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Toxic effects of dietary lead exposure and detoxification effects of ascorbic acid in juvenile rockfish *Sebastes schlegelii*

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치어기 조피볼락에서 급이를 통한 납 노출의 독성영향과
비타민 C의 해독작용

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요약

Chapter I: 납 노출에 따른 독성영향을 확인하기 위해, 조피볼락 (평균 전장 14.2±1.9 cm, 평균 무게 57.3±5.2 g)을 먹이를 통해 납 0, 30, 60, 120 그리고 240mg/L의 농도로 4주간 노출시켰다. 납 노출에 따른 생체 조직의 축적 중 신장에서 가장 높은 납 축적이 관찰되었다. 성장률과 간 중량지수는 120 mg/kg 이상의 노출에서 유의적으로 감소되었다. 적혈구 수, 적혈구 용적, 그리고 헤모글로빈 등과 같은 혈액학적 지표에서는 납 노출 60 mg/kg 이상에서 유의적인 감소가 나타났다. 혈장 무기성분인 칼슘과 마그네슘은 유의적으로 감소하였다. 혈장 유기성분인 글루코즈와 콜레스테롤은 증가한 반면, 총 단백은 감소를 나타냈다.혈장 효소성분인 GOT와 GPT는 유의적인 증가를 나타낸 반면, ALP는 변화가 없었다. 납 노출에 따른 항산화반응에서, 조피볼락 간과 아가미 조직에서의 SOD는 유의적 증가가 나타난 반면, GSH 수치는 유의적으로 감소되었다. 신경독성을 확인하기 위한, AChE 활성 측정에서 납 노출에 따른 뇌와 근육 조직에서 유의적인 억제가 관찰되었다. 중금속 노출 시 중금속을 결합하는 단백질인 metallothionein 유전자는 납 노출에 의해 유의적으로 증가되었다. 스트레스 측정지표로 널리 사용되는 HSP 70

유전자는 60 mg/kg 이상의 납 노출에 의해 유의적인 증가를 나타냈다. 납 노출에 따른 면역반응을 보기 위한 실험에서, 간 조직의 Ig M은 120 mg/kg 이상의 납 노출에서 유의적으로 증가하였고, 라이소자임 활성 또한 60 mg/kg 이상의 납 노출에 의해 유의적으로 증가하였다. 본 실험의 결과는 납 노출은 실험어인 조피볼락에서의 특정조직에서의 생체축적을 유발하였으며, 혈액학적 성상 변화, 산화스트레스에 따른 항산화효소 변화, 특정 환경독성 유전자 발현 및 면역학적 변화를 나타내었다.

Chapter II: 비타민 C의 차이에 따른 조피볼락에서의 영향을 확인하기 위해, 조피볼락 (평균 전장 13.6±1.4 cm, 평균 무게 53.6±4.2 g)을 먹이를 통해 비타민 C 0, 50, 100, 200 그리고 400mg/L의 농도로 4주간 실험을 진행하였다. 비타민 C 50 mg/kg 이상의 농도에서 성장률의 유의적 증가가 관찰되었다. 적혈구 수, 적혈구 용적, 그리고 헤모글로빈 등과 같은 혈액학적 지표에서는 비타민 C 100 mg/kg 이상을 투여한 구간에서 유의적인 증가가 나타났다. 그리고 혈장성분에서도 증가된 비타민 C의 투여는 혈장 글루코즈와 총 단백의 유의적 증가를 유발한 반면, 혈장 GOT와 GPT에서는 유의적 감소를 유발했다. AChE의 활성은 비타민 C 200 mg/kg 이상의 투여에서 유의적으로 증가하였다. 본 실험의 결과 조피볼락에서 비타민 C의 투여는 성장률의 증가뿐 만 아니라, 혈액성상 및 혈장성분의 유의적 변화를 가져왔으며, AChE의 활성에도 영향을 주었다.

Chapter III: 납 노출에 따른 조피볼락의 독성영향에서 비타민 C의 영향을 확인하기 위해, 조피볼락 (평균 전장 11.3±1.2 cm, 평균 무게 32.5±4.1 g)을 먹이를 통해 납 0, 120 그리고 240mg/L의 농도와 비타

민 C 100, 200, 그리고 400 mg/kg의 농도로 사료를 통한 급이 방법으로 4주간 노출시켰다. 납 노출에 따라 조피볼락 특정장기에 유의적으로 높은 축적이 관찰되었으며, 신장 조직에서 가장 높은 축적이 관찰되었다. 적혈구 수, 적혈구 용적, 그리고 헤모글로빈 등과 같은 혈액학적 지표에서는 납 노출에 따른 유의적 감소가 나타났고, 혈장 성상으로 칼슘, 마그네슘, 글루코즈, 콜레스테롤, GOT, 그리고 GPT의 유의적 변화가 관찰되었다. 납 노출은 실험어인 조피볼락의 혈액 성상에 유의적 변화를 나타내었으며, 높은 수준의 비타민 C의 투여는 납 노출에 의한 독성을 완화시키는데, 상당히 효과적이었다. 항산화효소에서, 납 노출에 따른 조피볼락의 간과 아기미 조직의 SOD와 GST는 유의적으로 증가한 반면, GSH는 유의적 감소를 나타내었다. 산화스트레스에 따른 항산화반응에서 대표적인 항산화물질인 비타민 C는 납 노출에 따른 항산화반응을 유의적으로 완화시켜주었다. Metallothionein 유전자와 HSP 70 유전자는 납 노출에 의해 유의적으로 모두 증가하였다. 높은 수준의 비타민 C의 투여는 metallothionein 유전자의 발현은 유의적으로 완화시켰지만, HSP 70의 발현에는 영향을 주지 않았다. 본 실험의 결과는, 납 노출이 조피볼락에서 생체축적과 그에 따른, 산화스트레스, 혈액학적 성상 등에 독성으로 작용하며, 비타민 C의 투여가 조직의 납 축적을 감소시키며, 다른 산화스트레스 반응, 혈액학적 성상 등에서 독성을 유의적으로 감소시키는데 효과적임을 나타낸다.

General Introduction

Heavy metals occur naturally in the aquatic environment, and anthropogenic activities have substantially increased the discharge of various heavy metals into aquatic ecosystems (Mekkawy et al., 2011). Heavy metal contamination in the aquatic environment is a major global environmental issue, because the heavy metals are transferred to humans via the food chain through fish, shellfish, and shrimp, increasing health risks in humans. Of the various heavy metals in the aquatic environment, lead is one of the most toxic substances, even at low concentrations. Lead occurs naturally in the earth's crust, rock, soil, and water. However, excessive doses of the lead exposure in the aquatic environment derived from anthropogenic activities such as mining and smelting, cement manufacture, batteries, and paint, induce lead poisoning in fishes (Rogers et al., 2003). In South Korea, lead contamination in the aquatic environment is mainly due to the anthropogenic activities. Marine lead contamination has recently been attributed to the leisure industry, e.g., fishing gear (Lloret et al., 2014). Lead from abandoned lead-containing waste on the coast is accumulated in the sediment, causing lead toxicity of the marine ecosystem.

Lead exposure in the marine environment negatively influences reproduction, development, growth, and behavior in fishes, and the consumed less feed at 201 ppb Pb after 12 day exposure was

observed in rainbow trout, *Oncorhynchus mykiss* (Burdena et al., 1998). Lead toxicity occurs through the interaction of lead and proteins; high affinity metal-binding proteins such as lead-binding proteins and metallothioneins can intervene in the interaction between lead and enzymes (Goering, 1992). Lead exposure higher than permissible concentrations results in damage to all tissues, the central nervous system (CNS), and blood (Palaniappan et al., 2008), and lead toxicity in fish can induce muscular atrophy, lordoscoliosis, numbness, black tail, caudal fin degeneration, hyperactivity, erratic swimming, and loss of equilibrium (Burdena et al., 1998). In South Korea, the level of the lead in coastal sediments ranges from 9.6 to 92.0 mg/L, and the toxic effects of lead exposure varies from the exposure concentrations, periods, environmental conditions, and species (Lim et al., 2007). In marine sediment, threshold effects level (TEL) in lead is 44 mg/kg, and probable effects level (PEL) is 119 mg/kg (Ministry of Land, Transport and Maritime Affairs, 2012).

Lead exposure generally induces bioaccumulation in specific tissues of fish, which can lead to toxic effects. The accumulation of non-essential metals in the aquatic environment occurs by various routes such as water, food, or sediment. Therefore, metal accumulation in the specific tissues of fishes through their feed can be a realistic and sensitive indicator to evaluate lead toxicity. The metal accumulation levels in fishes depend on species-specific indicators, including ecological needs, sex, and

size, as well as environmental factors such as water pH, salinity, temperature, and hardness (Kalay et al., 1999). In addition, the variations in metabolic activity in aquatic animals are a critical outcome of metal accumulation. Further, because metal accumulation in specific tissues of aquatic animals can be a major cause of toxic effects, this should be a reliable parameter to evaluate dietary lead exposure toxicity. Lead exposure in fishes commonly affects hematological parameters and blood components by inhibiting enzyme activities associated with heme biosynthesis (Vinodhini and Narayanan, 2009). A linear decrease in hematological parameters such as RBC count, hematocrit value, and hemoglobin concentrations in aquatic animals is noted because of lead toxicity (Vinodhini and Narayanan, 2009).

Lead exposure in aquatic animals induces the production of reactive oxygen species (ROS) such as hydrogen peroxide, and hydroxyl and superoxide radicals. Oxidative stress occurs when ROS generation overwhelms antioxidant responses (Pena-Llopis et al., 2003). Oxidative stress leads to osmoregulatory dysfunctions, tissue damage, and antioxidant balance disruption. Fish tissues include many polyunsaturated fatty acids (PUFAs) that are vulnerable to oxidative stress. Therefore, fishes have various antioxidant mechanisms for defense against oxidative stress. Superoxide dismutase (SOD) is a major antioxidant enzyme that decomposes superoxide anion to hydrogen peroxide, and catalase (CAT) decomposes H_2O_2 to molecular oxygen and water (Almeida

et al., 2007). Glutathione S-transferase (GST) also acts as a detoxification enzyme for oxidative stress by catalyzing the nucleophilic attack of the sulfur atom of the glutathione (GSH) tripeptide (Kim et al., 2001). GSH is the most abundant intracellular thiol-based antioxidant, and it detoxifies through conjugation reactions catalyzed by GST (Nordberg and Arner, 2001). Oxidative toxicity from lead exposure in aquatic animals should be a reliable biomarker to assess lead toxicity in environmental toxicology and ecotoxicology (Regoli et al., 2002). Tissue injury from lead-induced oxidative stress causes neuronal damage and neurotoxicity. In addition, the lead exposure can impair the CNS by decreasing cognitive and neurobehavioral functions. Among various neurotransmitters, acetylcholine plays a critical role in the central and peripheral nervous system, and it functions as stimulator of muscle in the peripheral nervous system, and activator of sensory perceptions in the CNS. Metal exposure causes neurochemical changes by penetrating the blood brain barrier, which induces alterations in the metabolism of some proteins associated with the neurodegeneration and oxidative stress (Monnet-Tshudi et al., 2006; Senger et al., 2006). Acetylcholinesterase (AChE) is a major component of cholinergic system in fish and controls the nervous impulse transmission in cholinergic synapses. AChE activity is generally inhibited by metal exposure, inducing acetylcholine accumulation at synaptic junctions, which in turn causes acute cholinergic syndrome, finally

leading to death (Hsieh et al., 2001). Therefore, the AChE inhibition can be a major biomarker to assess the toxicity in the aquatic animals from lead exposure.

Acute toxicity from metal exposure such as cadmium, lead, chromium, and arsenic, stimulates the expression of various metal-sensitive genes including metallothionein and heat shock protein 70 (HSP-70) as a stress response (Roh et al., 2006). In aquatic animals, metallothionein (MT), a metal-binding protein, functions in metal detoxification (Achard et al., 2004), and metal ion homeostasis (Chen et al., 2004). Increased intracellular metal accumulation in fishes from metal exposure induces increased MT concentrations in target tissues such as liver, kidney, and gills (Baudrimont et al., 2003). Environmental stress such as the exposure to metals generally induces the induction of HSPs; HSP-70 maintains protein integrity under stress (Basu et al., 2001; Maradonna and Carnevali, 2007). Given that MT and HSP 70 gene are the major biomarkers to monitor metal contamination in the aquatic environment, MT and HSP 70 gene expressions have been proposed as sensitive and reliable biomarkers to evaluate lead-induced toxicity (Linde et al., 2001; Rajeshkumar and Munuswamy, 2011).

Alterations of the immune responses can occur as a response to various stress factors such as metals, pesticides, hydrocarbons, and other chemicals, and metal exposure has been closely associated with the alterations of the immune systems in fish

(Arunkumar et al., 2000). Plasma immunoglobulins have been considered as a main component of the vertebrate humoral immune system, and immunoglobulin M (IgM) is a major immunoglobulin in teleosts (Wilson et al., 1995). Even though the effects of metal-induced stress on fish immune indicators are a critical in ecotoxicology and environmental toxicology, the changes in total plasma IgM levels for metal exposure to assess metal toxicity have been insufficiently analyzed (Cuesta et al., 2004). As IgM is commonly altered by stressors, it could be a reliable biomarker to evaluate metal toxicity. Among various immune responses, lysozyme is a major immune response in response to the metal exposure. Lysozymes are bacteriolytic enzymes, protecting fish from microbial invasion. Lysozyme activity in fishes can be increased by exposure to aquatic toxicants in addition to health condition, stress, sex, and temperature (Saurabh and Sahoo, 2008).

Ascorbic acid (AsA) is an essential nutrient in aquatic animals for their efficient growth, physiological function, and immunity (Roberts et al., 1995; Ai et al., 2004; Lin and Shiau, 2005; Ren et al., 2007). AsA has many functions in aquatic animals such as an immune-stimulating effect, histamine inhibition from allergic reactions, powerful antioxidant activity to neutralize free radicals, collagen synthesis, peptide hormones and neurotransmitters synthesis, and prevention of the formation of potentially carcinogenic nitrosamines. Dietary AsA supplementation through

feed is essential to maintain physiological homeostasis in fishes, because most fish cannot synthesize AsA due to lack of L-gulonolactone oxidase activity, which functions as a catalyst converting L-gulonolactone to AsA (Nishikimi and Yagi, 1991; Wang et al., 2003). AsA acts as a cofactor involved in biological hydroxylation of many metabolic processes in connective tissue, scar tissue, blood vessels, and bones (Fracalossi et al., 2001). Therefore, AsA deficiency in fishes causes many negative symptoms such as spine deformation, internal hemorrhaging, resorbed opercles, jaw and snout hyperplasia, abnormal pigmentation, malformation, anorexia, liver steatosis, and late wound repair, in addition to decreased growth and reduction in feed intake and utilization (Roberts et al., 1995). Correlations between dietary AsA supplementation in fish and various defense mechanisms for pathogens, environmental indicators, and general stress reactions have been analyzed (Sandnes et al., 1990). The optimum requirement of AsA differs depending on many factors, including the species and size of fish examined, in addition to their environment. For example, the AsA requirement in juvenile fish is greater than that of adult fish, and the AsA requirement increases three to ten times based on the disease status (NFRDI, 2007).

