of 0.05 grams. They were kept throughout the experiment at temperature 24°C, salinity level of 5 ppt, pH 8.5 and dissolved oxygen (D.O) 8 mg/L.

The fish were fed four times a day, at 10:00 am, 02:00 pm, 04:00 pm and 07:00 pm with artemia and an artificial diet designed for flounder larvae (Woosung Feed Corp., Daejeon, Korea). Larvae were fed with 100 μm and adult 350 μm sizes.

2.4. In vivo exposure of fish to ammonia stress

To understand the expression pattern of target genes in response to ammonia, adult fish were exposed to 1.5 ppm of ammonia chloride (NH_4cl) for 24, 48 and 96 hours. Then after end of each experiment respective experiment time, 5 fish were sampled, anaesthetized in 200 ppm lidocaine and surgical removal of gills and kidney tissues under dry ice was done. After that, tissues were stored in -80°C to be analyzed.

Also, 2-month-old juvenile fish; average total length, 1.4 cm and 0.05 grams) were immersed in a 300 ml containers supplemented with NH_4cl (4.5, 9.0 18 and 36 ppm total ammonia nitrogen). Treatment duration was fixed at 48 hours and 3 fish were sampled, anesthetized in 200 ppm lidocaine and whole body part which is posterior part of larvae fish containing brain, gills, kidney, intestine and liver were surgically obtained pooled together, dry frozen and stored in -80°C for future analysis.

In both experiments, fish were starved for 12 hours before experiment and non-exposed control groups of similar-sized were prepared in an identical manner, except that ammonia was not added to the tank instead 1 milliliter of distilled water was added.

2.5. Semi quantitative PCR analysis

Basal expression levels of each gene in *O. dancena* tissues were determined using semi quantitative PCR assay under the methodology used by (Rahman, 2016). This experiment stage was conducted by using 1 µl of cDNA template, 2 µl each forward and reverse primers, 15 µl distilled water added to AccuPower^{R} PCR-PreMix (dNTP, Taq DNA polymerase and $Mgcl_2$) (Bioneer Company, South Korea). Conditions were temperature of 94°C for 20 seconds, 60 °C for 20 seconds, and 72 °C for 20 seconds, with an initial denaturation step at 94 °C for 2 minutes. The reactions were completed in a thermos cycler with the following conditions:

Whole body sample CAT, GPX: 24 cycles GST, SOD: 22 cycles

CAT Gill: 26 cycles and Kidney: 26 cycles

GPX Gill: 24 cycles and Kidney: 24 cycles

GST Gill: 26 cycles and Kidney: 26 cycles

SOD Gill: 24 cycles and Kidney: 24 cycles

The PCR products were analyzed by electrophoresis in a 1% agarose gel stained with ethidium bromide (10 μg/ml) and finally the bands were quantified by determining gel density using image lab 5.2 software.

The expression of CAT, GPx, GST and SOD at between control and ammonia exposure group were evaluated by using paired t test. A significance level of $P < 0.05$ was used throughout the analyses. Statistical analyses were performed using SAS software (SAS Institute Inc. USA.).

3. Results

3.1. Target genes sequences and RNA isolation

DNA sequences of target genes which were obtained from our lab database (unpublished data), whereby all sequence clones of target genes were assembled and consensus sequences were made to get the correct sequences. Basic Local Alignment Tool (BLAST) was used for confirmation and results showed they match with other sequences found in the database.

After sequences were obtained, they were analyzed to get their related information.

CAT, **Figure 1** has got 2,157 base pairs, 61 base pairs 5′-untranslated region (UTR), 515 base pairs 3′-untranslated region (UTR) and 1,581 base pairs open reading frame (ORF) which encoded 527 amino acids with molecular weight 59.9 kDa and theoretical p.I value of 8.11.

The full length sequence of GPx, **Figure 2** has 925 base pairs, 37 base pairs 5′-untranslated region (UTR), 330 base pairs 3′-untranslated region (UTR) and 558 base pairs open reading frame (ORF) which encoded 185 amino acids, with molecular weight of 20.8 kDa and theoretical p.I value of 6.83.

The full length sequence of GST **Figure 3** has got 893 base pairs, 79 base pairs 5′-untranslated region (UTR), 145 base pairs 3′-untranslated region (UTR), 669 base pairs open reading frame (ORF) which encoded 223 amino acids with molecular weight valued 25.5 kDa and 7.71 theoretical p.I value.

SOD sequence, **Figure 4** has got 1,016 base pairs, 5 base pairs 5′-untranslated region (UTR), 336 base pairs 3′-untranslated region (UTR) and 675 base pairs open reading frame (ORF) which encoded 225 amino acids, molecular weight of 25.1 kDa and 7.73 p.I value.

Isolation of catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and superoxide dismutase (SOD) RNA was done in order to confirm the sequences of the target genes by obtaining full sequence from DNA molecule. Then, designed primers were used randomly to verify sequences used in our study and turned out to be proved.

Also isolated RNA sequence was used in determination of expression assay of the target genes. The primers used are shown in the **Table 1**.

ttgcgctacatcttctgctgttttgctaaggctagcaaacttccaggcaatttctgtcaac**ATG**GC
TGAGAACAGAGATAAAACTACCGACCAAATGAAGGCGTGGAAGGAGAACAGAGGTTCTCGGAAACC
AGACGTCCTGACCACTGGGGCTGGACATCCCATTGGAGACAAGCTGAACCTGCAGACAGCTGGACC
CAGAGGCCCTCTTCTGGTCCAAGATGTGGTCTTCACTGATGAAATGGCCCACTTTGACCGGGAGCG
GATCCCAGAGAGAGTGGTGCACGCCAAAGGAGCAGGGGCGTTCGGCTACTTTGAGGTCACCCATGA
CATCACCCGCTACTGCAAAGCCAAAGTGTTTGAACATATTGGAAAAACGACACCCATTGCTGTCCG
TTTCTCCACCGTTGCTGGAGAGTCTGGATCGGCTGACACAGTTCGAGATCCCAGAGGATTTGCTGT
CAAGTTTTACACTGACGAGGGCAACTGGGACCTCACGGGCAACAACACCCCTATTTTCTTCATCAG
GGACGCCCTACTGTTTCCGTCCTTCGTCCACACCCAGAAGCGTAACCCCCAGACTCACATGAAAGA
CCCTGACATGGTGTGGGACTTCTGGAGCCTGAGGCCTGAGTCTCTGCATCAGGTGTCCTTCCTGTT
CAGTGACCGGGGTCTGCCTGATGGTTACCGCCACATGAACGGCTACGGTTCCCACACTTTCAAGCT
GGTCAACGCTCACGGCGACCGCTTCTACTGCAAGTTCCACTACAAGACTGACCAAGGAATTAAGAA
TCTTTCGGTGGAGAAGGCGGCGCATTTGTCGTCTACCAACCCTGACTACGCCATTGGAGACCTGTT
CAATGCAATTGCTAATGGAAACTACCCATCCTGGACTTTTTACATTCAAGTCATGACTTTTGAGCA
GGCAGAGAAATTCCAGTTCAACCCCTTCGATCTGACAAAGGTGTGGTCCCACAAAGAATTCCCATT
GATCCCAGTGGGAAGAATGGTCCTCAACAGGAATCCCGTCAACTACTTTGCAGAGGTGGAGCAGCT
GGCCTTTGACCCCAGCAACATGCCCCCCGGCATTGAGCCCAGTCCTGACAAGATGCTGCAGGGCCG
GCTCTTTTCCTACCCCGACACCCACCGACACCGGCTGGGGGCCAACTACCTGCAGATCCCCGTCAA
CTGTCCTTTCAGGACCCGGGTTGCCAATTATCAGCGGGACGGCCCCATGTGCATGTTTGACAACCA
AGGCGGTGCTCCAAACTACTACCCAAACAGCTTCAGCGCCCCAGAGACCCAGCCTCAGTTTGTGGA
GTCCAAGTTCCAGGTGTCTCCTGATGTGGCACGGTACAACAGTGCAGATGACGACAATGTGACTCA
GGTTCGTGCCTTCTATACCAAAGTGCTAAATGAGGACGAGCGCCAAAGGCTTTGCCAGAACATGGC
GGGGGCCCTGAAGGAAGCGCAGCTGTTTATCCAGAAACGAATGGTGGAGAACTTGAATTCTGTCCA
TCCTGACTATGGAAACAGAGTTCAGGCCCTCCTGGATAAATATAATGCAGAGGCCAAAAAGAGCAC
CAGTGTGCACGTCTACAGCCGCCCAGGAGCCTCCGCCATCGCTGCTTCCTCCAAAATG**tga**agctc
cgcctcctcctcctctaacctcagaggaatcgtgattacctgatagaacaacagtcgactgaacct
gctaataatgaagtgtgtctttgctagcagttgattatctttgcagcgcaatactgtcaaaaacaa
gatgatcgtaatccagacaaacgtctggaattaatcctgtttctctggatctgtggacgtgtcgtc
ttctggaacaactgtctttcctgatgctgcgtcttcatgaacgtgtgtgagaactataactgtttt
attgtgtggaaggatgtgcacacgcacgtgcactcgcctttaaaacgttctgctgggatgatgaga
atcacactgttgtataggtgtgtgaacagtctgggtctgccacgcccactagaggcggggctatct
gatcgaagcttcaattatacagattttctttgtactctgtcaataatatattctaatgaaaacatt
tttataatcattaaaagttctaaacaaaattaaaaaaaaaaaaaa

Figure 1. Full length sequence of *O. dancena* CAT gene. The ORF is underlined and Poly-adenylation signal is highlighted with grey color.

gttttgtgcctttatagtggctaactgaacggcagcc**ATG**CTGCTGGCAGGCTGTACAGTGCTTTT
CTCTGTCCTGCTGCAGGCTATGTCTTCCCCGACTGAGGACTGGCAGAGGGCAACATCTATTTATGA
TTTCAATGCAACAGACATCGATGGCAATGTGATTCCCTTGGAAAAGTACAGGGGGAACGTTGTCAT
CATCACCAACGTTGCCTCCAAATGAGGCAAAACCGTAGTAAACTACTCTCAGTTTGCAAAGATGCA
CGCCGCGTATGCTGAGAGGGGTTTACGCATCCTCGCTTTCCCATCGAACCAGTTTGGGAATCAGGA
GCCGGGTAATGAAACTCAGATCAAGGAGTTTGCTGAGTCCTTCGATGCTCATTTTGATATGTTTAG
TAAGATTGACGTGAATGGACCCAACGCTCACCCTCTGTGGAAGTGGTTGAAGGAGCAGCCAAATGG
TAAAGGAATTTTTGGAAATAGCATCAAGTGGAACTTCACTAAGTTTTTGATCAACAGAGAGGGACA
GGTGATGAAAAGATACGGACCACTGGATGATCCAAGTGTGGTGGAAAAGGATCTTCCTAAATACCT
T**taa**ccttctgacggatggcctattcctgttttgacgctgtttatgctgtgtgtttccactgcttt
cttcatctcttcaagcaccggtggacatacttgtaactcccctccagaccaatgatggactgttcg
aaaatccactcgctgggcagaacagacctgataaaactggcgtgcaaacctgctgggaggatttaa
aagcttatgttcccttggtttcctgaagtgcagagagcatgagaacatcaatatttgaacagcttc
aaatcctgccttgtgttcttaataaatatattccttctactaaaaaaaaaaaaaaaaaaaaaaaaa

Figure 2. Full length sequence of *O. dancena* GPx gene. The ORF is underlined and grey highlighted part is Poly-adenylation signal.

gtttgttataaaagcagacgtgtgaagccgtcctgcgctccactccctccttcagctgaactttcc
tcaccctgcgacc**ATG**GCCAAGGACATGACTCTGCTGTGGGGCTCCGGCTCTCCCCCCTGCTGGAG
GGTGCAGATCGCTCTGGAGGAGAAGAACCTGCAGGGATACAACCAGAAGCTGCTGTCCTTTGAGAA
AGGGGAGCACAAATCCAAGCAGGTCCTGGAGATCAACCCCAGGGGTCAGCTTCCTGCTTTCAAACA
CAAGAACATAATTGTGAATGAGTCCTACGGCGCCTGCATGTACCTGGAGAACCAGTTCAAGTCTCA
GGGGAACCAGCTGATCCCTGAGGGCGCCGCCGAGATGGGCCTGATGTACCAGCGCATCTTTGAGGG
ACAGACTCTCTTCCAGAAACTGGCTGACGTTCTCTTCTACACCTGGAAAGTCCCAGAGGCGGAGAG
ACACAACTCAGCAGTGGAGAGAAACACTGAGGCCCTGAAACAGGAGATCCAGACGTGGGAGGGGTA
TTTGAAGGGACCCGGCGGTTTCCTGGGTGGGAAGTCCTTTTCTCTCGCTGATGTCATCGTTTACCC
AGTTATTGCTTTTTTCTTCCGATTTGGAGGAAATGAGAACCATTACCCAAACCTGGCAGCGTACTA
CAACAAACTGAAGGAAAGACCCAGCATCAAGAAGACCTGGCCTCCTACCTGGGAGGAGACACCTGG
ACAGGAGGCGCTGAAGGACCTC**taa**gacgtccacctgctccgttgtgttttcatgtttaaccactt
cctgtttcactgtgtaattctctagtcacatttgtcactgaaaatagattttgtgatgtgaatata
ataaatgtaaattgtagaaaaaaaaaaaaaaaaa

Figure 3. Full length sequence of *O. dancena* GST gene. The ORF is underlined and grey highlighted part is Poly-adenylation signal.

caaac**ATG**CTTTGCAGAGTCTGGCAAATGCGCAGTTGTGCATCAGTTCTTAACCAAGCAGCTTCTT
GGAAAGTCGGATCCAGACAGAAACATACACTTCCTGACCTGACCTATGACTATGGCGCCCTGGAGC
CCCACATCTGTGCAGAGATAATGCAGCTGCACCACAGCAAACACCATGCAACCTATGTCAACAACC
TCAATGTAACAGAGGAGAAGTATCAGGAGGCACTTGCTAAGGGAGACGTGACCGCACAGGTAGCTC
TTCAGGCGGCTTTGAAGTTCAACGGAGGAGGTCACATTAACCACACCATCTTTTGGACGAACCTTT
CTCCTAATGGTGGAGGCGAGCCACAAGGGGAGCTCATGGAGGCCATCAAACGAGACTTTGGCTCCT
TCCAGAAGATGCAGGAGAAGCTCTCCGCTGCCACGGTGGCGGTGCAGGGCTCAGGATGGGGATGGC
TAGGTTATGACAAAGAGAGTGGAAGATTACGCGTCGCCGCTTGTGCTAACCAAGACCCTCTGCAGG
GAACTACAGGTCTCATCCCCCTCCTCGGTATTGACGTTTGGGAGCACGCTTACTATCTTCAGTATA
AAAATGTCCGCCCTGACTATGTAAAGGCTATTTGGAATGTCGTTAACTGGGAAAATGTGAGTGACC
GTCTTCAGACGGCCAAAAAG**tag**aagcaaatctcctacaactttaatcaaataataatattcagat
caatagaatcacagtaaatgagtgttgctctgattgtattgtttttggaggatgtacattgaataa
acttaaagttatatgcaatttacttttttgtaaccagctgtcaacttttgtcagaacgcacaaaaaa
tggccgcctttaacgtcgaaggtggcttttgttttcgacgatggaagaacaaattcagagctggat
cgttgtctgacttgcatgacactaaaatcagattccagttcatgtttttttgatgtaataaattcaa
acatttcaataaaaaaaaaaaaaaaa

Figure 4. Full length sequence of *O. dancena* SOD gene. The ORF is underlined and grey highlighted part is Poly-adenylation signal.

Table 1. Primers used in this study

Oligo Name	Oligo Sequence	Mer (s)	Amount	Purification
qOD_CAT_FW1	GCT GTT TTG CTA AGG CTA GC	20	0.05	MOPC
qOD_CAT_RV1	GGT TCA GCT TGT CTC CAA TG	20	0.05	MOPC
qOD_GST_FW1	CCT CCT TCA GCT GAA CTT TC	20	0.05	MOPC
qOD_GST_RV1	CCT TTC TCA AAG GAC AGC AG	20	0.05	MOPC
qOD_SOD_FW1	CCA AGC AGC TTC TTG GAA AG	20	0.05	MOPC
qOD_SOD_RV1	GTT GCA TGG TGT TTG CTG TG	20	0.05	MOPC
qOD_GPX_FW1	CAG TGC TTT TCT CTG TCC TG	20	0.05	MOPC
qOD_GPX_RV1	CTC ATT TGG AGG CAA CGT TG	20	0.05	MOPC

3.2. Multiple sequence alignment and bioinformatics sequence analysis

The multiple alignment of *O. dancena*'s amino acid sequences with that of other close related species chosen from the first top 5 orthologues of each target gene's BLAST result. Our results showed that the species are related to one another as they exhibit similarities of their amino acid sequences and they seem to have common ancestors during evolutionary development. The results also showed that the most amino acid units are conserved among the group of analyzed sequences. Uniform colored columns show unspoiled conservation of either identical amino acids denoted with asterisk (*) or similar amino acids by which the score of similarity was shown in number out of 10.

The multiple alignment of marine medaka CAT amino acid sequences (**Figure 5**) with those from other species showed the highest level of identity to *O. melastigma* (99%), *O. sinensis* (96%), *O. latipes* (96%), *Lates calcarifer* (92%) and *Sparus aurata* (92%).

Multiple sequence alignment of marine medaka GPx amino acid sequences (**Figure 6**) with other species revealed the highest level of identity to *O. latipes* (91%), *Cyprinodon variegatus* (90%), *Xiphophorus maculatus* (89%), *Poecilia Mexicana* (88%) and *Poecilia reticulata* (87%).

For GST, the results of multiple sequence alignment marine medaka GST (**Figure 7**) and other species indicated similarity of 86% to *O. latipes*, 73% to *C. variegatus*, 72% to *Solea senegalensis*, 70% to *Pleuronectes platessa* and 67% to *Anoplopoma fimbria*.

The results of multiple alignment of SOD amino acid sequence (**Figure 8**) with other species showed that marine medaka is related to *O. latipes* by 96%, *Cynoglossus semilaevis*, *Austrofundulus limnaeus* and *Oplegnathus fasciatus* by 90% and finally 89% to *Lutjanus peru*.