AsA supplementation has many protective functions against toxicity from metal exposure by effectively decreasing metal accumulation and toxicity by chelation metal (Dalley et al., 1990;

West et al., 1994; Tandon et al., 2001). AsA supplementation enables excretion of the accumulated metal from the body by reducing ferric iron to ferrous iron in the duodenum, which competes with metal for intestinal absorption (Patrick, 2006). However, reduction in metal accumulation by AsA supplementation has not been reported in aquatic animals. Therefore, determining the AsA function to reduce metal accumulation in aquatic animals is valuable and novel.

AsA supplementation protects aquatic animals exposed to metals from decreased growth performance and changes in hematological parameters and plasma components (Grosicki, 2004; Yousef, 2004). In the aquatic environment, metal exposure induces ROS, and AsA supplementation effectively protects cells by scavenging the free radicals (Singh et al., 2014). AsA supplementation also effectively alleviates neurotoxicity caused by metal exposure, because it is a cofactor for the biosynthesis of neurotransmitters. Metal exposure causes an increase in specific gene expressions such as MT and HSP 70. AsA supplementation effectively alleviates metal-induced toxicity, thereby affecting specific gene expression.

In South Korea, the rockfish *Sebastes schlegelii* is one of the most largely cultured marine fish and is crucial to aquaculture due to its high demand, appreciated flesh, and rapid growth. Rockfish are widely distributed in temperate marine environments in Korea, China, and Japan, which inhabit coastal reefs from 10 to 100 m

depth. In addition, rockfish are carnivorous, consuming small fish and squid, and are ovoviviparous, spawning from April to June. To determine ecologically useful and desirable outcomes from aquatic toxicity experiments, appropriate animals and suitable experiments should be used. If possible, endemic animals should be used as representatives of the natural environment for the research (Rand et al., 1995). Therefore, rockfish could be a suitable experimental animal for this research.

Toxicological researches have shown the beneficial effects of AsA supplementation to overcome metal toxicity from metal exposure. However, research on the combined effect of experiments is insufficient. Moreover, no study, to our knowledge, has shown that AsA supplementation effectively reduces metal accumulation in aquatic animals, whereas some have assessed the effects of AsA supplementation in rodents such as rats and mice. The purpose of the present study was to assess dietary AsA effects on dietary lead exposure including bioaccumulation, hematological parameters, and antioxidant systems of *S. schlegelii*, in addition to assessing the effects of dietary lead exposure and AsA supplementation.

Chapter I. Toxic effects of dietary lead exposure on bioaccumulation, hematological parameters, antioxidant and immune responses in rockfish, *Sebastes schlegelii*.

1. Introduction

The exposure to toxic metals in aquatic environment is a critical environmental issue, because these metals unavoidably transfer to the human through food chain, which creates a health risk for human. The heavy metal exposure at toxic concentrations easily accumulates in aquatic animals leading to diseases and disorders (Bailey et al., 1999). Among heavy metals, lead is non-essential and harmful to animals even at low concentrations (Shah, 2006), and it is prevalent in aquatic environment by anthropogenic activity such as industries of batteries, paint production, and leaded gasoline (Monteiro et al., 2011).

The lead exposure in aquatic environment negatively affects reproduction, growth, and behavior in fish (Burdena et al., 1998). The lead toxicity occurs by the interaction of lead with protein, and the high affinity metal-binding proteins such as lead-binding proteins and metallothioneins can intervene in the interaction between lead and enzyme (Goering, 1992). The higher lead exposure than a permissible concentration result from damage to

all tissues, central nervous system, and blood (Palaniappan et al., 2008), and its exposure of the lead toxicity in fish can induce muscular atrophy, lordoscoliosis, numbness, black tail, and caudal fin degeneration, in addition to hyperactivity, erratic swimming, and loss of equilibrium (Burdena et al., 1998).

The bioaccumulation by the heavy metal exposure to fish is one of the causes to induce toxic effects, and it has been studied by many authors (Adami et al., 2002; Rasmussen and Anderson, 2000). The non-essential metal accumulation in fish occurs by their water, food, or sediment, and accumulates in their tissues, which is similar to the route of essential metals (Canli and Atli, 2003). The metal accumulation in tissues of fish depends on the concentrations and periods of the metal exposure, in addition to the species-specific factors such as ecological needs, sex, and size and the environmental factors such as pH, salinity, temperature, and hardness (Kalay et al., 1999). It is well known that the metabolic activity in marine animals is also one of the most crucial factors in heavy metal accumulation in their tissues, and the accumulation in young individuals is commonly higher than that of older individuals considering the metabolic activity in young individuals is much more active than older individuals (Widianarko et al., 2000). In aquatic environment, the prevalent heavy metals have been usually accumulated through the food chain, and the accumulation in the tissues may be toxic for fish (Dural et al., 2007). Therefore, the accumulation in the specific

tissues of fish can be a sensitive and reliable indicator to assess aquatic contamination.

The crucial aim of ecotoxicology in aquatic ecosystems is to assess the risks for aquatic animals and human populations (Oliverira Ribeiro et al., 2006). Hematological parameters such as RBC count, hematocrit value, and hemoglobin concentration is one of targets for lead toxicity (Gurer et al., 1998), and it can be a useful indicator to evaluate the metal toxicity in aquatic environment (Shah and Altindag, 2004). The lead exposure in fish has been widely known to effect the alterations of hematological parameters by restraining the enzyme activities linked to heme biosynthesis (Vinodhini and Narayanan, 2009). Iavicoli et al. (2003) suggested that the exposure to lead concentration was connected to a linear decrease in hematological parameters such as RBC count, hematocrit, and hemoglobin.

Exposure to metals generally induces the generation of reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical, and superoxide radical, which is leading to oxidative stress, as well as osmoregulatory dysfunctions and tissue damage (Dautremepuits et al., 2004). Considering lead is known to induce strong oxidative stress, which causes the disruption of antioxidant balance via oxidative damage to critical biomolecules (Jiun and Hsien, 1994), oxidative stress can be a reliable indicator to assess the lead-induced toxicity. Although oxygen is a critical element for aerobic cells, the reactive oxygen species (ROS) such

as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxide ions (OH^-), and singlet oxygen (O_2) is generated by oxygen in the respiratory process, which induces potential cytotoxic problems (Fuente and Victor, 2000). ROS can react with biological molecules and lead to enzyme inactivation, lipidperoxidation, and DNA damage. Generally, the balance between ROS production and antioxidant defenses affects the level of oxidative stress (Pena-Llopis et al., 2003). As the response to ROS production, superoxide dismutase (SOD) is one of anti-oxidant defense mechanisms of living organisms, which scavenges the superoxide anion. Glutathione S-transferase (GST) plays acritical role in catalyzing the conjugation of toxicants to remove them from the cellular system (Atli and Canli, 2010). GSH also scavenges ROS directly through the oxidation of two molecules of GSH to a molecule of glutathione disulphide (GSSG), and the relation between the decreased and oxidized state of glutathione is considered as an indicator of oxidative damage and cellular redox status (Pena-Llopis et al., 2003). The oxidative toxicity by xenobiotics in aquatic animals can be a sensitive and reliable biomarker in environmental toxicology and ecotoxicology (Regoli et al., 2002).

The lead toxicity also involves neuronal damage as neurotoxicity, because it is a neurotoxic agent that adversely affects the central nervous system. In nervous system, acetylcholine is a major neurotransmitter both in central and peripheral nervous system

such as activating muscle and enhancing sensory perceptions. Acetylcholinesterase (AChE) is a hydrolase of neurotransmitter acetylcholine, and the measurement of AChE activity has been generally conducted with brain and muscle tissue. The neuromuscular system in fish is vital for normal muscle behavior and function (Modesto and Martinez, 2010), and the inhibition AChE in brain induces alterations in behavior, which may result in tetania and paralysis by hypersimulation of muscle fibers (Kirby et al., 2000). Therefore, the determination of AChE is widely used as a diagnosis for the evaluation of ecotoxicological risk and monitoring program in aquatic systems.

Metal toxicity also induces the reactions in cytosol to inactivate the toxicity by binding metal through the non-specific binding of metals to non-thionein ligands, and the regulation of intracellular metal toxicity generally occur via the production of metal-binding ligands that sequester metals (Giguere et al., 2003). Metallothionein (MT) is a class of low molecular weight, cysteine-rich, heavy metal binding proteins, which acts as heavy metal ion homeostasis and detoxification (Chen et al., 2004). MT has been shown to be a reliable biomarker for metal contamination in aquatic environment (Langston et al., 2002). The environmental stresses such as chemicals and temperature cause a generalized stress response at the cellular level, and the stress response induces the induction of heat shock proteins (Basu et al., 2001). Heat shock protein 70 (HSP 70) functions to maintain

protein integrity under the various stresses such as chemicals and temperature (Maradonna and Carnevali, 2007), HSP 70 has been proposed as a sensitive and reliable biomarker to assess environmental stressors against toxicants (Ahamed et al., 2010).

The immune responses in aquatic animals can be enhanced by the toxicant exposure (Bols et al., 2001). Immunoglobulin M (Ig M) is one of the most major components in teleosts as a specific innate immune response, and the antibody levels in fish can be influenced by environmental factors such as xenobiotics and pathogens in addition to temperature, salinity, oxygen, and pH (Tellez-Banuelos et al., 2010). Koller et al. (1976) reported long term experiments induced immunosuppression of mice, whereas the single dose exposure for lead and cadmium enhanced Ig M antibody formation. Metal exposure can cause an immunotoxicity to animals, and the arsenic and mercury exposure to rats significantly affected the immune responses (Institoris et al., 2001). Lysozyme is a critical innate immune response in fish, which disrupts the cell walls of bacteria by splitting glycosidic linkages in the peptidoglycan layers. The levels of lysozyme have been modulated by the exposure to heavy metals (Bols et al., 2001). Lysozyme has been studies in various invertebrates, and the lysozyme activity can be considered as a critical parameter to evaluate immunotoxic effects of metals (Marcano et al., 1997). Given that the fish immune responses are affected by toxicant exposure such as metals, pesticides, and hydrocarbons, the

immune responses can be a reliable indicator to measure the effects of environmental toxicants on fish innate immunity (Kim and Kang, 2015).

Rock fish, *Sebastes schlegelii*, is one of the most largely cultured fish of marine net cages in South Korea, because it is one of the most crucial farming fish due to its high demand, appreciated flesh, and rapid growth. However, the insufficient study about the exposure to lead toxicity has been conducted. Therefore, the aim of this chapter is to assess the toxic effects of dietary lead exposure to the *S. schlegelii* on the accumulation, growth performance, hematological parameters, antioxidant systems, acetylcholinesterase activity, specific gene expression, and immune responses by exposing the *S. schlegelii*.

2. Materials and Methods

2.1. Experimental fish and culture conditions

Juvenile *S. schlegelii* were obtained from a local fish farm in Tongyeong, Korea. The fish were acclimatized for 2 weeks under laboratory conditions. During the acclimation period, the fish were fed a Pb-free diet twice daily and maintained on a 12-h:12-h light/dark cycle and constant condition at all times (Table 1-1). After acclimatization, 60 fishes (body length, 14.2±1.9 cm; body weight, 57.3±5.2 g) were randomly selected for the study. Dietary lead exposure took place in 500L circular tanks 6 fish per treatment group in duplicates. The dietary lead concentrations were 0, 30, 60, 120, and 240 mg/kg (Table 1-2), and fish were fed each lead concentration at a rate of 2% body weight daily (as two 1% meals per day). At the end of each period (at 2 and 4 weeks), fish were anesthetized in buffered 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma Chemical, St. Louis, MO).

Table. 1-1. The chemical components of seawater and experimental condition used in the experiments.

Item	Value
Temperature (°C)	20.0±0.5
pH	8.1±0.5
Salinity (‰)	33.2±0.5
Dissolved Oxygen (mg/L)	7.1±0.3
Chemical Oxygen Demand (mg/L)	1.21±0.1
Ammonia (μg/L)	11.7±0.8
Nitrite (μg/L)	1.6±0.2
Nitrate (μg/L)	10.31±1.0

2.2. Feed ingredients and diets formulation

Formulation of the diets is shown in Table 1-2. Lead (II) nitrate was obtained from Sigma Chemical Co., Ltd. All diets contained 33% casein, 23% fish meal, 5% corn starch, 2% vitamin premix, and 2% mineral premix. 10% fish oil was added to meet the essential fatty acids (EFA) requirements of rock fish. Lead premix was made up of 1 g lead with 99 g cellulose. Five isonitrogenous and isolipidic diets were formulated with supplementation of different dietary lead concentrations of 0, 30, 60, 120, and 240 mg/ kg diet. All ingredients were blended thoroughly. At last, water was added into the mixture to produce stiff dough. Then the dough was pelleted by experimental feed mill, and dried for 24 h at room temperature. After processing, all the diets were packed and kept - 20 °C until use.

Table. 1-2. Formulation of the experimental diet (% dry matter).

Ingredient (%)	Lead concentration (mg/kg)				
	0	30	60	120	240
Casein[1]	33.0	33.0	33.0	33.0	33.0
Fish meal[2]	23.0	23.0	23.0	23.0	23.0
Wheat flour[3]	20.0	20.0	20.0	20.0	20.0
Fish oil[4]	10.0	10.0	10.0	10.0	10.0
Cellulose[1]	5.0	4.7	4.4	3.8	2.6
Corn starch[3]	5.0	5.0	5.0	5.0	5.0
Vitamin Premix[5]	2.0	2.0	2.0	2.0	2.0
Mineral Premix[6]	2.0	2.0	2.0	2.0	2.0
Lead Premix[7]	0.0	0.3	0.6	1.2	2.4
Actual Pb levels	1.8	31.4	62.1	118.8	244.5

[1]United States Biochemical (Cleveland, OH).

[2]Suhyup Feed Co., Ltd., Gyeong Nam Province, Korea.

[3]Young Nam Flour Mills Co., Pusan, Korea.

[4]Sigma Chemical Co., St. Louis, MO.

[5]Vitamin Premix (mg/kg diet): ascorbic acid, 240; dl-calcium pantothenate, 400; choine chloride 200; inositol, 20; menadione, 2; nicotinamide, 60; pyridoxine·HCl, 44; riboflavin, 36; thiamine mononitrate, 120, dl-a-tocopherol acetate, 60; retinyl acetate, 20000IU; biotin, 0.04; folic acid, 6; vitamin B_{12},0.04;cholecalcifero,4000IU.

[6]Mineral Premix (mg/kg diet): Al, 1.2; Ca, 5000; Cl, 100; Cu, 5.1; Co, 9.9; Na, 1280; Mg, 520; P, 5000; K, 4300; Zn, 27; Fe, 40; I, 4.6; Se, 0.2; Mn, 9.1.

[7]Lead Premix (mg/kg diet): 10,000 mg Pb/ kg diet

2.3. Bioaccumulation

The tissue samples of liver, kidney, spleen, intestine, gill, and muscle of *S. schlegelii* were performed with freeze-dried to measure dry weight of the samples. The freeze-drying samples were digested by wet digestion method (Arain et al., 2008; Korai et al., 2008). The dried samples were digested in 65%(v/v) HNO3, and re-dried at 120 °C on hot plate. The procedure was repeated until total digestion. The entirely digested samples were diluted in 2%(v/v) HNO3. The samples were filtered through a 0.2 μm membrane filter (Advantec mfs, Ins.) under pressure for analysis. For determination of total lead concentrations, the digested and extracted solutions were analyzed by ICP-MS. The ICP-MS measurements were performed using an ELAN 6600DRC ICP-MS instrument with argon gas (Perkin-Elmer). Total lead concentrations were determined by external calibration. ICP multi-element standard solution VI (Merck) was used for standard curve. The lead bioaccumulation in tissue samples was expressed μg/g dry wt.

2.4. Growth Performance

No mortality was observed for the experimental periods. The weight and length of *S. schlegelii* was measured just before exposure, at 2 and 4 weeks. Daily length gain, daily weight gain,

condition factor, and hepatosomatic index (HIS) were calculated by the following method.

Daily growth gain = Wf-Wi/day

(Wf=Finallengthorweight,Wi=Initiallengthorweight)

Condition factor (%) = (W/L3)x100

(W = weight (g), L = length (cm))

HIS = (liver weight/ total fish weight) x 100

2.5. Hematological assay

Blood samples were collected within 35-40 seconds through the caudal vein of the fish in 1-ml disposable heparinized syringes. The blood samples were kept at 4°C until the blood parameters were completely studied. The total red blood cell (RBC) count, hemoglobin (Hb), concentration, and hematocrit (Ht) value were determined immediately. Total RBC counts were counted using optical microscope with hemo-cytometer (Improved Neubauer, Germany) after diluted by Hendrick's diluting solution. The Hb concentration was determined using Cyan-methemoglobin technique (Asan Pharm. co., Ltd.). The Ht value was determined by the microhematocrit centrifugation technique. The blood samples were centrifuged to separate serum from blood samples at 3000 g for 5 minutes at 4°C. The serum samples were analyzed for inorganic substances, organic substances, and

enzyme activity using clinical kit (Asan Pharm. Co.,Ltd.). In inorganic substances assay, calcium and magnesium were analyzed by the o-cresolphthalein-complexon technique and xylidyl blue technique. In organic substances assay, glucose and total protein were analyzed by GOD/POD technique and biuret technique. In enzyme activity assay, glutamic oxalate transaminase (GOT) and glutamic pyruvate transaminase (GPT) were analyzed by Kind-king technique and alkaline phosphatase (ALP) was analyzed using clinic al kit.

2.6. Antioxidant system analysis

Liver and gill tissues were excised and homogenized with 10 volumes of ice-cold homogenization buffer using Teflon-glass homogenizer (099CK4424, Glass-Col, Germany). The homogenate was centrifuged at 10,000 g for 30 min under refrigeration and the obtained supernatants were stored at - 80 °C for analysis.

Superoxide dismutase (SOD) activity was measured with 50% inhibitor rate about the reduction reaction of WST-1 using SOD Assay kit (Dojindo Molecular Technologies, Inc.). One unit of SOD is defined as the amount of the enzyme in 20 μl of sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%. SOD activity was expressed as unit mg protein-1.

Glutathione-S-transferase(GST) activity was measured according

to the method of modified Habig (1974). The reaction mixture consisted of 0.2 M phosphate buffer (pH 6.5), 10 mM GSH (Sigma) and 10 mM 1-chloro-2,-dinitrobenzene, CDNB (Sigma). The change in absorbance at 25 °C was recorded at 340 nm and the enzyme activity was calculated as 340 nm and the enzyme activity was calculated as nmol min-1mgprotein-1.

Reduced glutathione was measured following the method of Beutler et al.(1963). Briefly, 0.2ml fresh supernatant was added to 1.8 ml distilled water. Three ml of the precipitating solution (1.67 g metaphosphoric acid, 0.2 g EDTA and 30 g NaCl in 100 ml distilled water) was mixed with supernatants. The mixture was centrifuged at 4500 g for 10 min. 1.0mL of supernatant was added to 4.0ml of 0.3M NaHPO4 solution and 0.5mL DTNB (5,5`-dithiobis-2-nitrobenzoic acid) was then added to this solution. Reduced glutathione was measured as the difference in the absorbance values of samples in the presence and the absence of DTNB at 412 nm. GSH value was calculated as μmol mg protein-1inthetissues.

2.7. Inhibition of AChE activity

AChE activity was determined brain (1:25) and muscle (1:10) homogenate in 0.1 M phosphate buffer, pH 8.0. The homogenate were centrifuged 10,000 g for 20 min at 4 °C. The supernatant was removed and used to test AChE activity. AChE activity was

determined according to the method of Ellman et al. (1961). AChE activity was normalized to protein content and expressed as nmol min-1mgprotein-1. Briefly, the activity on the homogenate was measured by determining the rate of hydrolysis of acetylthiocholine iodide (ACSCh, 0.88mM) in final volume of 300µl, with 33 µl of 0.1 M phosphate buffer, pH 7.5 and 2 mM DTNB. The reaction was started with the addition of the substrate acetylthiocholine, as soon as the substrate was added the hydrolysis and the formation of the dianion of DTNB were analyzed in 412 nm for 5 min (in intervals of 1 min) using a microplate reader. Protein concentration was determined using Ellman's method (1961), with a bovine plasma albumin (Sigma, USA) as standard.

2.8. Specific gene expression

Total RNA was extracted from liver samples using RNA purification kit (Real Biotech Corporation, Taipei, Taiwan), and the quantity and quality of the total RNA were assessed using the Ultrospec 3100 pro (Amersham Bioscience, Amersham, UK). The 260/280 nm absorbance ratios of all samples ranged from 1.80 to 2.00, indicating a satisfactory purify of the RNA samples. Purified RNA was subjected to reverse transcription to cDNA by cDNA synthesis kit (Enzo Life Sciences Inc., NY, USA) according to the reagent's instructions. For real-time quantitative PCR analysis of

MT and HSP70 gene expression, the real-time qPCR primer of MT gene, HSP 70 and 18s rRNA gene are shown in Table 1-3. Real-time PCR assay were carried out in a quantitative thermal cycler (LightCycler® 480 II, Roche Diagnostics Ltd., Rotkreuz, Switzerland) in a final volume of 20 μl containing 10 μl 2 x Master Mix (LightCycler® 480 SYBR Green I Master, Roche Diagnostics Ltd., Rotkreuz, Switzerland), 1 μl of cDNA mix. MT gene-specific primers were applied to evaluate the mRNA levels of MT in liver. Reference 18s rRNA gene was used as internal control. The real-time qPCR amplification began with 5 min at 95 °C, followed by 45 cycles of denaturation of 10 s at 95 °C, annealing of 10 s at 60 °C, and extension of 10 s 72 °C. To analyze the mRNA expression level, the comparative CT methods (2 -ΔΔCT method) was used.

Table. 1-3. The primers used in this study for real-time qPCR.

Gene	Sequence	Product size
18s rRNA	Fw : TGAGAAACGGCTACCACATC Rv : CAATTACAGGGCCTCGAAAG	100 bp
MT	Fw : CAACTGCGGTGGATCCTG Rv : CCAGAGGCGCATTTAGGG	102 bp
HSP 70	Fw : GATGCAGCCAAGAACCAGGTGG Rv : CTTCCCTCCATCTCCGATCACC	144 bp

2.9. Immune responses

To assess the level of the plasma immunoglobulin M, the primary endpoint of the study was to evaluate the modulation of the immune response induced by diets containing different levels of ascorbic acid of *S. schlegelii*. Ig M concentrations in plasma of *S. schlegelii* were measured by enzyme-linked immunosorbent assay (ELISA) quantification kits (MyBioSource Inc. San Diego, CA, USA) as published. Briefly, add standard 50 ul (25, 50, 100, 200, 400, and 800 ng/ml) in each standard well and sample 50 ul to testing sample well, and nothing is added in blank well. HRP- conjugate reagent is added to each well except the blank well. After that, cover with an adhesive strip and incubate for 60 minutes at 37 °C. Wash the microtiter plate 4 times to remove incubation mixture using wash solution (1x), and dry until no moisture appears. And then, add chromogen solution A 50 ul and chromogen solution B 50 ul to each well successively, and protect from light to incubate for 15 minutes at 37 °C. Add 50 ul stop solution to each well. The color should change from blue to yellow in the wells. Lastly, the optical density was determined at 450 nm within 15 minutes. The measurements were performed in triplicate.

To analyze the lysozyme activity of the plasma and kidney, the plasma for analysis was separated from the blood sample, and kidney tissues were excised and homogenized with 10 volumes of

ice-cold homogenization buffer (0.004 M phosphate buffer, pH 6.6) using Teflon- glass homogenizer (099CK4424, Glass-Col, Germany). The homogenate was centrifuged at 10,000 g for 10 min under refrigeration and the obtained supernatant was stored at -70 ℃ (MDF-U53V, SANYO Electric Co. Ltd., Japan) for analysis. Protein content was determined by the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories GmbH, Munich, Germany) based on the Bradford dye-binding procedure, using bovine serum albumin as standard. Lysozyme concentration was calculated through the measure of its enzyme activity. Lysozyme activity was determined by a turbidimetric method (Ellis, 1990) using Micrococcus lysodeikticus (Sigma) as substrate (0.2 mg/ml 0.05M phosphate buffer, pH 6.6 for kidney sample and pH 7.4 for plasma). A standard curve was made with lyophilized hen egg white lysozyme (sigma) and the rate of change in turbidity was measured at 0.5-min and 4.5-min intervals at 530 nm. The results were expressed as μg/ml and μg/g equivalent of hen egg white lysozyme activity (Anderson and Siwicki, 1994).

2.10. Statistical analysis

The experiment was conducted in exposure period for 4 weeks and performed triplicate. Statistical analyses were performed using the SPSS/PC+ statistical package (SPSS Inc, Chicago, IL, USA). Significant differences between groups were identified using

one-way ANOVA and Duncan's test for multiple comparisons or Student's t-test for two groups (Duncan, 1955). The significance level was set at $P < 0.05$.

3. Results

3.1. Bioaccumulation

The lead accumulation in kidney, liver, spleen, intestine, gill, and muscle of *S. schlegelii* exposed to the dietary lead concentrations is demonstrated in Fig. 1-1. It was observed that the highest lead accumulation in kidney. A considerable increase was shown over 30 mg/kg at 2 and 4 weeks after the dietary lead exposure. The Pb accumulation values in kidney was 4.87±1.48 μg/g at 30 mg/kg, 8.02±1.02 μg/g at 60 mg/kg, 9.41±1.25 μg/g at 120 mg/kg, and 14.57±1.43 μg/g at 240 mg/kg dietary lead exposure after 2 weeks, and 6.24±1.27 μg/g at 30 mg/kg, 9.60±1.21 μg/g at 60 mg/kg, 12.89±1.62 μg/g at 120 mg/kg, and 17.11±1.52 μg/g at 240 mg/kg dietary lead exposure after 4 weeks, respectively. For the liver tissue, the Pb accumulation significantly increased over 30 mg/kg at 2 and 4 weeks, and the highest Pb accumulation value in liver was 14.61±1.64 μg/g for exposure to 240 mg/kg after 4 weeks. The Pb accumulation in spleen was also notably increased, reaching a value of 10.44±1.23 μg/g at dose for exposure to 240 mg/kg in 4 weeks. For the tissues of intestine and gill, significant accumulations was observed over 60 mg/kg, but the levels of Pb accumulation were much lower than the kidney, liver, and spleen. The highest Pb accumulation values were 5.72±0.72 μg/g for exposure to 240 mg/kg in intestine and

3.87±0.41 μg/g for exposure to 240 mg/kg in gill after 4 weeks. In the muscle Pb accumulation, a significant increase was observed at 240 mg/kg after 2 and 4 weeks. The Pb accumulation depended upon the dietary lead concentration and exposure periods. After 4 weeks of the dietary lead exposure, the profile of tissue Pb accumulation was kidney > liver > spleen > intestine > gill > muscle.

Fig. 1-1. Pb accumulation of rockfish, *Sebastes schlegelii* exposed to the different concentration of dietary lead for 4 weeks. Values with different superscript are significantly different in 2 and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.2. Growth performance

The growth rate and hepatosomatic index of *S. schlegelii* is shown in Fig. 1-2. A significant decrease in daily length gain was observed over 120 mg/kg at 2 and 4 weeks, compared to control. In daily weight gain, a clear decreasing trend noted over 120 mg/kg at 2 weeks and over 60 mg/kg at 4 weeks. A notable decrease in condition factor was observed at 240 mg/kg after 2 and over 120 after 4 weeks. Hepatosomatic index was significantly decreased at 240 mg/kg after 2 weeks and over 120 mg/kg after 4 weeks.

Fig. 1-2. Daily length gain, daily weight gain, condition factor, and hepatosomatic index factor of rockfish, *Sebastes schlegelii* exposed to the different concentration of lead for 4 weeks. Vertical bar denotes a standard error. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.3. Hematological parameters

The RBC count, hematocrit value, and hemoglobin concentration of *S. schlegelii* exposed to different levels of dietary lead are demonstrated in Table 1-4. In hematological parameters, considerable decreases in RBC count over 120 mg/kg, hematocrit vale and hemoglobin over 60 mg/kg of *S. schlegelii* were observed by the dietary lead exposure.

The blood plasma components of *S. schlegelii* exposed to the dietary lead are demonstrated in Table 1-5. In inorganic components, a significant increase in calcium and magnesium was observed over 120 mg/kg after 4 weeks and over 60 mg/kg after 4 weeks. In organic components, glucose and cholesterol were considerably elevated over 120 mg/kg after 4 weeks by the dietary lead exposure, whereas there was a notable decrease in total protein. In enzyme components, GOT and GPT were significantly increased over 120 mg/kg, but there was no change in ALP.

Table 1-4. Changes of RBC count, Hematocrit and Hemoglobin in rockfish, *Sebastes schlegelii* exposed to lead for 4 weeks.