Jalview 2.10.1 Multiple sequence alignment results

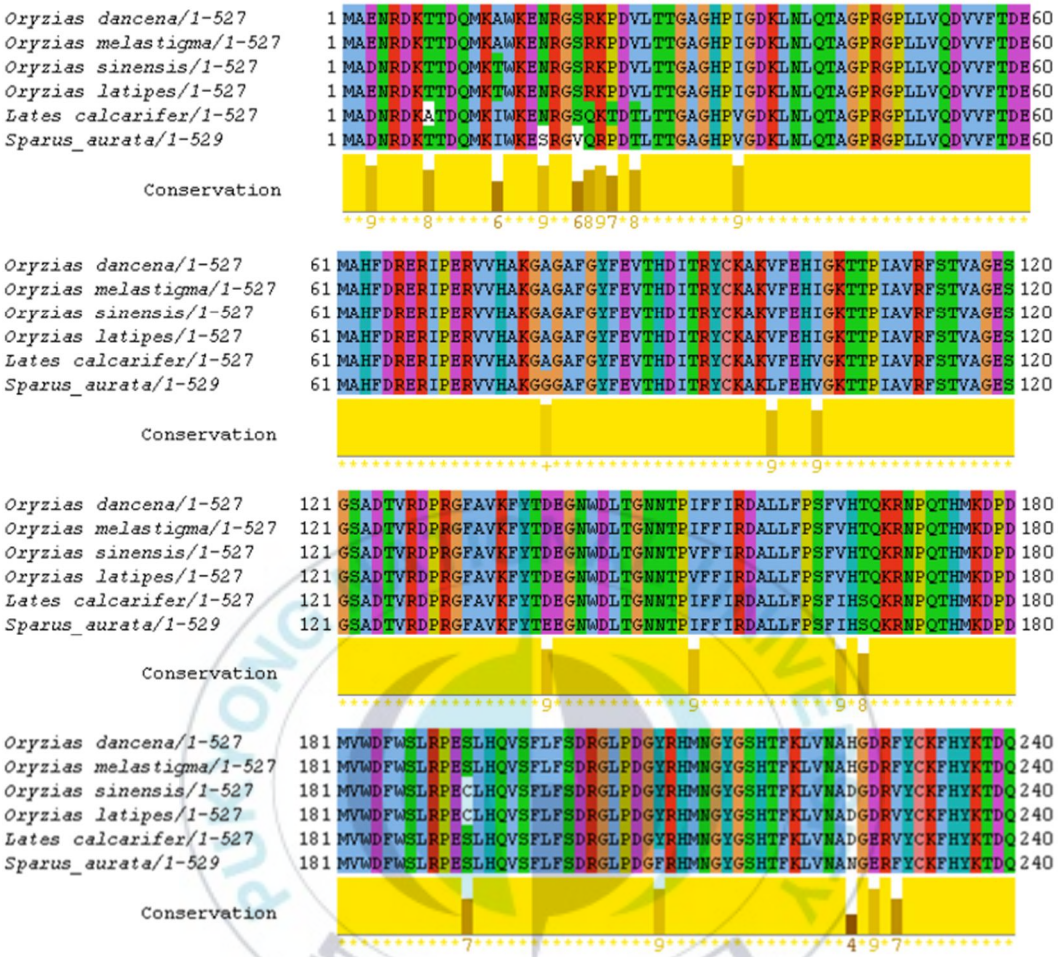

Figure 5. Multiple sequence alignment of CAT gene of marine medaka fish and other close related species. Identical amino acids are denoted with asterisk (*) and similar amino acid residues scores were computed out of 10.

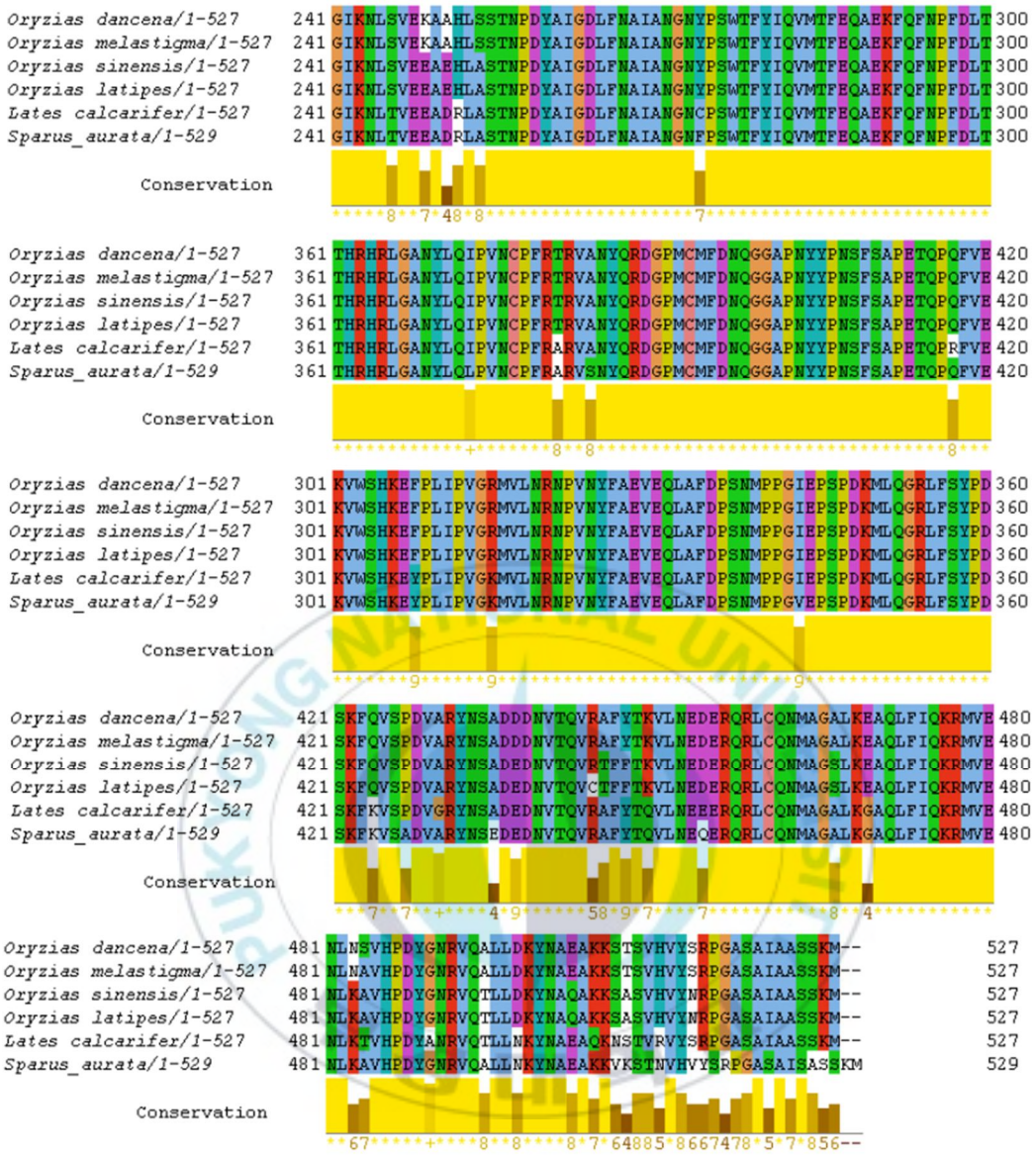

Figure 5 (Continued)

Jalview 2.10.1 Multiple sequence alignment results

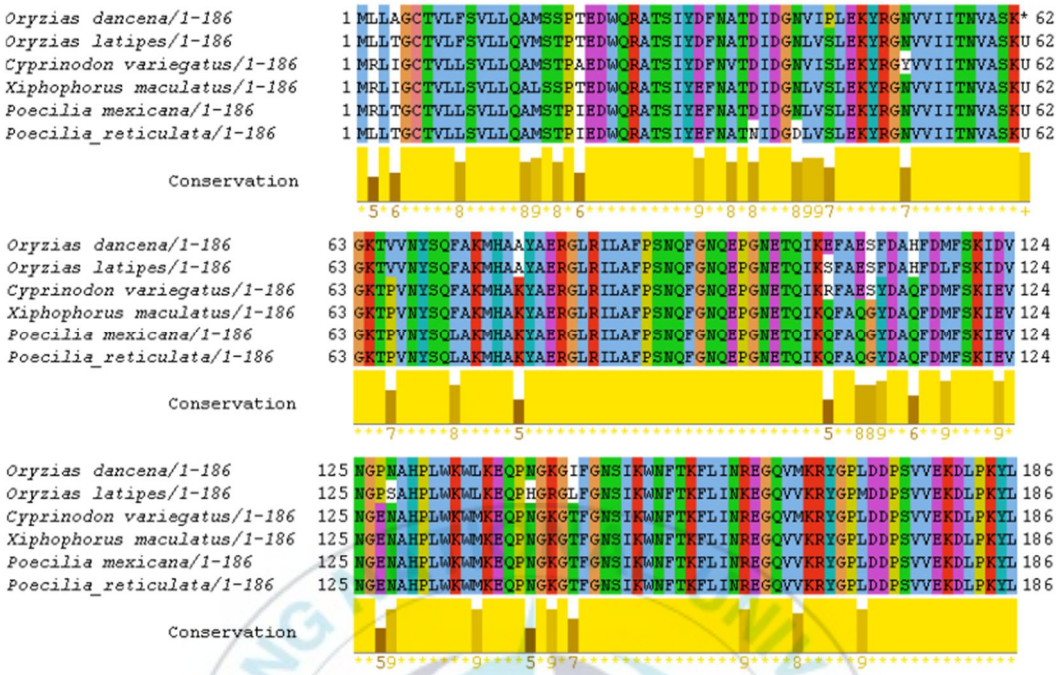

Figure 6. Multiple sequence alignment GPx gene of marine medaka fish with other close related species. Identical amino acids are denoted with asterisk (*) and comparison of amino acid residue scores were computed out of 10.