Parameters	Period (week)	Lead concentration (mg/kg)				
		0	30	60	120	240
RBC count ($\times 10^4 mm^3$)	2	230.5±14.3^{a}	224.6±16.9^{a}	217.3±15.4^{a}	205.8±16.4^{ab}	185.2±12.6^{b}
	4	235.8±16.2^{a}	218.9±14.6^{ab}	226.7±10.7^{a}	192.4±13.1^{bc}	179.7±15.3^{c}
Hematocrit (%)	2	42.5±2.3^{a}	39.3±2.8^{ab}	36.6±3.2^{ab}	37.5±2.6^{ab}	34.2±3.3^{b}
	4	43.7±3.5^{a}	40.1±3.4^{ab}	34.8±2.8^{bc}	34.6±3.2^{bc}	31.9±2.7^{c}
Hemoglobin (g/dL)	2	7.46±0.37^{a}	7.12±0.42^{ab}	6.76±0.38^{bc}	6.44±0.35^{bc}	6.18±0.31^{c}
	4	7.58±0.33^{a}	7.03±0.34^{ab}	6.52±0.31^{bc}	6.37±0.28^{bc}	5.86±0.27^{c}

Values are mean±S.E. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

Table 1-5. Changes of serum parameters in rockfish, *Sebastes schlegelii* exposed to lead for 4 weeks.

Parameters	Period (week)	Lead concentration (mg/kg)				
		0	30	60	120	240
Calcium (mg/dL)	2	23.3±1.9^{a}	22.8±2.2^{a}	23.1±2.0^{a}	21.4±1.7^{ab}	19.3±2.2^{b}
	4	23.6±1.6^{a}	23.3±1.8^{a}	22.6±2.3^{ab}	19.3±2.4^{b}	18.8±1.9^{b}
Magnesium (mg/dL)	2	3.95±0.18^{a}	3.87±0.26^{a}	3.62±0.28^{ab}	3.41±0.23^{b}	3.21±0.31^{b}
	4	3.88±0.23^{a}	3.81±0.31^{a}	3.48±0.30^{b}	3.16±0.27^{bc}	2.85±0.26^{c}
Glucose (mg/dL)	2	78.6±3.8^{a}	76.7±4.4^{a}	81.4±3.1^{ab}	83.2±3.9^{ab}	86.7±3.3^{b}
	4	77.9±4.2^{a}	77.1±5.3^{a}	83.3±4.7^{ab}	87.5±4.2^{b}	89.4±2.7^{b}
Cholesterol (mg/dL)	2	169.3±10.9^{a}	168.5±9.6^{a}	173.1±8.3^{a}	188.4±11.2^{ab}	201.8±10.4^{b}
	4	168.2±9.5^{a}	170.7±11.8^{a}	181.6±9.4^{ab}	194.4±10.8^{bc}	204.0±8.4^{c}
Total protein (g/dL)	2	4.32±0.33^{a}	4.40±0.37^{a}	4.37±0.26^{a}	4.58±0.41^{a}	3.83±0.32^{b}
	4	4.48±0.29^{a}	4.58±0.41^{a}	4.52±0.37^{a}	4.13±0.26^{ab}	3.65±0.38^{b}
GOT (KU)	2	75.2±3.8^{a}	74.9±3.2^{a}	75.7±4.6^{a}	83.8±3.7^{b}	86.2±4.2^{b}
	4	76.0±4.4^{a}	76.4±4.1^{a}	79.1±2.9^{ab}	84.1±2.8^{bc}	89.5±5.1^{c}
GPT (KU)	2	38.9±2.2^{a}	41.5±1.7^{a}	41.1±2.7^{a}	43.3±1.6^{ab}	47.7±2.5^{b}
	4	39.4±2.4^{a}	40.5±2.1^{a}	40.3±2.3^{a}	44.7±2.1^{b}	48.7±1.9^{c}
ALP (K-A)	2	5.27±0.41^{a}	5.31±0.43^{a}	5.39±0.46^{a}	5.30±0.29^{a}	5.44±0.53^{a}
	4	5.14±0.37^{a}	5.33±0.41^{a}	5.30±0.35^{a}	5.26±0.33^{a}	5.45±0.41^{a}

Values are mean±S.E. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.4. Antioxidant system

Antioxidant system analysis (SOD, GST, and GSH) in liver and gill tissues of *S. schlegelii* is shown in Fig. 1-3. Liver and gill SOD activity of the *S. schlegelii* was substantially increased. The liver SOD activity was notably increased at 240 mg/kg of dietary Pb exposure after 2 weeks and over 60 mg/kg of dietary Pb exposure after 4 weeks. In case of gill SOD activity, a considerable increase was observed over 120 mg/kg of Pb exposure after 2 weeks and over 60 mg/kg of dietary Pb exposure after 4 weeks. Liver and gill GST activity of the *S. schlegelii* was also considerably increased. The liver GST activity was considerably elevated at 240 mg/kg of dietary Pb exposure after 2 weeks and over 60 mg/kg of dietary Pb exposure after 4 weeks. The gill GST activity was increased over 120 mg/kg of Pb exposure after 2 weeks and at 60, 240 mg/kg of dietary Pb exposure after 4 weeks. On the contrary to SOD and GST activity, liver and gill GSH level were considerably decreased. The liver GSH was notably decreased over 60 mg/kg of dietary Pb exposure after 2 weeks and 30 mg/kg of dietary Pb exposure after 4 weeks. In case of gill GSH, a significant decrease was observed at 240 mg/kg of dietary Pb exposure after 2 weeks and over 120 mg/kg of dietary Pb exposure after 4 weeks.

Fig. 1-3. Antioxidant system analysis (SOD, GST, and GSH) of rockfish, *Sebastes schlegelii* exposed to the different concentration of lead for 4 weeks. Vertical bar denotes a standard error. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.5. Inhibition of AChE activity

AChE activities of brain and muscle tissues exposed to dietary Pb are shown in Fig 1-4. AChE activity in brain tissue was noticeably inhibited in the concentration at 240 mg/kg after 2 weeks and over 120 mg/kg after 4 weeks, compared to control. Brain AChE inhibition levels were 29% at 240 mg/kg after 2 weeks and 28% at 240 mg/kg after 4 weeks. In muscle tissue, AChE activity was inhibited at 240 mg/kg after 2 weeks and over 60 mg/kg after 4 weeks. Muscle AChE inhibition levels were 39% at 240 mg/kg after 2 weeks and 45% at 240 mg/kg after 4 weeks, respectively.

Fig. 1-4. AChE activity of rockfish, *Sebastes schlegelii* exposed to the different concentration of lead for 4 weeks. Vertical bar denotes a standard error. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.6. Specific gene expression

Relative MT and HSP 70 mRNA gene expression of *S. schlegelii* by the dietary lead exposure is demonstrated in Fig. 1-5. The dietary Pb exposure significantly induced the increase of MT gene expression in liver, compared to the control. MT gene expression in liver was considerably increased over 60 mg/kg both at 2 weeks and 4 weeks. The noticeable increase trend in MT gene expression was observed by the dietary Pb exposure. The HSP 70 gene expression of *S. schlegelii* was notably increased over 60 mg/kg after 2 and 4 weeks exposed to the dietary lead. The highest gene expression appeared in the concentration of 240 mg/kg at 4 week.

Fig. 1-5. Relative MT and HSP 70 mRNA of rockfish, *Sebastes schlegelii* exposed to the different concentration of dietary lead for 4 weeks. Values with different superscript are significantly different in 2 and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.7. Immune responses

Plasma immunoglobulin M of *S. schlegelii* by the dietary lead exposure is demonstrated in Fig. 1-6. A substantial increase in the plasma Ig M of *S. schlegelii* was observed over 120 mg/kg at 2 weeks by the dietary lead exposure. After 4 weeks, the level of plasma Ig M was increased over 120 mg/kg. The notable increase in plasma Ig M of *S. schlegelii* was observed by the dietary lead exposure.

Lysozyme activity in plasma and kidney of *S. schlegelii* exposed to dietary lead is shown in Fig. 1-7. A considerable increase in plasma lysozyme activity of *S. schlegelii* was observed over 120 mg/kg after 2 weeks and over 60 mg/kg after 4 weeks by the dietary lead exposure. The kidney lysozyme activity of *S. schlegelii* was increased at 240 mg/kg after 2 weeks and over 120 mg/kg after 4 weeks by the dietary lead exposure.

Fig. 1-6. Immunoglobulin M of rockfish, *Sebastes schlegelii* exposed to the different concentration of lead for 4 weeks. Vertical bar denotes a standard error. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

Fig. 1-7. Lysozyme activity of rockfish, *Sebastes schlegelii* exposed to the different concentration of lead for 4 weeks. Vertical bar denotes a standard error. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

4. Discussion

4.1. Bioaccumulation

The dietary lead accumulation in the kidney of *S. schlegelii* was significantly increased by the dietary lead exposure, which is the highest accumulation among the tissues. Likewise, the highest accumulation in kidney of rainbow trout, *Oncorhynchus mykiss* was observed by the exposure to dietary lead (Alves et al., 2006; Alves and Wood, 2006), which is owing to its properties to precipitate of protein-bound lead as a detoxicification mechanism (Loumbourdis, 2003). The liver is one of the most important tissue in ecotoxicological studies due to the main accumulation tissue for metal exposure in fish, and it functions amino acid and energy metabolism, blood composition, and detoxification of toxic substances (Reynders et al., 2006). Similar with the tissue of kidney, the significant accumulation was observed in the liver of *S. schlegelii* exposed to the dietary lead. It is well known that kidney and liver tissues have been recognized as major lead accumulation tissues in fish (Tulasi et al., 1992). A considerable lead accumulation was also observed in the spleen of *S. schlegelii*. The spleen has been considered as a crucial tissue early removal of particulate substances from the blood (Handy et al., 2011). Alves and Wood (2006) suggested that a notable accumulation in the spleen of rainbow trout, *Oncorhynchus mykiss*

exposed to the dietary lead, and it accumulated a substantial portion of lead burden, compared to the relatively low weight of spleen tissue. It is commonly accepted that the metabolic activities of tissues in aquatic animals affect metal accumulation in tissues (Shukla et al., 2007). The high metabolic activity in tissues such as kidney, liver, and spleen may contribute to the higher lead accumulation in those tissues of *S. schlegelii*.

The accumulation in aquatic animals has been occur through different mechanisms via the direct uptake from water by gills and ingestion from food by intestine (Oost et al., 2003). Between two pathways, the metal accumulation in fish is mainly from their diet (Hall et al., 1997). The lead accumulation in intestine of *S. schlegelii* was observed by the dietary lead exposure, which is higher than that of gill tissue. Ojo and Wood (2007) also reported that a much higher accumulation in intestine of rainbow trout, *Oncorhynchus mykiss* exposed to dietary lead, than the accumulation exposed to waterborne lead. The high accumulation in intestine may result from the secretions into the intestine from other organs (Mager et al., 2010). The gill in fish is one of the most vital organ to function gaseous exchange, ionic transport, acid-base regulation, and nitrogenous waste excretion, as well as detoxification (Goss et al., 1998). A notable accumulation in gill tissue of *S. schlegelii* was observed by the exposure to the dietary lead. The accumulation of gill in aquatic animals by the dietary metal exposure induces a switch from the bloodstream to

the basolateral cell membranes (Szebedinsyky, et al., 2001). In the muscle tissue of *S. schlegelii*, there was no considerable accumulation except for the highest exposure concentration of the dietary lead. Ay et al. (1999) reported the lowest accumulation in muscle of *Tilapia zillii* exposed to lead exposure.

The study about bioaccumulation patterns in fish tissues exposed to metals can be used as effective and reliable indicators of environmental metal pollution (Kim et al., 2004). The experimental fish, *S. schlegelii* accumulated lead from the dietary lead exposure resulting in accumulation in their tissues and redistribution among tissues. The relative accumulation exposed to the dietary lead in specific tissues was: kidney > liver > spleen > intestine > gill > muscle. In the present study, the dietary lead exposure results from a considerable bioaccumulation in specific tissues of *S. schlegelii*.

4.2. Growth performance

The growth rate and reproduction in fish is generally retarded in the response of the exposure to toxicants because the allocation of energy for growth and reproduction transfers to the use to compensate tissue repair (Wendelaar Bonga, 1997). Actually, the metal accumulation at high concentration can induce retarded growth in fish development, which has influence on fish size (Friedmann et al., 1996). The dietary lead exposure caused a

significant inhibition of growth of *S. schlegelii*, and the condition factor was considerably decreased by the lead exposure. The hepatosomatic index in aquatic animals is commonly regarded as a crucial indicator of toxic effects by the metal toxicity exposure (Datta et al., 2007). It was observed that a significant decrease in hepatosomatic index of *S. schlegelii* exposed to the dietary lead, which is a similar observation with Maceda-Veiga et al. (2012). The dietary lead exposure induces a significantly negative effect on the growth and hepatosomatic index of *S. schlegelii*.

4.3. Hematological parameters

Hematological parameters such as RBC count, Ht value, and Hb concentration can be a good indicator to assess the metal contamination in the aquatic environment as well as the status for the oxygen carrying ability (Shah and Altindag, 2004; Seriani et al., 2015). In the present study, a considerable reduction in the RBC count, Ht value, and Hb concentration of *S. schlegelii* was observed by the dietary lead exposure, which may be thought to result in reduced heme synthesis and anemia (Gurer et al., 1998). The alterations of the parameters may be occur osmotic changes induced by metal toxicity resulting in hemo-dilution or hemoconcentration and red blood cell fragility, as well as impaired hematopoietic tissues (Shah, 2006). Gill and Epple (1993) suggested that anemia induced metal toxicity results from

increased erythroclasis according to changed membrane permeability and elevated mechanical fragility, in addition to damage to erythropoiesis owing to a direct toxic influence of metal on hematopoietic tissues such as kidney and spleen.

In the inorganic components in plasma, calcium and magnesium are crucial indicators of metal toxicity, which also function as the ion regulator for homeostasis (Bijvelds et al., 1998). The lead exposure can induce an ionoregulatory toxicity, which has an effect on Ca^{2+} influx and homeostasis as well as the balance of Na^{+} and Cl^{-} (MacDonald et al., 2002; Rogers et al., 2005). The calcium and magnesium of *S. schlegelii* was considerably decreased by the dietary lead exposure. Among organic components, the major components such as glucose, cholesterol, and total protein of *S. schlegelii* was analyzed. The glucose and cholesterol of *S. schlegelii* was significantly increased by the dietary lead exposure, whereas the total protein was notably decreased. The plasma glucose can be a reliable indicator to assess environmental stress in fish, and it is usually increased due to gluconeogenesis (Saravanan et al., 2011). The cholesterol is a critical structural component of cell membranes and a precursor of all steroid hormones (Yang and Chen, 2003), and it usually used as a stress indicator (Firat and Kargin, 2010). The plasma protein has been dramatically altered under stress situations (Gopal et al., 1997). In the enzyme components, the GOT and GPT were measured in this study. These enzyme

components have been considered as sensitive and reliable indicators for the exposure to metals and xenobiotics (Ozman et al., 2006). The GOT and GPT of *S. schlegelii* were notably increased by the dietary lead exposure. The ALT levels in plasma have been considered as a crucial indicator of liver damage, which results from its high storage in the liver (Oost et al., 2003). Reynders et al. (2006) reported that a considerable increase in ALT of common carp, *Cyprinus carpio* exposed to cadmium, causing the increased membrane permeability of the liver cells by liver damage, which results from a leakage of the enzyme into the plasma. However, there was no significant change in ALP of *S. schlegelii* exposed to the dietary lead, which indicates there was no considerable effect of the Pb exposure in *S. schlegelii* in this study. Therefore, significant alterations in plasma components of *S. schlegelii* were observed by the dietary lead exposure.