Jalview 2.10.1 Multiple sequence alignment results

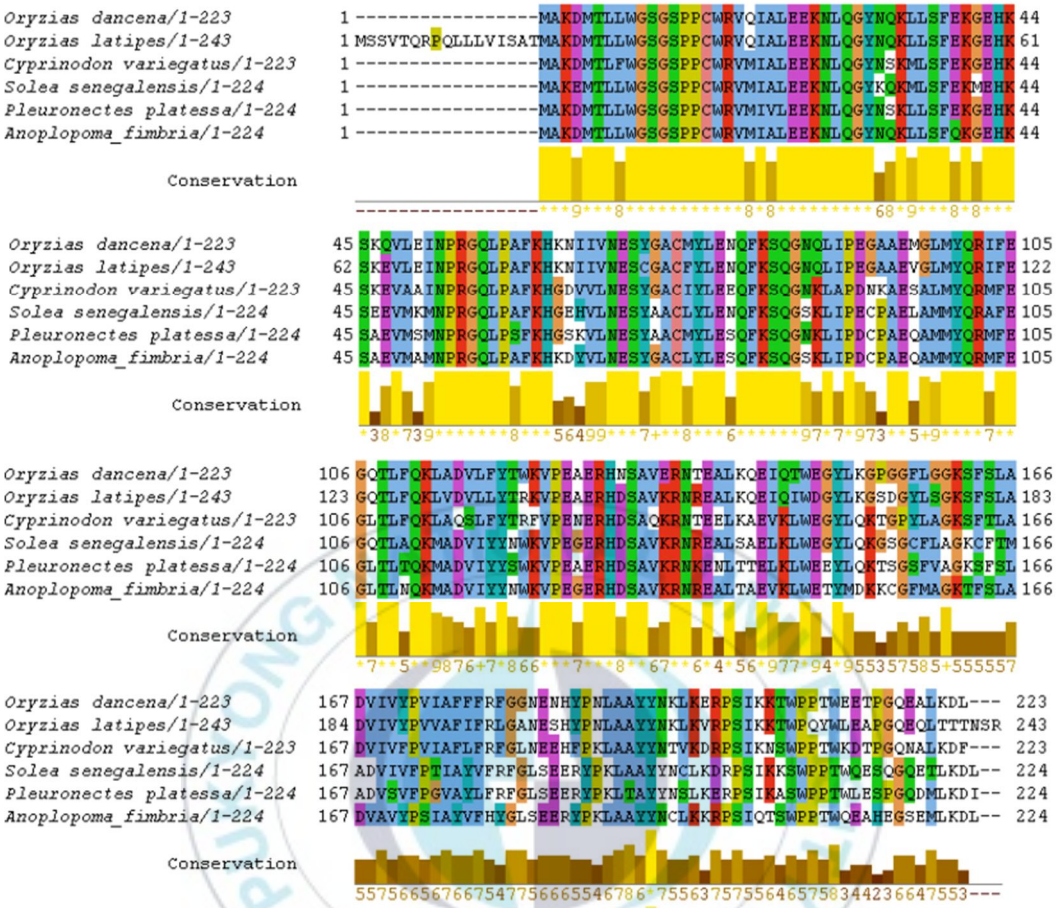

Figure 7. Multiple sequence alignment GST gene of marine medaka fish with other close related species. Identical amino acids are denoted with asterisk (*) and comparison of amino acid residue scores were computed out of 10.

Jalview 2.10.1 Multiple sequence alignment results

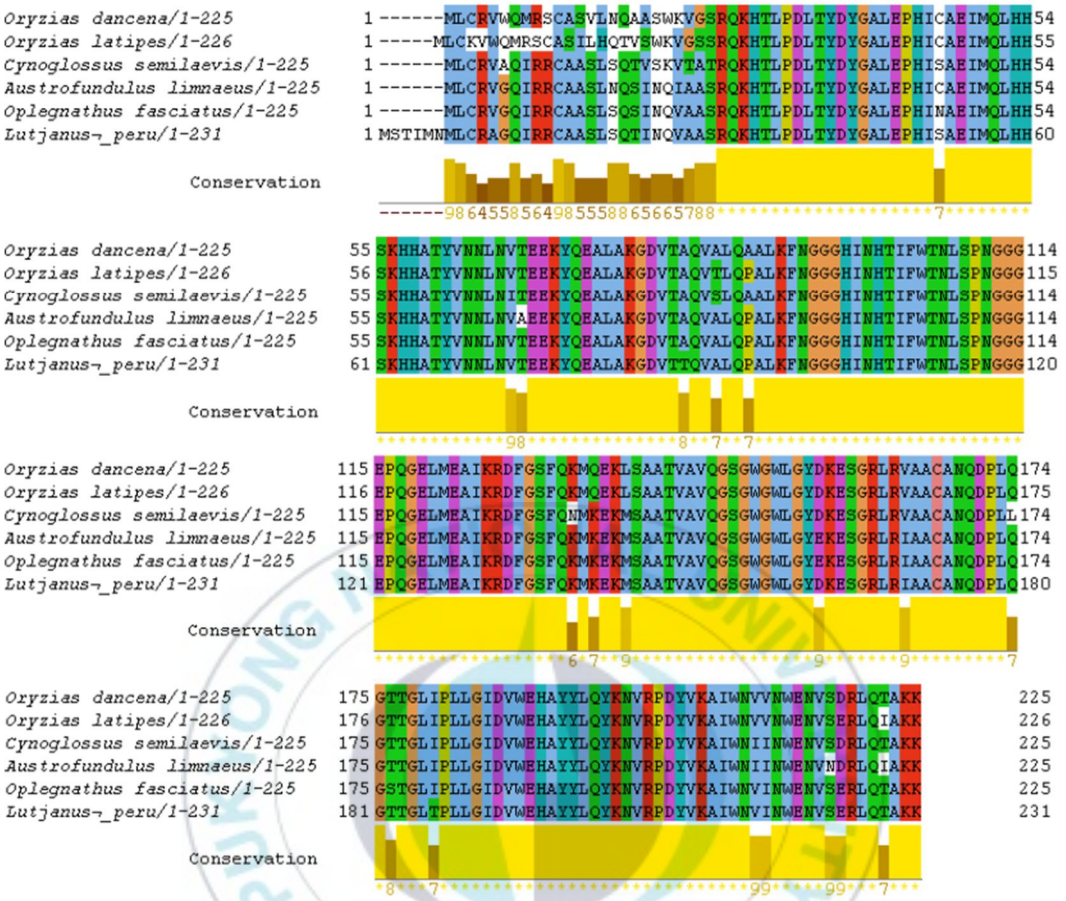

Figure 8. Multiple sequence alignment SOD gene of marine medaka fish with other close related species. Identical amino acids are denoted with asterisk (*) and comparison of amino acid residue scores were computed out of 10.

Phylogenetic trees

The trees have been constructed to verify multiple sequence alignment as result suggested that the fish selected were closely related and share common ancestors.

Figure 9 and **Figure** 10 show the relation of the selected species by using target genes amino acid molecules.

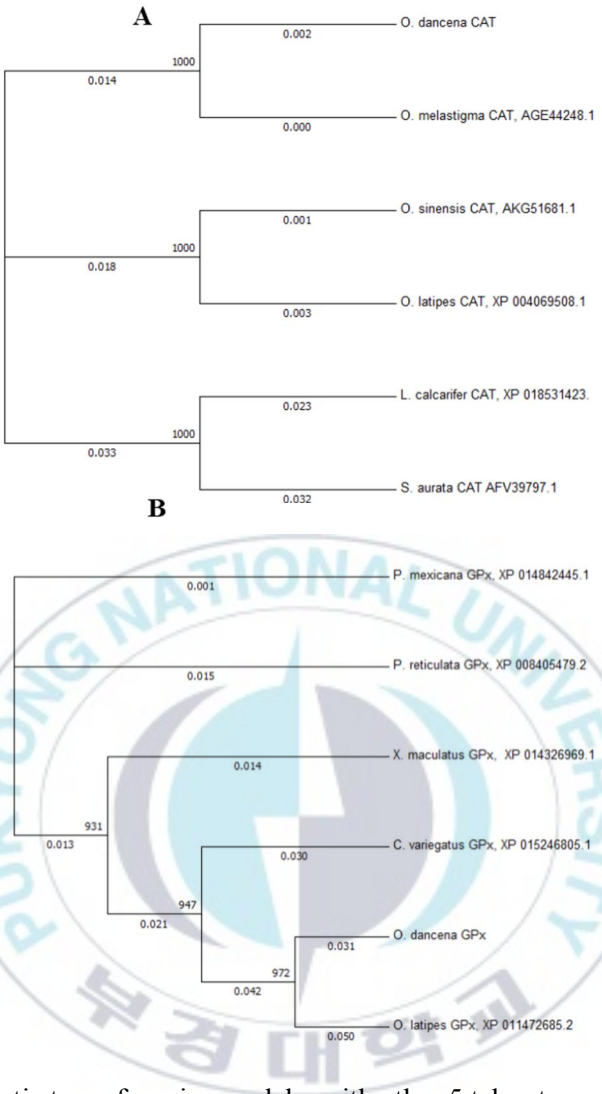

Figure 9. Phylogenetic tree of marine medaka with other 5 teleost species. A shows CAT and B represents GPx.