4.4. Antioxidant system

Aquatic toxicants induce the intracellular formation of ROS, which causes oxidative damage to the organ systems of aquatic animals. Superoxide anion radicals in redox cycling can be generated by various toxicants (Ferreira et al., 2005). The fish uptake of theses toxicants can occur from sediments, suspended particulate material, waterborne and food sources. Among various routes,

dietary input is a major route in toxicant uptake (Livingstone, 1998).

Oxidative damage can occur when the active intermediates produced by xenobiotics and their metabolites cannot be neutralized by antioxidant and detoxifying systems (Kim and Kang, 2015). In this study, the oxidative stress of the experimental fish was monitored by evaluating the levels of SOD, GST, and GSH. The SOD activity both in liver and gill of *S. schlegelii* was significantly increased by the dietary lead exposure. Superoxide anions are dismutased by superoxide dismutase (SOD) to H_2O_2, as a first defense mechanism for oxygen toxicity. Zhang et al. (2007) also reported the SOD activity increase of tadpoles (*Bufo raddei*) by high concentrations of Pb exposure. The increases in SOD activity may occur by its activity to catalytically scavenges superoxide radical against the production of superoxide radical to H_2O_2 (Doherty et al., 2010). Glutathione S-transferase (GST) catalyzes the conjugation reaction of xenobiotics with GSH as anantioxidant enzyme (Baysoy et al., 2012), and alterations in GST activity according to the metal exposure are accompanied by GSH depletion (Elia et al., 2003). GST has been also recognized as a biomarker for oxidative stress by heavy metal exposure (Durou et al., 2007). The GST activity in the liver and gill of *S. schlegelii* was considerably increased by the dietary lead exposure. Yilmaz et al. (2014) also reported a notable GST increase in *Cyprinus carpio* exposed to lead toxicity. The

stimulation of GST activity may be caused by the metal-induced decrease in glutathione content, because GSH is essential for proper functioning of GST (Elia et al., 2003). Glutathione (GSH) is a primary molecule in cellular antioxidant systems due to its function to detoxify agent for endogenous radical species, as well as enzymatic detoxification reactions as a cofactor or a coenzymes (Shelly, 2009). The oxidative stress by Pb exposure can also induce depleted glutathione levels by binding exclusively to the sulfhydryl group, which can interfere with the antioxidant activity of GSH (Stohs and Bagghi, 1995; Gurer and Ercal, 2000). Depleted GSH caused by Pb exposure was observed in many study (Baysoy et al., 2012; Dai et al., 2012). GSH level both in liver and gill of *S. schlegelii* was also decreased by increasing dietary Pb exposure concentration. The decreased GSH levels may reflect an aggravation status caused by reduced cell protection ability, because Pb has a high affinity for sulfhydryl (SH) groups that inhibits functional SH groups (Atli and Canli, 2008; Dai et al., 2012). The alterations in GSH level of *S. schlegelii* may result in oxidative damage by dietary Pb. These results demonstrated that dietary Pb exposure to *S. schlegelii* considerably affects antioxidant activity as oxidative stress. Considering the alterations in antioxidant enzymes (SOD and GST) and GSH, the dietary Pb exposure may cause oxidative tissue damage in *S. schlegelii* by increased ROS production.

4.5. Inhibition of AChE activity

As one of the most important neurotransmitters, acetylcholine acts on both the central nervous system (CNS) by improving sensory perceptions and peripheral nervous system (PNS) by activating skeletal muscles. Acetylcholinesterase (AChE) acts as a hydrolase that hydrolyzes the acetylcholine mainly in cholinergic brain synapses and neuromuscular junctions. There is considerable evidence of the interaction between the exposure concentration of heavy metals and AChE inhibition due to its permeability across the blood brain barrier and accumulation in the brain (Richetti et al., 2011). When inhibiting AChE by heavy metals, acetylcholine is not hydrolyzed both in nerve synapses and neuromuscular junctions, which induces overactivation of muscular tissue by an abnormal amount of acetylcholine. This overactivation can cause behavioural effects such as hyperactivity, asphyxia, and finally death (Roex et al., 2003). The Pb exposure is closely associated with neurotoxicity due to its risk for impairment in the central nervous system (CNS) by depleting cognitive and neurobehavioral functions (She et al., 2009). Devi and Fingerman (1995) reported that a significant inhibition in AChE of red swamp crayfish, *Procambarus clarkia* exposed to waterborne Pb. Similarly, Richetti et al. (2011) also reported the lead exposure to zebrafish induced inhibition of AChE. In this study, the dietary Pb exposure of *S. schlegelii* led to a notable inhibition of AChE. Considering

acetylcholinesterase is an important biomarker in neurotoxicity for several environmental toxicants, the dietary Pb exposure may substantially influence *S. schlegelii* as neurotoxicity.

4.6. Specific gene expression

Metallothioneins (MTs) have mechanisms to provide protection against oxidative damage caused by metals (Atli and Canli, 2008), and also function as homeostasis of essential metals (Kelly et al., 1998). The induction of MT as a metal-binding protein is closely connected with heavy metal exposure due to its function to bind metals for detoxification (Ay et al., 1999). Recently, there has been much attention in MT as a reliable indicator of warning system for heavy metal contamination (Boeck et al., 2003). The large amount of metallothionein induction occurs in the liver tissue of fish, because liver tissue is a main site in the uptake and storage of heavy metals as well as a major producer of metal-binding proteins against the heavy metal exposure (Allen, 1994). Woo et al. (2006) reported a considerable increase in hepatic MT gene of Japanese medaka, *Oryzias javanicus* exposed to heavy metals such as Ag, Cd, Cu, and Zn. The dietary Pb exposure to *S. schlegelii* caused a significant increase in hepatic MT gene expression. The result indicates the dietary Pb exposure should affect the experimental fish, *S. schlegelii*, considering MT is a sensitive and reliable indicator to assess metal toxicants in

the aquatic environment.

Environmental and physiological stress is well known to cause the gene expression of stress protein such as HSP 70 and HSP 90 (Rajeshkumar and Munuswamy, 2011). Especially, the increase in HSP 70 is induced by the toxic environment such as heat shock and heavy metals (Voznesensky et al., 2004). Therefore, heat shock protein 70 (HSP 70) can be a reliable indicator to assess the toxic effects on fish exposed to toxicants (Boone and Vijayan, 2002). Singer et al. (2005) reported a significant increase in HSP 70 gene expression of zebra mussel, *Dreissena polymorpha* exposed to Pb. In this study, a considerable increase in the mRNA gene expression of HSP 70 was observed by the dietary lead exposure to *S. schlegelii*, which implicates the protective role for HSP 70 against the dietary lead exposure. Considering the increases in HSP 70 of *S. schlegelii*, the dietary lead exposure induced the stress reaction of *S. schlegelii*.

4.7. Immune responses

The immunological capacity in fish has been known to be caused by the stress from environmental toxicants. Among various xenobiotics, heavy metals such as lead, mercury, and cadmium have been considered to affect the immune responses in fish (Zelikoff et al., 1995). Immunoglobulin M (Ig M) is one of the most critical components in teleost innate immune system, and it

has a major function to defend and control fish disease (Nikoskelainen et al., 2003). Generally, the levels of Ig M in several fish species range between 0.25 and 23.5 mg/ml, and the levels in fish can be altered by environmental conditions and disease status as well as size and age (Cuesta et al., 2004). In this study, the dietary lead exposure to *S. schlegelii* caused the considerable increase in the plasma Ig M, which may indicate that the lead exposure stimulates the immune response of *S. schlegelii*. Lysozyme activity is also a major innate immune response in fish; it excretes to prevent them from xenobiotics. Kong et al. (2012) also reported the substantial increase in the lysozyme activity of goldfish, *Carassius auratus* exposed to mercury. In this study, the dietary lead exposure to *S. schlegelii* induced the significant elevation of the lysozyme activity in plasma and kidney, which may reflect that the lead exposure seemed to induce the modulation in humoral immune response. To evaluate the immunity of fish exposed to the dietary lead, the Ig M and lysozyme activity of *S. schlegelii* were analyzed, and the lead exposure resulted in the significant immune stimulations.

Chapter II. Effects of dietary ascorbic acid supplementation on growth performance, hematological parameters, and a neurotransmitter in rockfish, *Sebastes schlegelii*.

1. Introduction

Proper nutrition in cultured fish is essential for normal growth and resisting disease (Sealey and Gatlin, 1999). Among many essential nutrients, ascorbic acid (vitamin C, AsA) is one of the most important nutrient components for metabolic function and immunity in addition to growth and development (Ai et al., 2004; Lin and Shiau, 2005). AsA functions as a cofactor in the enzymatic hydroxylation in collagen and connective tissue of fish in addition to a reducing agent and an antioxidant. Moreover, it is also required to maintain the physiological homeostasis in most fishes (Wang et al., 2003). However, fish cannot synthesize AsA due to the lack of the enzyme L-gulonolactone oxidase (Sato et al., 1976).

Therefore, supplementing with an optimum concentration of ascorbic acid in feed should be a reliable and effective way to improve the health status in fish as well as improving disease resistance (Anbarasu and Chandran, 2001) and immunity (Ai et al., 2004). The beneficial effects of AsA supplement on immunological

parameters (Verlhac et al., 1998; Cuesta et al., 2002) include improved resistance to stress and disease (Montero et al., 1999). In addition, AsA is also involved in biological hydroxylation reactions of various metabolic pathways in connective tissue, scar tissue, blood vessel, and bone matrix (Fracalossi et al., 2001).

Most teleosts cannot synthesize AsA owing to a lack of L-gulonolactone oxidase activity which acts as a catalyst changing L-gulonolactone to AsA (Nishikimi and Yagi, 1991). AsA is not stored in the body of fish, because it is excrete through urine (NFRDI, 2007). The depletion of vitamin C can alter immune function resulting in an increase in mortality due to bacterial disease (Hardie et al., 1991). Therefore, it is necessary to supply an adequate concentration of AsA in the diet. The required concentration of AsA may vary depending upon a number of factors including the species and size of fish examined as well as their environment. For example, the requirement in juvenile fish is greater than that of adult fish and it increases three to ten times when the fish is under stress due to disease (NFRDI, 2007). A deficiency of AsA in teleosts can cause structural malformation (scoliosis, lordosis, and abnormal support cartilage in eye, gill, and fins), abnormal pigmentation, and liver steatosis, as well as reduced growth and immune responses (Roberts et al., 1995; Fracalossi et al., 1998). Dabrowski et al. (1988) reported that gill arch pathology and caudal fin erosion in carp larvae was induced by a deficiency in dietary AsA. Lovell (1973) also suggested that

fin malformation, scoliosis, lordosis, depigmentation were observed in fish fed AsA depleted diets. AsA deficiency can also cause darkening, anorexia, and opercular deformity (Dabrowski et al., 1996).

The assessment of hematological parameters in fish can be a good indicator to monitor physiological and pathological changes, because it offers fundamental information about the degrees of stress, metabolic abnormalities, reproductive dysfunctions, and diseases (Huffman et al., 1997; Clauss et al., 2008; Buscaino et al., 2010; Fazio et al., 2012). Many authors emphasized that the evaluation of hematological values is crucial to assess the health status in wild and cultured fish (Knowles et al., 2006; Fanouraki et al., 2007; Tavares-Dias and Moraes, 2007). However, it is difficult to interpret the blood parameters due to variations caused by both internal and external factors such as blood sampling, laboratory techniques, size, sex, population density, and environmental effects (Rey Vazquez and Guerrero, 2007).

Studies have scarcely been conducted about the functions of AsA in the nervous system, whereas it is well known that dietary AsA supplement is essential for immunity in addition to growth and development. AsA has been considered as a neuromodulator both dopamine- and glutamate- mediated neurotransmission (Grunewald, 1993; Rebec and Pierce, 1994). In the animals, the AsA deficiency has influence on neurotransmitter levels, although there has been no clear mechanism between AsA deficiency and neurotransmitter

levels (Harrison and May, 2009). Among various neurotransmitters, acetylcholinesterase (AChE) is one of the most important neurotransmitters, which acts as a terminator of neurotransmission process so as not to accumulate acetylcholine that may excessively stimulate nicotinic or muscarinic receptors (Taylor and Radic, 1994).

Rockfish, *S. schlegelii* are carnivorous and widely inhabit the coast of Korea, China, and Japan. *S. schlegelii* are one of the most important farming species of cage-aquaculture in Korea due to its appreciated flesh and rapid growth in addition to high demand. However, little study has been conducted on the effects of dietary AsA in *S. schlegelii*. Therefore, the aim of the present study is to evaluate the effects of dietary AsA on growth, hematological parameters, and neurotransmitters in juvenile *S. schlegelii*.

2. Materials and Methods

2.1. Experimental fish and culture conditions

Juvenile *S. schlegelii* were obtained from a local fish farm in Tongyeong, Korea. The fish were acclimatized for 2 weeks under laboratory conditions was evaluated prior to the dietary AsA supplement. During the experimental period, the fish were fed diets containing different levels of ascorbic acid diet twice daily and maintained on a 12-h:12-h light/dark cycle and constant condition at all times (Table 2-1). After acclimatization, 60 fishes (body length, 14.3 ±1.2 cm; body weight, 54.9±5.3 g) were randomly selected for the study. Dietary ascorbic acid study took place in 500L circular tanks containing 6 fish per treatment group in duplicates. The dietary ascorbic acid concentrations were 0, 50, 100, 200, and 400 mg/kg (Table 2-2), and fish were fed each ascorbic acid feed at a rate of 2% body weight daily (as two 1% meals per day). At the end of each period (at 2 and 4 weeks), fish were anesthetized in buffered 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma Chemical, St. Louis, MO).

Table 2-1. The chemical components of seawater and experimental condition used in the experiments.

Item	Value
Temperature (°C)	20.0±0.5
pH	8.1±0.5
Salinity (‰)	33.2±0.5
Dissolved Oxygen (mg/L)	7.1±0.3
Chemical Oxygen Demand (mg/L)	1.21±0.1
Ammonia (μg/L)	11.7±0.8
Nitrite (μg/L)	1.6±0.2
Nitrate (μg/L)	10.31±1.0

2.2. Feed ingredients and diets formulation

Formulation of the diets is shown in Table 2-2. AsA was obtained from Sigma Chemical Co., Ltd. All diets contained 33% casein, 23% fish meal, 5% corn starch, 2% vitamin premix (vitamin C free), and 2% mineral premix. 10% fish oil was added to meet the essential fatty acids (EFA) requirements of rock fish. Ascorbic acid premix was made up of 1 g AsA with 99 g cellulose. Five isonitrogenous and isolipidic diets were formulated with supplement of different dietary AsA levels of 0, 50, 100, 200, and 400 mg AsA/ kg diet. All ingredients were blended thoroughly. Water was added into the mixture to produce a stiff dough. The the dough was pelleted by an experimental feed mill and dried for 24 h at room temperature. After processing, all the diets were packed and kept at - 20 °C until use.