Phylogenetic trees

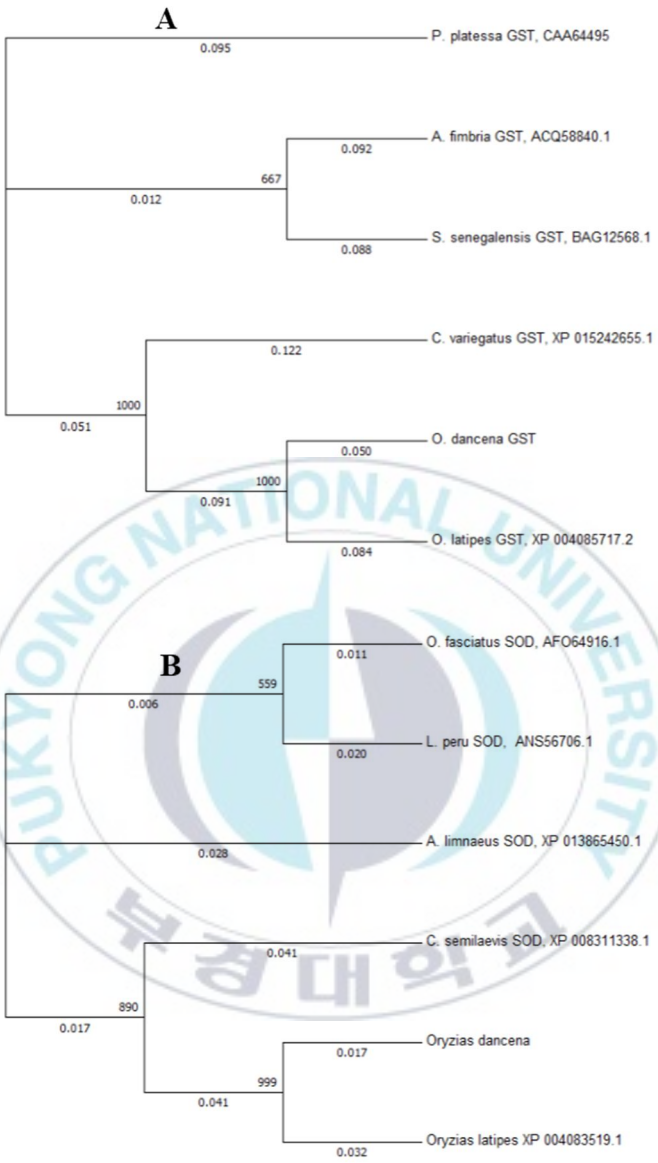

Figure 10. Phylogenetic tree of marine medaka with other 5 teleost species. A shows GST and B represents SOD.

3.3. Semi quantitative PCR results

Expression of CAT in the gills of adult marine medaka fish when exposed to 1.5 ppm showed no significant difference compared to the control ($P>0.05$) but in the kidney, CAT revealed significant difference between control and treatment group ($P<0.05$) **Figure 11.** Expression in the gills was slightly high compared to the kidney. No remarkable difference in expression of gill and kidney CAT noticed among exposure days.

Expression of GPx in the gills and kidney of adult marine medaka fish exposed to 1.5 ppm showed that there is significant difference compared to the control ($P<0.05$) **Figure 12.** In gills, no significant difference of expression was seen but in kidney, expression was progressively up regulated as exposure time increases.

Expression of GST in the gills and kidney of adult marine medaka fish exposed to 1.5 ppm showed that there is significant difference compared to the control ($P<0.05$) **Figure 13.** But no difference was seen among 24, 48 and 96 hours.

SOD results of both gills and kidney of adult marine medaka fish exposed to 1.5 ppm showed that there is significant difference between control and experiment group ($P<0.05$) **Figure 14.** But for exposure duration no much differences was noticed.

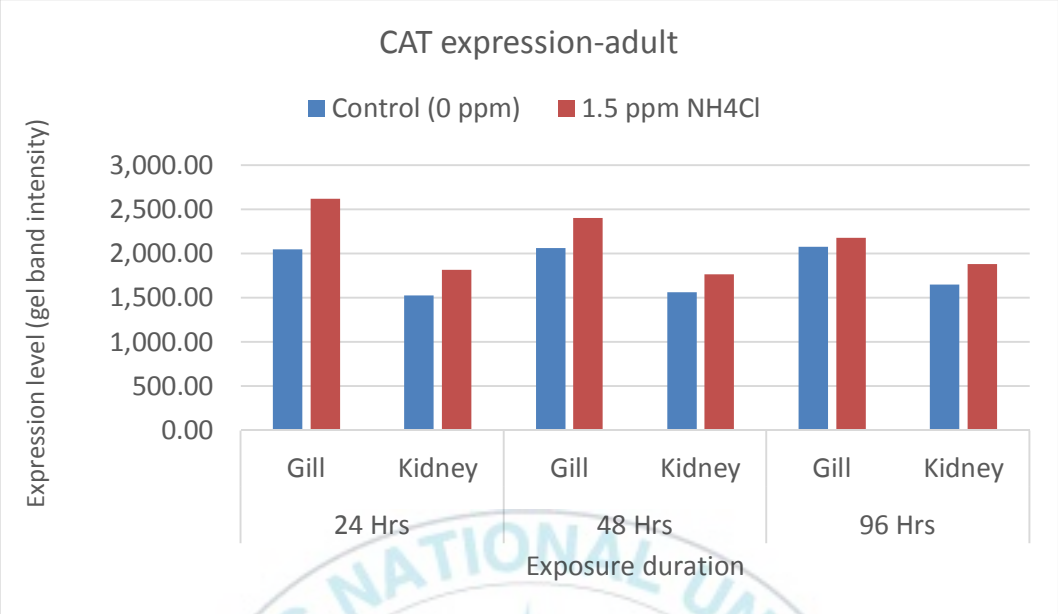

Figure 11. CAT expression result of adult medaka fish gills and kidney.

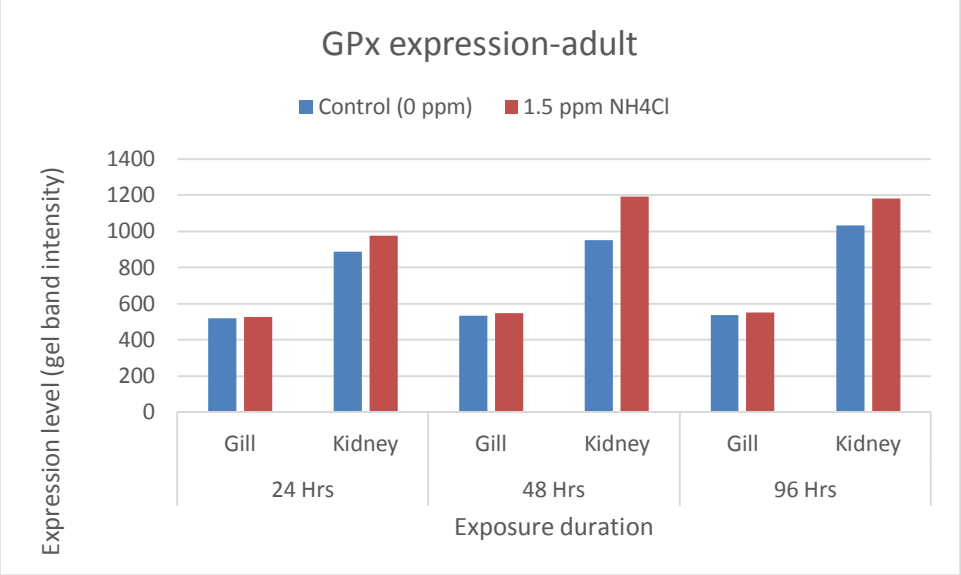

Figure 12. GPx expression result of adult medaka fish gills and kidney.

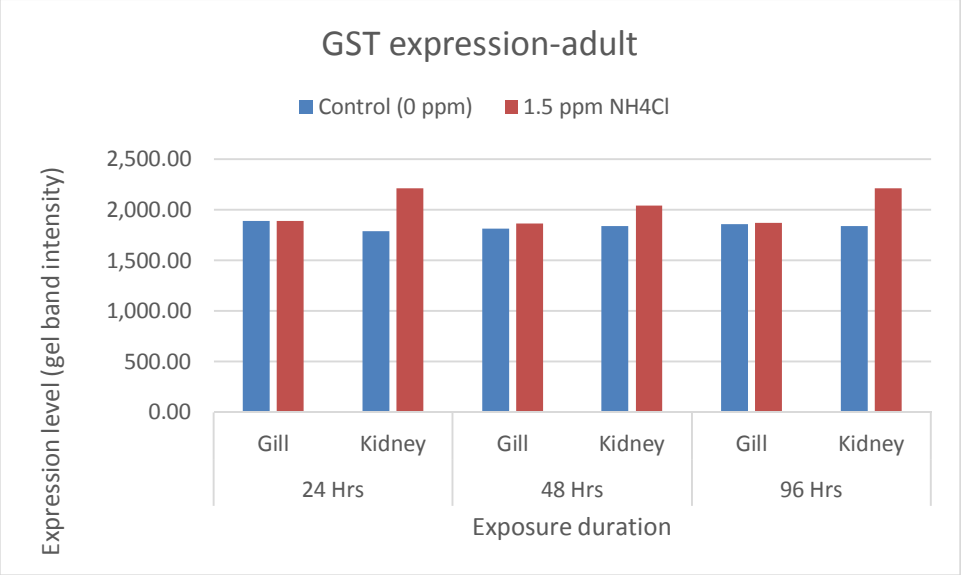

Figure 13. GST expression result of adult medaka fish gills and kidney.

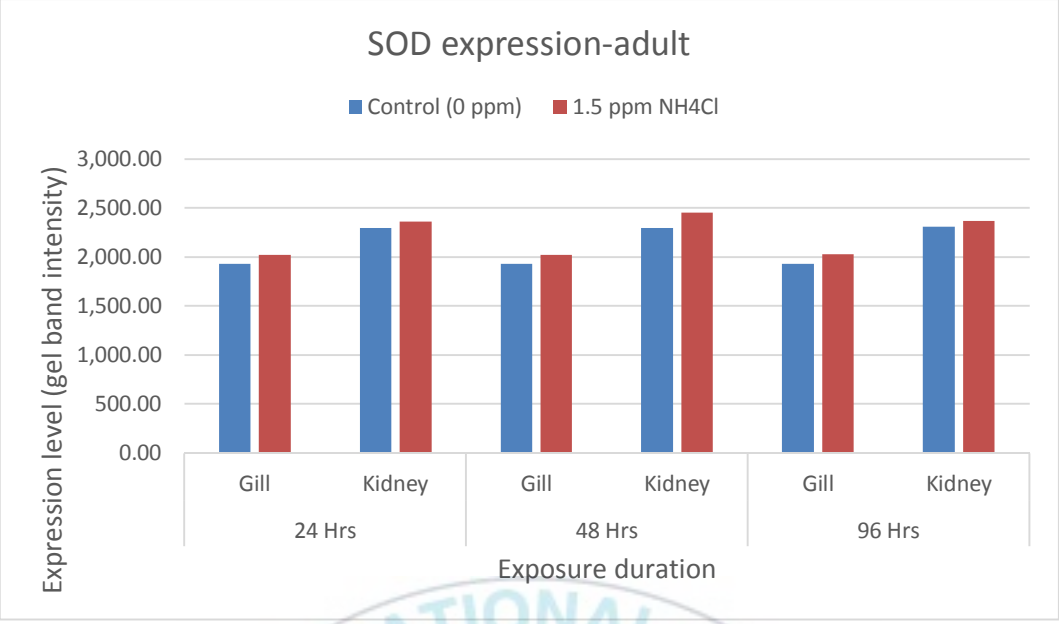

Figure 14. SOD expression result of adult medaka fish gills and kidney.

For juvenile marine medaka fish, **Figure 15**, the expression of CAT was upregulated in the concentration 4.5, 9 and 18 ppm but in 36 ppm it decreased compared to the first three groups but showed higher expression compared to control (o ppm).

The expression pattern of juvenile marine medaka fish's GPx (**Figure 16)** increased slightly from concentration 4.5 and 9 ppm then increased to the abruptly in the concentration 18 ppm and down regulated to the nearly same level as 0 ppm which is regarded as control.

The expression of GST gene of juvenile marine medaka fish shown in **Figure 17** was high in experiment groups compared to control (0 ppm) and the highest expression was observed in concentration of 4.5 ppm compared to others.

The result of SOD expression of juvenile marine medaka fish (**Figure 18)** showed gradual expression level in the concentrations 4.5, 9, 18 and 36 ppm. All these experiment groups are higher compared to control group.

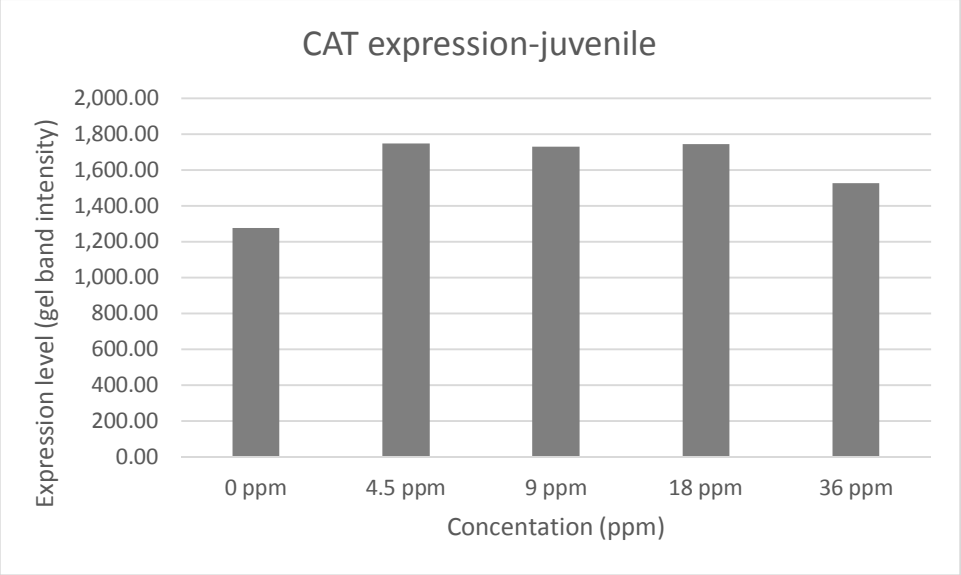

Figure 15. CAT expression of whole the body juvenile medaka fish exposed to different ammonia concentrations for 48 hours.

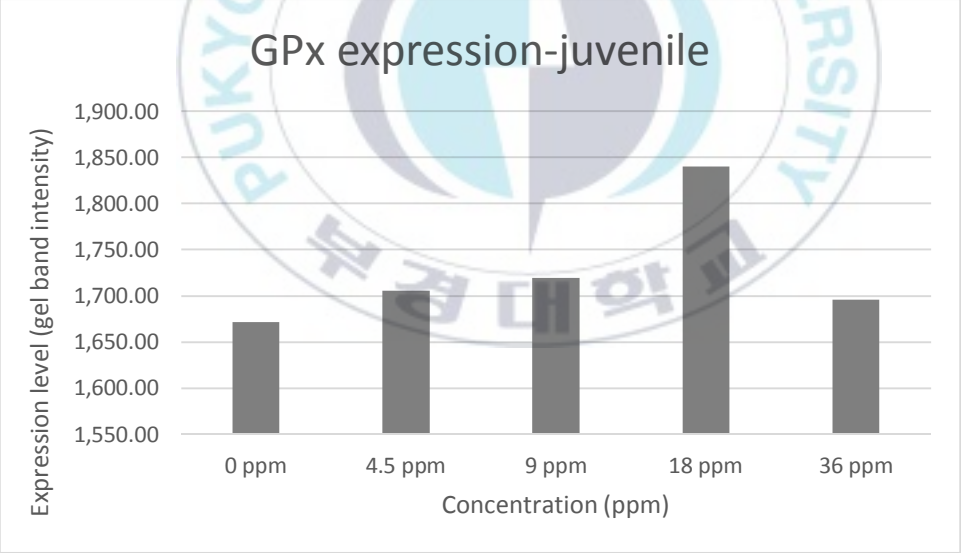

Figure 16. GPx expression of whole the body juvenile medaka fish exposed to different ammonia concentrations for 48 hours.

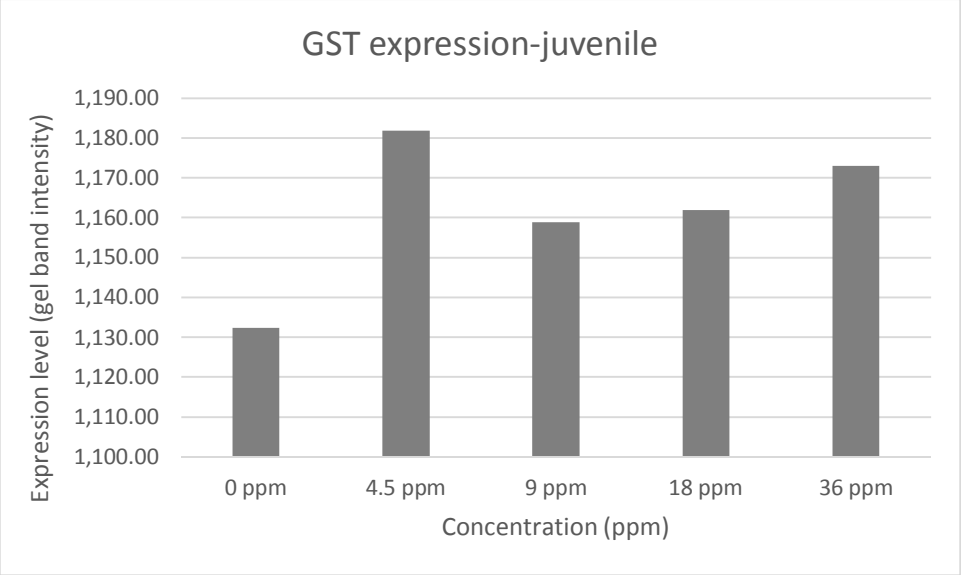

Figure 17. GST expression of whole the body juvenile medaka fish exposed to different ammonia concentrations for 48 hours.

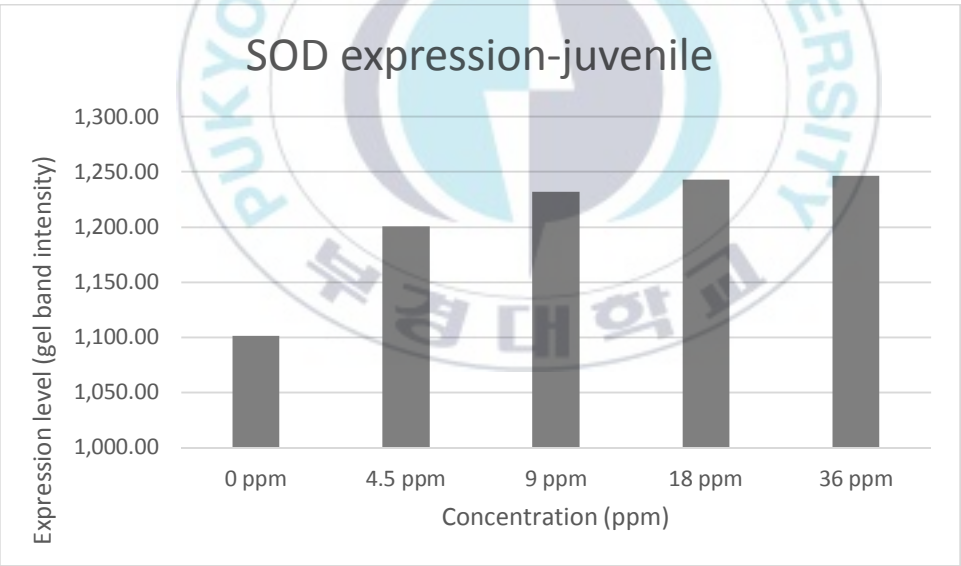

Figure 18. SOD expression of whole the body juvenile medaka fish exposed to different ammonia concentrations for 48 hours.