Table 2-2. Formulation and chemical proximate composition of the experimental diet (% dry matter).

Ingredient (%)	Ascorbic acid concentration (mg/kg)				
	0	50	100	200	400
Casein[1]	33.0	33.0	33.0	33.0	33.0
Fish meal[2]	23.0	23.0	23.0	23.0	23.0
Wheat flour[3]	20.0	20.0	20.0	20.0	20.0
Fish oil[4]	10.0	10.0	10.0	10.0	10.0
Cellulose[1]	5.0	4.5	4.0	3.0	1.0
Corn starch[3]	5.0	5.0	5.0	5.0	5.0
Vitamin Premix (vitamin C-free)[5]	2.0	2.0	2.0	2.0	2.0
Mineral Premix[6]	2.0	2.0	2.0	2.0	2.0
Ascorbic acid Premix[7]	0.0	0.5	1.0	2.0	4.0

[1]United States Biochemical (Cleveland, OH).

[2]Suhyup Feed Co., Ltd., Gyeong Nam Province, Korea.

[3]Young Nam Flour Mills Co., Pusan, Korea.

[4]Sigma Chemical Co., St. Louis, MO.

[5]Vitamin Premix (vitamin C-free) (mg/kg diet): dl-calcium pantothenate, 400; choine chloride 200; inositol, 20; menadione, 2; nicotinamide, 60; pyridoxine·HCl, 44; riboflavin, 36; thiamine mononitrate, 120, dl-a-tocopherol acetate, 60; retinyl acetate, 20000IU; biotin, 0.04; folic acid, 6; vitamin B_{12},0.04;cholecalcifero,4000IU.

[6]Mineral Premix (mg/kg diet): Al, 1.2; Ca, 5000; Cl, 100; Cu, 5.1; Co, 9.9; Na, 1280; Mg, 520; P, 5000; K, 4300; Zn, 27; Fe, 40; I, 4.6; Se, 0.2; Mn, 9.1.

[7]Ascorbic acid Premix (mg/kg diet): 10,000 mg ascorbic acid/ kg diet

2.3. Growth Performance

No mortality was observed during the 4 week period. The weight and length of *S. schlegelii* was measured just before feeding the diet, and at 2 and 4 weeks of feeding the diet. Daily length gain, daily weight gain, body weight gain (BWG), specific growth rate (SGR), and feed conversion ratio (FCR) were calculated by the following method.

Daily growth gain = (Wf-Wi)/day
Body weight gain (BWG) (%) = ((Wf-Wi)/Wi)x100
Specific growth rate (SGR) = ((ln Wf-lnWi)/T2-T1)x100
Feed conversion ratio (FCR) = feed fed / live weight gain

Where: Wf = Final length or weight, Wi = Initial length or weight, and T1 and T2 = time (day)

2.4. Hematological assay

Blood samples were collected within 35-40 seconds through the caudal vein of the fish in 1-ml disposable heparinized syringes. The blood samples were kept at 4°C until the blood parameters were completely studied. The total red blood cell (RBC) count, hemoglobin (Hb), concentration, and hematocrit (Ht) value were determined immediately. Total RBC counts were counted using

optical microscope with hemo-cytometer (Improved Neubauer, Germany) after diluted by Hendrick's diluting solution. The Hb concentration was determined using Cyan-methemoglobin technique (Asan Pharm. co., Ltd.). The Ht value was determined by the microhematocrit centrifugation technique. The blood samples were centrifuged to separate plasma from blood samples at 3000 g for 5 minutes at 4°C. The plasma samples were analyzed for inorganic substances, organic substances, and enzyme activity using clinical kit (Asan Pharm. Co.,Ltd.). In inorganic substances assay, calcium and magnesium were analyzed by the o-cresolphthalein-complexon technique and xylidyl blue technique. In organic substances assay, glucose and total protein were analyzed by GOD/POD technique and biuret technique. In enzyme activity assay, glutamic oxalate transaminase (GOT) and glutamic pyruvate transaminase (GPT) were analyzed by Kind-king technique.

2.5. AChE activity

AChE activity was determined brain(1:25) and muscle(1:10) homogenate in 0.1 M phosphate buffer, pH 8.0. The homogenate were centrifuged 10,000 g for 20 min at 4 °C. The supernatant was removed and used to test AChE activity. AChE activity was determined according to the method of Ellman et al. (1961). AChE activity was normalized to protein content and expressed as nmol

min-1mgprotein-1. Briefly, the activity on the homogenate was measured by determining the rate of hydrolysis of acetylthiocholine iodide (ACSCh, 0.88mM) in final volume of 300µl, with 33 µl of 0.1 M phosphate buffer, pH 7.5 and 2 mM DTNB. The reaction was started with the addition of the substrate acetylthiocholine, as soon as the substrate was added the hydrolysis and the formation of the dianion of DTNB were analyzed in 412 nm for 5 min (in intervals of 1 min) using a microplate reader. Protein concentration was determined using Ellman's method (1961), with a bovine plasma albumin (Sigma, USA) as standard.

2.6. Statistical analysis

The experiment was conducted in exposure period for 4 weeks and performed triplicate. Statistical analyses were performed using the SPSS/PC+ statistical package (SPSS Inc, Chicago, IL, USA). Significant differences between groups were identified using one-way ANOVA and Duncan's test for multiple comparisons or Student's t-test for two groups (Duncan, 1955). The significance level was set at $P < 0.05$.

3. Results

3.1. Growth performance

Daily weight gain and length gain according to the different levels of ascorbic acid of *S. schlegelii* is shown in Fig. 2-1. In daily weight gain, a clear increasing trend was noted in the concentration over 50 mg/kg at 2 and 4 weeks. Daily length gain in fish fed diets containing the concentrations higher than 100 mg/kg at 2 weeks and 50 mg/kg at 4 weeks was significantly increased. BWG, SGR, and FCR of *S. schlegelii* fed diets containing different levels of ascorbic acids were demonstrated in table 2-3. BWG was considerably increased over 50 mg/kg at 2 and 4 weeks, and FCR was also significantly elevated over 200 mg/kg at 2 and 4 weeks. In contrast, there was no notable alteration in SGR.

Fig. 2-1. Daily length gain and daily weight gain of rockfish, *Sebastes schlegelii* fed diets containing different levels of ascorbic acids for 4 weeks. Vertical bar denotes a standard error. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

Table 2-3. BWG, SGR, and FCR of rockfish, *Sebastes schlegelii* fed diets containing different levels of ascorbic acids for 4 weeks.

Parameters	Period (week)	Ascorbic acid concentration (mg/kg)				
		0	50	100	200	400
BWG	2	7.37±0.69^{a}	9.62±1.01^{b}	10.2±1.0^{b}	12.5±1.9^{bc}	12.9±1.5^{c}
	4	12.2±1.2^{a}	15.7±1.2^{b}	17.6±1.6^{bc}	24.7±2.5^{c}	25.1±2.3^{c}
SGR	2	0.48±0.06^{a}	0.46±0.05^{a}	0.43±0.03^{a}	0.45±0.04^{a}	0.43±0.05^{a}
	4	0.82±0.10^{a}	0.81±0.08^{a}	0.78±0.11^{a}	0.81±0.07^{a}	0.79±0.09^{a}
FCR	2	1.84±0.22^{a}	1.61±0.17^{ab}	1.54±0.19^{ab}	1.36±0.15^{b}	1.27±0.14^{b}
	4	2.06±0.18^{a}	1.74±0.20^{ab}	1.63±0.14^{ab}	1.39±0.14^{b}	1.24±0.15^{b}

Values are mean±S.E. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.2. Hematological parameters

The RBC count, Ht value, and Hb concentration of *S. schlegelii* fed diets containing the different levels of AsA are summarized in Table 2-4. The major hematological findings were considerable increases in RBC count and Hb concentration in *S. schlegelii* over 100 mg/kg AsA supplement at 2 and 4 weeks. The Ht value following the AsA supplement for 4 weeks was significantly increased at 200 and 400 mg/kg.

The blood plasma components of *S. schlegelii* supplemented with AsA are shown in Table 2-5. In inorganic components, it has no notable alteration in calcium and magnesium for 4 weeks. In organic components, glucose was considerably increased at 400 mg/kg after 2 weeks and at the higher concentrations of 200 mg/kg after 4 weeks, and a significant increase in total protein was observed at 400 mg/kg after 2 weeks and over 200 mg/kg after 4 weeks. In the change of enzyme components, GOT and GPT were gradually decreased by increasing the dietary AsA supplement. A considerable decrease in GOT was observed over 100 mg/kg at 2 and 4 weeks, and GPT decreased notably over 100mg/kg after 2 weeks and 50 mg/kg after 4 weeks.

Table 2-4. Changes of RBC count, Hematocrit and Hemoglobin in rockfish, *Sebastes schlegelii* fed diets containing different levels of ascorbic acids for 4 weeks.

Parameters	Period (week)	Ascorbic acid concentration (mg/kg)				
		0	50	100	200	400
RBC count ($\times 10^4 mm^3$)	2	197.6±10.5^{a}	214.8±12.5^{a}	246.6±14.8^{b}	259.4±13.1^{b}	261.7±10.4^{b}
	4	183.5±14.1^{a}	205.3±16.4^{ab}	228.7±10.9^{b}	267.5±11.5^{c}	263.8±15.7^{c}
Hematocrit (%)	2	36.4±4.7^{a}	38.4±5.1^{ab}	41.7±4.2^{ab}	45.6±3.5^{b}	46.2±5.2^{b}
	4	34.2±3.9^{a}	35.5±4.2^{a}	42.3±3.5^{ab}	44.3±5.3^{b}	45.7±5.7^{b}
Hemoglobin (g/dL)	2	5.72±0.52^{a}	6.19±0.46^{ab}	6.86±0.33^{bc}	7.35±0.42^{c}	7.48±0.68^{c}
	4	5.81±0.68^{a}	6.32±0.37^{ab}	7.08±0.49^{bc}	7.60±0.38^{c}	7.57±0.41^{c}

Values are mean±S.E. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

Table 2-5. Changes of plasma parameters in rockfish, *Sebastes schlegelii* fed diets containing different levels of ascorbic acids for 4 weeks.

Parameters	Period (week)	Ascorbic acid concentration (mg/kg)				
		0	50	100	200	400
Calcium (mg/dL)	2	19.4±2.2^{a}	19.8±2.0^{a}	20.2±2.2^{a}	20.4±1.9^{a}	19.8±2.1^{a}
	4	20.3±1.8^{a}	19.9±2.4^{a}	19.6±2.5^{a}	21.3±2.4^{a}	20.1±2.0^{a}
Magnesium (mg/dL)	2	3.71±0.36^{a}	3.70±0.26^{a}	3.94±0.29^{a}	3.85±0.35^{a}	3.87±0.25^{a}
	4	3.76±0.28^{a}	3.72±0.33^{a}	3.84±0.31^{a}	3.82±0.33^{a}	3.83±0.27^{a}
Glucose (mg/dL)	2	76.2±4.8^{a}	73.3±5.4^{a}	76.2±4.6^{a}	79.3±4.3^{ab}	82.8±4.9^{b}
	4	73.9±5.1^{a}	75.7±4.5^{a}	76.6±3.6^{a}	83.2±4.1^{b}	83.6±3.7^{b}
Total protein (g/dL)	2	4.46±0.24^{a}	4.37±0.31^{a}	4.58±0.30^{a}	4.98±0.21^{ab}	5.13±0.21^{b}
	4	4.39±0.18^{a}	4.50±0.27^{a}	4.41±0.24^{a}	5.10±0.18^{b}	5.52±0.29^{b}
GOT (KU)	2	93.6±5.7^{a}	89.4±4.5^{a}	80.6±4.4^{b}	75.6±3.9^{b}	76.4±4.2^{b}
	4	96.4±4.3^{a}	94.9±3.8^{a}	84.1±5.2^{b}	75.1±4.5^{c}	74.7±3.7^{c}
GPT (KU)	2	46.1±2.4^{a}	43.2±2.9^{ab}	40.7±2.0^{bc}	37.5±2.8^{c}	36.2±2.6^{c}
	4	49.3±1.7^{a}	45.7±2.7^{b}	39.3±1.8^{c}	36.4±1.4^{c}	37.8±1.9^{c}

Values are mean±S.E. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.3. AChE activity

AChE activities of brain and muscle tissues in *S. schlegelii* fed diets containing the different levels of AsA supplement are demonstrated in Fig. 2-2. AChE activity in brain was significantly increased at the higher concentrations of 200 mg/kg at 2 and 4 weeks, compared to control. Brain AChE increasing levels were 19.7% at 400 mg/kg after 2 weeks and 41.9% at 400 mg/kg after 4 weeks, compared to control. Similarly, a considerable increase of muscle AChE activity was observed at 400 mg/kg after 2 weeks and over 200 mg/kg after 4 weeks. Muscle AChE increasing levels were 21.4% at 400 mg/kg after 2 weeks and 34.8% at 400 mg/kg after 4 weeks, respectively.

Fig. 2-2. AChE activity of rockfish, *Sebastes schlegelii* fed diets containing different levels of ascorbic acids for 4 weeks. Vertical bar denotes a standard error. Values with different superscript are significantly different at 2 weeks and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

4. Discussion

4.1. Growth performance

L-gulonolactone oxidase is necessary for the biosynthesis of ascorbate from glucose (Roberts et al., 1995). A shortage of ascorbic acid in the diet leads to retarded growth (Gouillou-Coustans et al., 1998). A considerable growth increase as the result of AsA supplement in fish has been reported by many authors. Ai et al. (2004) reported a significant growth increase in Japanese seabass, *Lateolabrax japonicas* fed a diet of 47.6 mg ascorbic acid/ kg diet with the greatest growth achieved with over 53.5 mg ascorbic/ kg diet. Gouillou-Coustans et al. (1998) also suggested that dietary AsA supplement encourages the growth of common carp, *Cyprinus carpio* with the minimum requirement being 45 mg ascorbic/ kg diet. Similarly, parrot fish, *Oplegnathus fasciatus* fed a diet of 118 mg ascorbic acid/ kg diet induced a significant increase in optimal growth (Wang et al., 2003). In addition, significant growth was achieved in tilapia, *Oreochromis spilurus* fed a diet of 100 mg ascorbic acid/ kg diet (Al-Amoudi et al., 1992). Kumari and Sahoo (2005) suggested that high dietary AsA supplementation at a dose of 500 mg ascorbic acid/ kg diet for 4 weeks induced a significant growth in Asian catfish, *Clarias batrachus*. These results indicated that adequate exogenous AsA supplement is required to maintain normal growth

and physiological functions. In newly hatched Indian major carp, *Cirrhina mrigala*, the optimum requirement of AsA supplement level was 650-700 mg AsA/ kg diet (Mahajan and Agrawal, 1980). These differences may result from the fish species, size, and experimental conditions. Metabolic rate is one of the most important factors to control ascorbic acid requirement (Dabrowski, 1991), and metabolic rate varies with fish species, which is the reason for the different AsA requirement according to fish species (Jobling, 1985). In this study, the growth in *S. schlegelii* fed diets containing different levels of ascorbic acid was significantly increased with AsA in a dose-dependent manner. The minimum supplement for optimized growth performance was the concentration of 200 mg AsA/kg diet. The various factors such as feeding, absorption, and energy use may influence fish growth. The AsA supplement might drive the increase of growth in *S. schlegelii*, whereas the deficient levels of AsA cause the decrease of growth performance. These results demonstrated that the supply of adequate exogenous AsA is required for normal growth and physiological functions.

4.2. Hematological parameters

The hematological parameters indicate the physiological effects of dietary ascorbic acid supplement (Sandnes et al., 1990), and it can also be a good indicator to assess the condition of fish such

as stress, metabolic abnormalities, reproductive dysfunctions, and diseases (Fazio et al., 2012). In addition, AsA is a potent antioxidant which protects various fish tissues against oxidative damage, and it also improves resistance in red blood cell membranes (Sahoo and Mukherjee, 2002; Pearce et al., 2003; Sau et al., 2004). Therefore, red blood cell values such as hematocrit, hemoglobin, and RBC count should be a good indicator of oxidative status. Many authors demonstrated that there is a positive relation between AsA supplement and hematological parameters in fish. Andrade et al. (2007) reported a significant increase in the red blood cells count of *Arapaima gigas* fed diet containing the dietary AsA of 800 and 1200 mg/kg, whereas the deficient AsA supplement causes the decrease in hematocrit (Chagas and Val, 2003). Henrique et al. (1998) and Montero et al. (2001) reported alterations of hematological parameters in gilthead seabream, *Sparus aurata* by the vitamin deficient diets. It is also reported that a considerable increase in hematocrit, hemoglobin, and RBC count of *Arapaima gigas* by the dietary AsA supplementation (Menezes et al., 2006). In this study, we observed a significant increase in the RBC count, Ht value, and Hb concentration of *S. schlegelii*. Similar with previous studies, the dietary AsA supplement to *S. schlegelii* causes a considerable increase in the RBC count, Ht value, and Hb concentration.

The inorganic components in plasma, calcium and magnesium can be affected by the alteration of osmotic pressure in plasma

(Waring et al., 1996; Hur et al., 2001). But, the change of calcium and magnesium in plasma of *S. schlegelii* was not observed in this study. In the organic components in plasma of *S. schlegelii*, glucose was increased at the high dietary AsA supplement, and total protein was elevated by increasing the levels of the dietary AsA supplement. Paolisso et al. (1994) suggested that the dietary ascorbic acid intake increases plasma ascorbic acid levels, and glucose disposal was elevated by vitamin C-mediated improvement of insulin action, which increases non-oxidative glucose metabolism. The dietary AsA supplement of *S. schlegelii* may affect the modulation of insulin action. But, the AsA supplementation to *S. schlegelii* increased the plasma glucose, which may be a modulation of glucose homeostasis. The total protein concentration can be an indicator of the non-specific immune response in fish fed the diets containing the dietary AsA supplementation, because the supplementation elevates the protein activity in the complement system (Sahoo and Mukherjee, 2002; Ai et al., 2004; Lin and Shiau, 2005). Therefore, the total plasma level of *S. schlegelii* may be influenced by the dietary AsA supplement. Menezes et al. (2006) also reported a notable increase in the total plasma of pirarucu, *Arapaima gigas*, whereas opposite results that there was no considerable alteration in the total protein levels in fish fed diets containing the dietary AsA supplement were reported (Hardie et al., 1991; Roberts et al., 1995). Plasma GOT and GPT are indicators to assess liver

damage. Many authors reported that lower GOT level of Japanese flounder, *Paralichthys olivaceus* fed diets containing the dietary AsA supplementation over 500 mg/kg (Gao et al., 2014) and reduced GOT value of Japanese eel, *Anguilla japonica* feeding higher dietary AsA supplementation (Ren et al., 2007). On the other hand, the excess dietary AsA supplementation can induce the increase in GOT level by a poor condition of liver tissue, because it causes lipid peroxidation (Gao et al., 2014). The dietary AsA supplement of *S. schlegelii* caused a considerable decrease in GOT and GPT level. Generally, the dietary AsA supplement of *S. schlegelii* has influenced on the plasma parameters such as glucose, total protein, GOT, and GPT, whereas there was no alteration in calcium and magnesium.

4.3. AChE activity

Acetylcholine is a major activator of sensory perceptions in the central nervous system and muscle in the peripheral nervous system, and acetylcholinesterase (AChE) is well known as a modulator to control acetylcholine secretion. Among various ascorbate functions, it is known as a role to be concerned in the regulation of acetylcholine release from synaptic vesicles (Kuo et al., 1979; Harrison and May, 2009). Mor and Ozmen (2010) reported that ascorbate also alleviates the AChE inhibition by a toxicant, which is a neuroprotective action of ascorbate. The

dietary AsA supplement of *S. schlegelii* induce the increase in AChE activity, although Dhingra et al. (2006) suggest that ascorbate has been considered in an effective AChE inhibitor.

Chapter III. Detoxification effects of dietary ascorbic acid supplementation for the dietary lead exposure on bioaccumulation, hematological parameters, and antioxidant responses in rockfish, *Sebastes schlegelii*.

1. Introduction

The exposure to heavy metals in marine environment is a crucial environmental issue, because these metals can be easily accumulated in the human through fishery products such as fish, shrimp, and shellfish, which creates a health risk for human. Among various heavy metals, lead is one of the most toxic substances, which can be highly toxic to aquatic organisms even at low doses of lead exposure. Lead is a naturally occurring metal existent in the rock, soil, and water resulting from the weathering phenomena and volcanic activities. But, the excessive amount of lead exposure occurs by anthropogenic activities such as mining and smelting, cement manufacturing, batteries, and paint, which induces the lead poisoning in aquatic animals (Rogers et al., 2003).

The lead exposure in aquatic environment induces the accumulation in the specific tissues of aquatic animals, and the bioaccumulation is influenced by the environmental factors such as

temperature, salinity, pH, and hardness, in addition to the species-specific factors such as feeding, metabolism, digestion, and excretion (McCarty and Mackay, 1993). Rabitto et al. (2005) reported a considerable metal accumulation in the specific tissues of fish result from the different physiological processes according to the functions of tissues.

The lead accumulation in aquatic animals induces a non-specific toxicant affecting physiological systems (Rabitto et al., 2005). The toxic symptoms of lead involve cognitive dysfunction, neurological damage, calcium homeostasis alterations, and oxidative damage, as well as muscular atrophy, black tail, fin degeneration, and hyperactivity (Patrick, 2006).

The toxicity exposure causes the homeostasis disturbance in aquatic animals that leads to a reallocation of energy resources for growth to compensatory, adaptive and pathological process (Knops et al., 2001). Many authors indicate that the reductions of growth rate in aquatic animals occur by the metal exposure, and the toxic effects on growth performance have been metallic dose-dependent manner (Campbell et al., 2002; Clearwater et al., 2002; Shaw and Handy, 2006).

Given that fish blood is closely related to the external environment via the circulatory system (Cazenave et al., 2005), hematological parameters have been sensitive and reliable indicators to assess the physiological status in aquatic animals under metallic stress by metal exposure (Vutukura, 2005; Kim and

Kang, 2014). Blood parameters have also been considered as pathophysiological indicators in diagnosing the structural and functional status in fish under toxicant exposure (Ribeiro et al., 2006). Jacob et al. (2000) suggest that the lead exposure induces the interference in heme and hemoglobin syntheses and alterations in erythrocyte morphology, which leads to anemia and depleted hematocrit by blood system damage.

Oxygen is essential in aerobic organisms to carry out various metabolic processes. But, reliance on oxygen forces aerobic organisms to withstand its notable toxicity like increased reactive oxygen species (ROS) resulting in considerable damage to cell (Ahmad et al., 2004). Oxidative stress by the ROS attack occurs when antioxidant defenses are not be effective in detoxifying ROS. The exposure to toxic substances in aquatic animals generally induces oxidative stress within intracellular space of aquatic animals by generating reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radical (Kim and Kang, 2015). Alterations in the levels of antioxidants can be reliable and sensitive biomarkers for toxicants (Ferreira et al., 2005). Among various toxicants, lead (Pb) is considered as a critical oxidative stress inducer (Mucha et al., 2003). The accumulation of heavy metals in aquatic animals may catalyze the production in ROS, which can cause the oxidative stress in aquatic animals (Farombi et al., 2007). Defensive mechanisms including various antioxidant defense enzymes have been activated

to counteract the ROS production (Tjalkens et al., 1998). Among various antioxidant mechanisms, superoxide dismutase (SOD) is a first defense system to counteract the oxidative stress by the ROS production, which converts superoxide anion into hydrogen peroxide (Vlahogianni et al., 2007). Glutathione S-transferase (GST) is also an antioxidant enzyme in phase II detoxifying enzyme, which can be a reliable biomarker to assess the oxidative damage in aquatic animals (Regoli and Principato, 1995). Glutathione (GSH) protects cells against the ROS production by the toxic substance exposure as the most abundant intracellular thiol-based antioxidant, and it also serve as a cofactor for antioxidant enzymes (Pandey et al., 2008).

The lead exposure to fish can also cause a neuronal injury as neurotoxicity, which has a harmful effect on the central nervous system in fish. Many authors reported significant alterations in learning and memory as well as locomotor activity and sensorimotor responses in vertebrate species including fish by the lead exposure (Kuhlmann et al., 1997; Weber et al., 1997; Carvan et al., 2004). Among various neurotransmitters, acetylcholine (ACh) is connected to cognitive processes via the stimulation of muscarinic and cholinergic receptors, and acetylcholinesterase (AChE) functions as the maintenance of ACh by catalyzing ACh. The metal exposure causes the neurodegeneration by oxidative stress (Senger et al., 2006). Lead is a neurotoxic substance, which influences the developing central nervous system, and the

lead exposure even at low concentration can induces the behavioral abnormalities and impaired cognitive abilities in animals (Xu et al., 2005). A significant AChE inhibition is generally shown in fish exposed to various toxicants, and the inhibition of AChE can be a sensitive biomarker to assess the neurotoxicity in fish (Modesto and Marinez, 2010).

As a metal binding protein induced by the metal toxicity, metallothionein (MT) functions as heavy metal ion homeostasis and detoxification, and it is well known that the elevated intracellular metal accumulation in fish by metal exposure causes the MT increase of target organs like liver, kidney, and gill (Baudrimont et al., 2003). Given that the MT is closely associated with metal exposure due to its ability to bind metals for detoxification (Ay et al., 1999), it can be a sensitive and reliable biomarker to assess metal contamination in aquatic environment (Chen et al., 2004).

Heat shock protein 70 (HSP 70) have been considered as sensitive and reliable indicators to assess the stress responses for the lead exposure. HSP 70 is a highly conserved cellular protein existent in all organisms including fish, which functions as a molecular chaperone mediating the repair and degradation of altered or denatured proteins (Basu et al., 2002). A correlation between elevated HSP 70 and exposure to toxicants has been demonstrated by many studies, and the exposure to heavy metals causes the increased levels of HSP 70 as environmental

contaminations (Duffy et al., 1999). Therefore, the HSP 70 can be proposed as a good biomarker to evaluate the environmental stress of aquatic animals against toxic substances.

Ascorbic acid (vitamin C, AsA) is an essential nutrient for the proper metabolic function and immunity as well as growth and development of teleost, which is also connected to the metabolism and toxic action of various metals (Grosicki, 2004). Many studies have indicated AsA reduces the lead tissue levels by chelating lead under the lead exposure (Dalley et al., 1990; West et al., 1994; Tandon et al., 2001). AsA inhibits the absorption of lead by decreasing ferric iron to ferrous iron in the duodenum, which competes with lead for intestinal absorption (Patrick, 2006). In addition to the AsA effect on the metal accumulation, AsA supplementation also significantly affects the decrease in the stress-induced cortisol levels and other stress indicators in animals (O'Keefe et al., 1999; Brody et al., 2002). Although the AsA effects on the metal accumulation and stress indicators in fish under the metal exposure have been scarcely reported, the AsA supplementation may affect the metal accumulation and stress indicators of fish against the metal exposure.

The AsA supplementation may protect most animals from metal-induced harmful effects on depression in growth, alterations in blood hematology, and changes in plasma biochemical components in addition to lipid peroxidation, free radicals, and neurotoxicity (Grosicki, 2004; Yousef, 2004).

Fish tissues contain lots of polyunsaturated fatty acids (PUFAs), which is vital for proper membrane function. Fish must possess effective antioxidant defenses, because the PUFAs are highly vulnerable to oxidative attack. Therefore, the dietary supply of essential antioxidants such as vitamins by their feed in fish is partly necessary to protect them from oxidative damage (Martinez-Alvarez et al., 2005). Singh et al. (2014) suggest that AsA functions as an antioxidant protecting the cell from ROS in vivo and in vitro by scavenging aqueous ROS by rapid electron transfer that reduces lipid per oxidation. AsA is also a cofactor for the biosynthesis of neurotransmitters, in particular the transformation of dopamine to norepinephrine, which is catalyzed by dopamine β-monooxygenase (Yousef, 2004)

In Korea, the experimental animal in this study, rock fish (*Sebastes schlegelii*) is a major cultured fish in marine net cages due to its high demand by appreciated flesh as well as rapid growth. But, the study about the toxic effects of dietary lead exposure on this fish has been insufficiently conducted. The AsA supplementation of *S. schlegelii* through their feed may protect the animal from the harmful effect of the dietary lead exposure. The role of AsA against lead-induced alterations in accumulation, growth performance, hematological parameters, antioxidant responses, AChE activity, and specific gene expression such as MT and HSP70 of *S. schlegelii* has not so far been studied. Therefore, the purpose of this study is to evaluate the toxic

effects of dietary lead exposure and the AsA effect for the lead toxicity.

2. Materials and Methods

2.1. Experimental fish and culture conditions

Juvenile *S. schlegelii* were obtained from a local fish farm in Tongyeong, Korea. The fish were acclimatized for 2 weeks under laboratory conditions. During the acclimation period, the fish were fed a Pb-free diet twice daily and maintained on a 12-h:12-h light/dark cycle and constant condition at all times (Table 3-1). After acclimatization, 90 fishes(bodylength,11.3±1.2 cm; body weight, 32.5±4.1 g) were randomly selected for the study. Dietary lead exposure took place in 500L circular tanks 5 fish per treatment group in duplicates. The dietary lead and ascorbic acids concentrations were 0, 120, and 240 mg/kg and 100, 200 and 400 mg/kg (Table 3-2), and fish were fed each lead concentration at a rate of 2% body weight daily (as two 1% meals per day). At the end of each period (at 2 and 4 weeks), fish were anesthetized in buffered 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma Chemical, St. Louis, MO).