4. Discussion

Antioxidant enzymes like CAT, GPx, GST and SOD play important role in the body system to stabilize physiological functions when the body is exposed to stress. Ammonia is toxic to aquatic animals including fish and is regarded as stress inducer. Hence, Fish have evolved a number of different strategies to defend against ammonia toxicity at the cellular and the subcellular level (Ip et al., 2005).

The expression of gill CAT in adult showed no significant difference between control and experiment group ($P>0.05$) **Figure 11** probably because of downregulation in the expression pattern between control and experiment group was not significant as ammonia concentration or short exposure duration could not affected the expression pattern. The antioxidant system has shown gradual declining of CAT expression as time goes on suggesting that the saturation level was reached and mechanism was stopped and could not mitigate the stress induction effect any longer.

For expression of CAT in the kidney, we observed significant difference between control and experiment group ($P<0.05$) **Figure 11.** This is because the gill responded to ammonia stress treatment induce high expression of CAT enzymes to act up on the designated stressor. Hegazi et al. (2010) reported a significant increase in the CAT activity of Nile tilapia, *Oreochromis niloticus* exposed to ammonia. For this result, we can get clear picture

that for the concentration used, the kidney responded successfully compared to gill which showed no significant difference between control and experiment group. This signifies the effect of ammonia and antioxidant response shown by the fish.

For GPx of adult medaka in the kidney organ, the result showed no significance difference was observed between control and treatment group ($P<0.05$) **Figure 12.** This is probably because of low induction dose and short exposure duration. This has shown that the gill responded efficiently due to ammonia stress compared to kidney and significance difference was observed compared to kidney which its expression could not show significant difference between the two compared groups of control and experiment. Generally, GPx catalyzes the reduction of H_2O_2 and a variety of lipid peroxides by using GSH which is further oxidized to GSSH and this expression (Sinha et al, 2015). Hence its expression is resultant feature of homeostatic mechanism to mitigate adverse effects of ammonia stress.

For GST, both gill and kidney showed significant difference between control and experiment group signifying the effect of ammonia treatment to adult medaka fish ($P<0.05$) **Figure 13**. This also indicate the moderation mechanism of the fish to respond to ammonia stress was initiated after exposure. Glutathione-S-transferases (GST) functions a main role in detoxification of deleterious electrophilic xenobiotics such as environmental toxicants (Keen and Jakoby, 1978). Mari (2001) suggested that oxidative stress can increase GST, which is a defense mechanism against oxidative stress or cellular damage.

In the gill of adult medaka fish, the expression of SOD showed significant difference between control and experiment group ($P<0.05$) **Figure 14**. This is illustration of how SOD in the gills responded to ammonia stress and help to stabilize body metabolism.

Turning on the kidney, no significant difference was observed between control and experiment group ($P>0.05$) **Figure 14**. This is because of low response rate of kidney SOD due to ammonia exposure.

On the other side, the expression of CAT in juvenile medaka fish showed upregulation in 4.5, 9, and 18 ppm and slightly downregulated but was higher compared to control group **Figure 15**. This is probably the antioxidant mechanism was effectively working but after reaching 36 ppm, the system was saturated and expression was decreased compared to other experiment groups.

The expression of GPx in the juvenile medaka fish was gradually upregulated and eventually reached highest expression level at the concentration 18 ppm **Figure 16**. This suggests that the concentration caused significant effect to the fish.

For GST of juvenile medaka fish, the expression increased significantly at 4.5 ppm and decreased at 9, 18 and slightly increased at 36 ppm but could not reach the level of 4.5 ppm **Figure 17**.This suggests that 4.5 ppm resulted to higher response rate but thereafter the response mechanism was saturated and could neither maintain nor increase the expression level.

For SOD, the expression level was increasing in the course of ammonia concentration **Figure 18**. As concentration increased from 4.5 to 36 ppm, the expression level was also increasing signifying that the more the concentration the more SOD level was observed.

Oxidative stress exposed to ammonia stress is indicated by changes in ROS production in pufferfish. A major defense mechanism for reducing the production of ROS is achieved by raising the levels of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione reductase (GR). The superoxide anion, the parental form of intracellular ROS, is a highly active molecule, but it can be converted to H_2O_2 by SOD (Jin et al., 2011). GPx and CAT eliminate H_2O_2 effectively, thus reducing its toxic effect.

In juvenile yellow catfish, *Pelteobagrus fulvidraco* it was observed significant difference in 5.7 ppm for 28 days (Li et al., 2016). In the study of rockfish *Sebastes schlegelii*, which concentration was 0, 0.1, 0.5 and 1 ppm but duration was 14 and 28 days, the CAT, GST and SOD activities were substantially increased by the ammonia exposure depending on water temperature, which may be induced in the process of xenobiotic detoxification for the ammonia exposure depending on water temperature (Kim et al., 2015).

Relative expression levels of antioxidant enzyme genes SOD, CAT, GPx indicated increase of expression after ammonia challenge of 78 ppm for 24 and 48 hours in in pufferfish (*Takifugu obscurus*) (Cheng et al., 2015). In goldfish and carp, SOD activity increased respectively from 24 h and 48 h up to 84 h when exposed to 72 ppm NH_4HCO3 (Sinha, et al., 2014). Also CAT activity of liver increased after 48 hours, 84 hours and 180 hours.

In grass carp, which was exposed in 144 ppm, the SOD activity was significantly increased as early as 2 hours of HEA exposure (p <0.05), but was slowly declined until 8 hours and showed no significant difference with control (p > 0.05). However, the activity of SOD

rose again and reached the highest peak after 24 hours exposure ($p < 0.05$). Significant effects were found on liver CAT activity after fish exposed to HEA. HEA treatment group had the highest CAT activity at 2 hours ($p > 0.05$), and then significant decline of CAT activity occurred as the exposure time prolonged to 4 hours ($p < 0.05$). The CAT activity in the samples with longer than 4 hour exposure were significantly lower than that in the control ($p < 0.05$) (Jin, et al. 2017).

5. Conclusion

Conclusively, the results of this study has shown that ammonia chemical is an agent of stress and can sauce oxidative stress to marine medaka fish and inturn the body system undergo response mechanism to prevent the effects of the stress by the use of enxymes like CAT, GPx, GST and SOD. As seen in this study, gills and kidney play important role in the antioxidant mechanism as they are evolutionary evolved to protect the body against chemical stressors like ammonia through excretion pathways.

From the findings we got from the current study, we can use the information to develope antioxidant chemical compounds from aquatic resources like seaweed and measure the degree of effectiveness in helping fish body to adjust homeostatic balance after being exposed to ammonia stress. The future coming studies should focus on measuring ammonia stress response of fish exposed in a particular concentration and analysis should be between fish fed with supplemented antioxidant and non antioxidant feeds.

6. References

Atli, G., & Canli, M. (2010). Response of antioxidant system of freshwater fish *Oreochromis niloticus* to acute and chronic metal (Cd, Cu, Cr, Zn, Fe) exposures. Ecotoxicology and Environmental Safety, 73(8), 1884-1889.

Batista, M. T. O., Rodrigues Junior, E., Feijó-Oliveira, M., Ribeiro, A. C., Rodrigues, E., Suda, C. N. K., & Vani, G. S. (2014). Tissue levels of the antioxidant enzymes superoxide dismutase and catalase in fish *Astyanax bimaculatus* from the Una River Basin. Revista Ambiente & Água, 9(4), 621-631.

Barton, B. A. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integrative and comparative biology, 42(3), 517-525.

Bonga, S. W. (1997). The stress response in fish. Physiological reviews, 77(3), 591-625.

Cheng, C. H., Yang, F. F., Ling, R. Z., Liao, S. A., Miao, Y. T., Ye, C. X., & Wang, A. L. (2015). Effects of ammonia exposure on apoptosis, oxidative stress and immune response in pufferfish (*Takifugu obscurus*). Aquatic Toxicology, 164, 61-71.

Cho, Y. S., Lee, S. Y., Kim, Y. K., Kim, D. S., & Nam, Y. K. (2011). Functional ability of cytoskeletal β-actin regulator to drive constitutive and ubiquitous expression of a

fluorescent reporter throughout the life cycle of transgenic marine medaka *Oryzias dancena*. Transgenic research, 20(6), 1333-1355.

Cummins, I., Cole, D. J., & Edwards, R. (1999). A role for glutathione transferases functioning as glutathione peroxidases in resistance to multiple herbicides in black□grass. The Plant Journal, 18(3), 285-292.

Eladari, D., Cheval, L., Quentin, F., Bertrand, O., Mouro, I., Cherif-Zahar, B., ... & Chambrey, R. (2002). Expression of RhCG, a new putative NH3/NH4+ transporter, along the rat nephron. Journal of the American Society of Nephrology, 13(8), 1999-2008.

Halliwell, B., & Gutteridge, J. M. (2015). Free radicals in biology and medicine. Oxford University Press, USA.

Hegazi, M. M., Attia, Z. I., & Ashour, O. A. (2010). Oxidative stress and antioxidant enzymes in liver and white muscle of Nile tilapia juveniles in chronic ammonia exposure. Aquatic toxicology, 99(2), 118-125.

Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. L., & Korsmeyer, S. J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell, 75(2), 241-251.

Hoffmann, A. A., & Parsons, P. A. (1997). Extreme environmental change and evolution. Cambridge University Press.

Hoffmann, A. A., & Hercus, M. J. (2000). Environmental stress as an evolutionary force. AIBS Bulletin, 50(3), 217-226.

Ip, Y. K., & Chew, S. F. (2010). Ammonia production, excretion, toxicity, and defense in fish: a review. Frontiers in Physiology, 1.

Iwama, G. K., Vijayan, M. M., Forsyth, R. B., & Ackerman, P. A. (1999). Heat shock proteins and physiological stress in fish. American Zoologist, 39(6), 901-909.

Jin, Y., Zheng, S., & Fu, Z. (2011). Embryonic exposure to cypermethrin induces apoptosis and immunotoxicity in zebrafish (*Danio rerio*). Fish & shellfish immunology, 30(4), 1049-1054.

Jin, J., Wang, Y., Wu, Z., Hergazy, A., Lan, J., Zhao, L., ... & Lin, L. (2017). Transcriptomic analysis of liver from grass carp (*Ctenopharyngodon idellus*) exposed to high environmental ammonia reveals the activation of antioxidant and apoptosis pathways. Fish & shellfish immunology, 63, 444-451.

Kayanoki, Y., Fujii, J., Islam, K. N., Suzuki, K., Kawata, S., Matsuzawa, Y., & Taniguchi, N. (1996). The protective role of glutathione peroxidase in apoptosis induced by reactive oxygen species. The Journal of Biochemistry, 119(4), 817-822.

Keen, J. H., & Jakoby, W. B. (1978). Glutathione transferases. Catalysis of nucleophilic reactions of glutathione. Journal of Biological Chemistry, 253(16), 5654-5657.

Kim, B. M., Kim, J., Choi, I. Y., Raisuddin, S., Au, D. W., Leung, K. M., ... & Lee, J. S. (2016). Omics of the marine medaka (*Oryzias melastigma*) and its relevance to marine environmental research. Marine environmental research, 113, 141-152.

Kim, S. H., Kim, J. H., Park, M. A., Hwang, S. D., & Kang, J. C. (2015). The toxic effects of ammonia exposure on antioxidant and immune responses in Rockfish, *Sebastes*

schlegelii during thermal stress. Environmental toxicology and pharmacology, 40(3), 954-959.

Kim, Y. K., Lee, S. Y., Kim, B. S., Kim, D. S., & Nam, Y. K. (2014). Isolation and mRNA expression analysis of aquaporin isoforms in marine medaka Oryzias dancena, a euryhaline teleost. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 171, 1-8.

Landis, G. N., & Tower, J. (2005). Superoxide dismutase evolution and life span regulation. Mechanisms of ageing and development, 126(3), 365-379.

Li, M., Gong, S., Li, Q., Yuan, L., Meng, F., & Wang, R. (2016). Ammonia toxicity induces glutamine accumulation, oxidative stress and immunosuppression in juvenile yellow catfish *Pelteobagrus fulvidraco*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 183, 1-6.

Li, S., Yan, T., Yang, J. Q., Oberley, T. D., & Oberley, L. W. (2000). The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. Cancer research, 60(14), 3927-3939.

Liu, Z., Peng, J., Mo, R., Hui, C. C., & Huang, C. H. (2001). Rh type B glycoprotein is a new member of the Rh superfamily and a putative ammonia transporter in mammals. Journal of Biological Chemistry, 276(2), 1424-1433.

Livingstone, D. R., O'hara, S. C. M., Frettsome, F., & Rundle, J. (2001). Contaminant-mediated pro-/anti-oxidant processes and oxidative damage in early life-stages of fish. Environment and animal development. Genes, life histories and plasticity, 173-201.

Lou, Z. (2001). Cytochrome P450 enzymes in channel catfish, *Ictalurus punctatus*, and metabolism of testosterone by catfish intestinal microsomes. PhD thesis.

Lushchak, V. I. (2012). Review article Glutathione Homeostasis and Functions: Potential Targets for Medical Interventions. Journal of Amino Acids Volume 2012 . Article ID 736837, 26 pages.

Margis, R., Dunand, C., Teixeira, F. K., & Margis□Pinheiro, M. (2008). Glutathione peroxidase family–an evolutionary overview. The FEBS journal, 275(15), 3959-3970.

Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K., & Hirose, S. (2007). Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 293(4), R1743-R1753.

Nawata, C. M., & Wood, C. M. (2009). mRNA expression analysis of the physiological responses to ammonia infusion in rainbow trout. Journal of Comparative Physiology B, 179(7), 799-810.

Pinto, M.R., Lucena, M.N., Faleiros, R.O., Almeida, E.A., McNamara, J.C., Leone, F.A. (2016). Effects of ammonia stress in the Amazon river shrimp *Macrobrachium amazonicum* (Decapoda, Palaemonidae) J. Aquatic Toxicology (170) 13–23.

Pottinger, T. G. (2008). The stress response in fish-mechanisms, effects and measurement. Fish Welfare. Blackwell Publishing Ltd, UK, 32-48.

Prunet, P., Cairns, M. T., Winberg, S., & Pottinger, T. G. (2008). Functional genomics of stress responses in fish. Reviews in Fisheries Science, 16(sup1), 157-166.

Qin, C., Shao, T., & Duan, H. (2016). The cloning of a heat shock protein 90β gene and expression analysis in *Botia reevesae* after ammonia-N exposure and Aeromonas hydrophila challenge. Aquaculture Reports, 3, 159-165.

Quentin, F., Eladari, D., Cheval, L., Lopez, C., Goossens, D., Colin, Y., ... & Chambrey, R. (2003). RhBG and RhCG, the putative ammonia transporters, are expressed in the same cells in the distal nephron. Journal of the American Society of Nephrology, 14(3), 545-554.

Rahman, S.M.A. (2016). Molecular cloning and mRNA expression analysis of interleukin-1β, cathepsin B and cathepsin D from Siberian sturgeon (*Acipenser baerii*). Master thesis Pukyong National University.

Rankin, J. C., Jensen, F. B. (1993) Fish ecophysiology. Volume 9 of the series Chapman & Hall Fish and Fisheries Series pp 105-135.

Rao, J. V. (2006). Biochemical alterations in euryhaline fish, *Oreochromis mossambicus* exposed to sub-lethal concentrations of an organophosphorus insecticide, monocrotophos. Chemosphere, 65(10), 1814-1820.

Sen, A., & Semiz, A. (2007). Effects of metals and detergents on biotransformation and detoxification enzymes of leaping mullet (*Liza saliens*). Ecotoxicology and environmental safety, 68(3), 405-411.

Sinha, K.A., AbdElgawad, T., Giblen, T., Zinta, G., De Rop, Asard, Blust, H., & Boeck, G. "Anti-Oxidative Defences Are Modulated Differentially in Three Freshwater Teleosts in Response to Ammonia-Induced Oxidative Stress", PLoS ONE, 2014

Stults-Kolehmainen, M. A., & Sinha, R. (2014). The effects of stress on physical activity and exercise. Sports medicine, 44(1), 81-121.

Sørensen, J. G., & Loeschcke, V. (2007). Studying stress responses in the post-genomic era: its ecological and evolutionary role. Journal of biosciences, 32(3), 447.

Stoliar, O. B., & Lushchak, V. I. (2012). Environmental pollution and oxidative stress in fish. In Oxidative Stress-Environmental Induction and Dietary Antioxidants. InTech.

Sturtz, L. A., Diekert, K., Jensen, L. T., Lill, R., & Culotta, V. C. (2001). A fraction of yeast Cu, Zn-superoxide dismutase and its metallochaperone, ccs, localize to the intermembrane space of mitochondria a physiological role for sod1 in guarding against mitochondrial oxidative damage. Journal of Biological Chemistry, 276(41), 38084-38089.

Tort, L. (2010). Stress in farmed fish. Its consequences in health and performance. Recent Advances in Aquaculture Research. Transworld Research Network, Trivandrum, Kerala, India. ISBN, 978-81.

Tort, L., & Teles, M. (2012). Genomic responses to stress challenges in fish. Functional Genomics in Aquaculture, 7, 147-168.

Tiligada, E., Giannoulaki, V., Sitaras, N., & Varonos, D. (2002). Heparin Inhibits the Effects of Compound 48/80 and Fluoxetine on Conjunctival Histamine Content in Vivo. Inflamm Res 51 Suppl 1, S07-S08. 4

Vasylkiv, O. Y., Kubrak, O. I., Storey, K. B., & Lushchak, V. I. (2011). Catalase activity as a potential vital biomarker of fish intoxication by the herbicide aminotriazole. Pesticide biochemistry and physiology, 101(1), 1-5.

Verlander, J. W., Miller, R. T., Frank, A. E., Royaux, I. E., Kim, Y. H., & Weiner, I. D. (2003). Localization of the ammonium transporter proteins RhBG and RhCG in mouse kidney. American Journal of Physiology-Renal Physiology, 284(2), F323-F337.

Wang, C., Yue, X., Lu, X., & Liu, B. (2013). The role of catalase in the immune response to oxidative stress and pathogen challenge in the clam *Meretrix meretrix*. Fish & shellfish immunology, 34(1), 91-99.

Wang, Z., & Leung, K. M. (2015). Effects of unionised ammonia on tropical freshwater organisms: implications on temperate-to-tropic extrapolation and water quality guidelines. Environmental Pollution, 205, 240-249.

Wright, P. A., & Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. Journal of Experimental Biology, 212(15), 2303-2312.

Wu, B., & Beitz, E. (2007). Aquaporins with selectivity for unconventional permeants. Cellular and molecular life sciences, 64(18), 2413-2421.

Yamashita, Y., Yabu, T., Touhata, K., & Yamashita, M. (2012). Purification and characterization of glutathione peroxidase 1 in the red muscle of Pacific bluefin tuna *Thunnus orientalis*. Fisheries science, 78(2), 407-413.

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