Table 3-1. The chemical components of seawater and experimental condition used in the experiments.

Item	Value
Temperature (°C)	19.0±1.0
pH	8.1±0.5
Salinity (‰)	33.2±0.5
Dissolved Oxygen (mg/L)	7.1±0.3
Chemical Oxygen Demand (mg/L)	1.15±0.2
Ammonia (μg/L)	10.3±0.7
Nitrite (μg/L)	1.4±0.2
Nitrate (μg/L)	9.27±1.0

2.2. Feed ingredients and diets formulation

Formulation of the diets is shown in Table 3-2. Lead (II) nitrate and ascorbic acids were obtained from Sigma Chemical Co., Ltd. All diets contained 33% casein, 23% fish meal, 20% wheat flour, 5% corn starch, 2% vitamin premix (vitamin C-free), and 2% mineral premix. 10% fish oil was added to meet the essential fatty acids (EFA) requirements of rock fish. Lead premix was made up of 2 g lead with 98 g cellulose. Three isonitrogenous and isolipidic diets were formulated with supplementation of different dietary lead concentrations of 0, 120, and 240 mg/ kg diet. Ascorbic acid premix was made up of 2 g ascorbic acids with 98 g cellulose. Three isonitrogenous and isolipidic diets were

formulated supplementation of different dietary ascorbic acid concentrations of 100, 200 and 400 mg/kg. All ingredients were blended thoroughly. At last, water was added into the mixture to produce stiff dough. Then the dough was pelleted by experimental feed mill, and dried for 24 h at room temperature. After processing, all the diets were packed and kept - 20 °C until use. For determination of total lead concentrations in diets, ICP-MS measurements were performed using an ELAN 6600DRC ICP-MS instrument with argon gas (Perkin-Elmer). Total lead concentrations were determined by external calibration. ICP multi-element standard solution VI (Merck) was used for standard curve. The lead bioaccumulation in diet samples was expressed mg/kg dry wt. For determine of total ascorbic acid concentrations in diets, HPLC measurements were performed using an Agilent 1200 series.

Table 3-2. Formulation of the experimental diet (% dry matter).

Ingredient (%)	Concentration (mg/kg)								
	M0V1	M0V2	M0V3	M1V1	M1V2	M1V3	M2V1	M2V2	M2V3
Casein[1]	33.0	33.0	33.0	33.0	33.0	33.0	33.0	33.0	33.0
Fish meal[2]	23.0	23.0	23.0	23.0	23.0	23.0	23.0	23.0	23.0
Wheat flour[3]	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Fish oil[4]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Cellulose[1]	4.5	4.0	3.0	3.9	3.4	2.4	3.3	2.8	1.8
Corn starch[3]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin Premix (Vitamin C-free)[5]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Mineral Premix[6]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Lead Premix[7]	0.0	0.0	0.0	0.6	0.6	0.6	1.2	1.2	1.2
Ascorbic acid Premix[8]	0.5	1.0	2.0	0.5	1.0	2.0	0.5	1.0	2.0

[1]United States Biochemical (Cleveland, OH).

[2]Suhyup Feed Co., Ltd., Gyeong Nam Province, Korea.

[3]Young Nam Flour Mills Co., Pusan, Korea.

[4]Sigma Chemical Co., St. Louis, MO.

[5]Vitamin Premix (vitamin C-free) (mg/kg diet): dl-calcium pantothenate, 400; choine chloride 200; inositol, 20; menadione, 2; nicotinamide, 60; pyridoxine·HCl, 44; riboflavin, 36; thiamine mononitrate, 120, dl-a-tocopherol acetate, 60; retinyl acetate, 20000IU; biotin, 0.04; folic acid, 6; vitamin B_{12},0.04;cholecalcifero,4000IU.

[6]Mineral Premix (mg/kg diet): Al, 1.2; Ca, 5000; Cl, 100; Cu, 5.1; Co, 9.9; Na, 1280; Mg, 520; P, 5000; K, 4300; Zn, 27; Fe, 40; I, 4.6; Se, 0.2; Mn, 9.1.

[7]Lead Premix (mg/kg diet): 20,000 mg Pb/ kg diet

[8]Ascorbic acid Premix (mg/kg diet): 20,000 mg ascorbic acid/ kg diet

(M0: Pb 0 mg/kg, M1: Pb 120 mg/kg, M2: Pb 240 mg/kg, V1: AsA 100 mg/kg, V2: AsA 200 mg/kg, V3: AsA 400 mg/kg)

2.3. Bioaccumulation

The tissue samples of liver, kidney, spleen, intestine, gill, and muscle of *S. schlegelii* were performed with freeze-dried to measure dry weight of the samples. The freeze-drying samples were digested by wet digestion method (Arain et al., 2008; Korai et al., 2008). The dried samples were digested in 65%(v/v) HNO3, and re-dried at 120 °C on hot plate. The procedure was repeated until total digestion. The entirely digested samples were diluted in 2%(v/v) HNO3. The samples were filtered through a 0.2 μm membrane filter (Advantec mfs, Ins.) under pressure for analysis. For determination of total lead concentrations, the digested and extracted solutions were analyzed by ICP-MS. The ICP-MS measurements were performed using an ELAN 6600DRC ICP-MS instrument with argon gas (Perkin-Elmer). Total lead concentrations were determined by external calibration. ICP multi-element standard solution VI (Merck) was used for standard curve. The lead bioaccumulation in tissue samples was expressed μg/g dry wt.

2.4. Growth Performance

The weight and length of *S. schlegelii* was measured just before exposure, at 2 and 4 weeks. Daily length gain, daily weight gain, condition factor, and hepatosomatic index (HIS) were calculated

by the following method.

Daily growth gain = Wf-Wi/day

(Wf=Finallengthorweight,Wi=Initiallengthorweight)

Condition factor (%) = (W/L3)x100

(W = weight (g), L = length (cm))

HIS = (liver weight/ total fish weight) x 100

2.5. Hematological assay

Blood samples were collected within 35-40 seconds through the caudal vein of the fish in 1-ml disposable heparinized syringes. The blood samples were kept at 4°C until the blood parameters were completely studied. The total red blood cell (RBC) count, hemoglobin (Hb), concentration, and hematocrit (Ht) value were determined immediately. Total RBC counts were counted using optical microscope with hemo-cytometer (Improved Neubauer, Germany) after diluted by Hendrick's diluting solution. The Hb concentration was determined using Cyan-methemoglobin technique (Asan Pharm. co., Ltd.). The Ht value was determined by the microhematocrit centrifugation technique. The blood samples were centrifuged to separate plasma from blood samples at 3000 g for 5 minutes at 4°C. The serum samples were analyzed for inorganic substances, organic substances, and enzyme activity using clinical kit (Asan Pharm. Co.,Ltd.). In

inorganic substances assay, calcium and magnesium were analyzed by the o-cresolphthalein-complexon technique and xylidyl blue technique. In organic substances assay, glucose and total protein were analyzed by GOD/POD technique and biuret technique. In enzyme activity assay, glutamic oxalate transaminase (GOT) and glutamic pyruvate transaminase (GPT) were analyzed by Kind-king technique and alkaline phosphatase (ALP) was analyzed using clinic al kit.

2.6. Antioxidant system analysis

Liver and gill tissues were excised and homogenized with 10 volumes of ice-cold homogenization buffer using Teflon-glass homogenizer (099CK4424, Glass-Col, Germany). The homogenate was centrifuged at 10,000 g for 30 min under refrigeration and the obtained supernatants were stored at -80 °C for analysis.

Superoxide dismutase (SOD) activity was measured with 50% inhibitor rate about the reduction reaction of WST-1 using SOD Assay kit (Dojindo Molecular Technologies, Inc.). One unit of SOD is defined as the amount of the enzyme in 20 μl of sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%. SOD activity was expressed as unit mg protein-1.

Glutathione-S-transferase(GST) activity was measured according to the method of modified Habig (1974). The reaction mixture

consisted of 0.2 M phosphate buffer (pH 6.5), 10 mM GSH (Sigma) and 10 mM 1-chloro-2,-dinitrobenzene, CDNB (Sigma). The change in absorbance at 25 °C was recorded at 340 nm and the enzyme activity was calculated as 340 nm and the enzyme activity was calculated as nmol min-1mgprotein-1.

Reduced glutathione was measured following the method of Beutler et al. (1963). Briefly, 0.2ml fresh supernatant was added to 1.8 ml distilled water. Three ml of the precipitating solution (1.67 g metaphosphoric acid, 0.2 g EDTA and 30 g NaCl in 100 ml distilled water) was mixed with supernatants. The mixture was centrifuged at 4500 g for 10 min. 1.0mL of supernatant was added to 4.0ml of 0.3M NaHPO4 solution and 0.5mL DTNB (5,5`-dithiobis-2-nitrobenzoic acid) was then added to this solution. Reduced glutathione was measured as the difference in the absorbance values of samples in the presence and the absence of DTNB at 412 nm. GSH value was calculated as μmol mg protein-1inthetissues.

2.7. Inhibition of AChE activity

AChE activity was determined brain(1:25) and muscle(1:10) homogenate in 0.1 M phosphate buffer, pH 8.0. The homogenate were centrifuged 10,000 g for 20 min at 4 °C. The supernatant was removed and used to test AChE activity. AChE activity was determined according to the method of Ellman et al. (1961). AChE

activity was normalized to protein content and expressed as nmol min-1mgprotein-1. Briefly, the activity on the homogenate was measured by determining the rate of hydrolysis of acetylthiocholine iodide (ACSCh,0.88mM) in final volume of 300μl, with 33 μl of 0.1 M phosphate buffer, pH 7.5 and 2 mM DTNB. The reaction was started with the addition of the substrate acetylthiocholine, as soon as the substrate was added the hydrolysis and the formation of the dianion of DTNB were analyzed in 412 nm for 5 min (in intervals of 1 min) using a microplate reader. Protein concentration was determined using Ellman's method (1961), with a bovine plasma albumin (Sigma, USA) as standard.

2.8. Specific gene expression

Total RNA was extracted from liver samples using RNA purification kit (Real Biotech Corporation, Taipei, Taiwan), and the quantity and quality of the total RNA were assessed using the Ultrospec 3100 pro (Amersham Bioscience, Amersham, UK). The 260/280 nm absorbance ratios of all samples ranged from 1.80 to 2.00, indicating a satisfactory purify of the RNA samples. Purified RNA was subjected to reverse transcription to cDNA by cDNA synthesis kit (Enzo Life Sciences Inc., NY, USA) according to the reagent's instructions. For real-time quantitative PCR analysis of MT and HSP70 gene expression, the real-time qPCR primer of

MT gene, HSP 70 and 18s rRNA gene are shown in Table 3-3. Real-time PCR assay were carried out in a quantitative thermal cycler (LightCycler® 480 II, Roche Diagnostics Ltd., Rotkreuz, Switzerland) in a final volume of 20 μl containing 10 μl 2 x Master Mix (LightCycler® 480 SYBR Green I Master, Roche Diagnostics Ltd., Rotkreuz, Switzerland), 1 μl of cDNA mix. MT gene-specific primers were applied to evaluate the mRNA levels of MT in liver. Reference 18s rRNA gene was used as internal control. The real-time qPCR amplification began with 5 min at 95 °C, followed by 45 cycles of denaturation of 10 s at 95 °C, annealing of 10 s at 60 °C, and extension of 10 s 72 °C. To analyze the mRNA expression level, the comparative CT methods (2 $^{-\Delta\Delta CT}$ method) was used.

Table. 3-3. The primers used in this study for real-time qPCR.

Gene	Sequence	Product size
18s rRNA	Fw : TGAGAAACGGCTACCACATC Rv : CAATTACAGGGCCTCGAAAG	100 bp
MT	Fw : CAACTGCGGTGGATCCTG Rv : CCAGAGGCGCATTTAGGG	102 bp
HSP 70	Fw : GATGCAGCCAAGAACCAGGTGG Rv : GCTTCCCTCCATCTCCGATCACC	144 bp

2.10. Statistical analysis

The experiment was conducted in exposure period for 4 weeks and performed triplicate. Statistical analyses were performed using the SPSS/PC+ statistical package (SPSS Inc, Chicago, IL, USA). Significant differences between groups were identified using one-way ANOVA and Duncan's test for multiple comparisons or Student's t-test for two groups (Duncan, 1955). The significance level was set at $P < 0.05$.

3. Results

3.1. Bioaccumulation

The Pb accumulation in kidney, liver, spleen, intestine, gill, and muscle of *S. schlegelii* exposed to the dietary Pb concentrations depending on AsA supplementation is demonstrated in Fig. 3-1. It was observed that the highest Pb accumulation in kidney. The significant accumulation in kidney was observed over 120 mg/kg of the dietary Pb exposure after 2 weeks and 4 weeks. In the 120 mg/kg Pb at 4 weeks, the level of bioaccumulation in kidney supplementing 200 and 400 mg/kg AsA was much lower than the accumulation supplementing 100 mg/kg AsA.

For the liver tissue, the Pb accumulation notably increased over 120 mg/kg Pb exposure at 2 and 4 weeks. At 2 weeks, there was no alteration at 120 mg/kg of the dietary Pb exposure depending on the levels of dietary AsA supplementation. But, the accumulation at the level of 400 mg/kg AsA for 240 mg/kg Pb exposure was much lower than those of 100 and 200 mg/kg AsA. At 4 weeks, the accumulation at the level of 400 mg/kg for 120 mg/kg of the dietary Pb exposure was significantly lower than those of 100 and 200 mg/kg AsA, and there was an obvious AsA dose dependent decrease depending on the AsA supplementation in the accumulation for 240 mg/kg of the dietary Pb exposure.

The Pb accumulation in spleen was also considerably increased by

the dietary Pb exposure at 2 and 4 weeks. At 2 weeks, the accumulation levels at 200 and 400 mg/kg AsA for 240 mg/kg of the Pb exposure were substantially lower than the accumulation at 100 mg/kg AsA. At 4 weeks, the levels of AsA supplementation significantly affected the decrease in the accumulation for 120 and 240 mg/kg of the Pb exposure.

For the tissue of intestine, the considerable accumulations were observed over 120 mg/kg of the Pb exposure. Both of 2 and 4 weeks, the levels of AsA supplementation notably affected the accumulation. The group supplementing 400 mg/kg AsA for 120 mg/kg Pb exposure at 2 and 4 weeks was much lower than those of 100 and 200 mg/kg AsA. The group supplementing 200 and 400 mg/kg AsA for 240 mg/kg Pb exposure was much lower than the group with 100 mg/kg AsA supplementation.

For the gill tissue, a considerable accumulation was observed by the dietary Pb exposure. At 2 weeks, there was no effect of the accumulation depending on the AsA supplementation. However, the levels of 200 and 400 mg/kg AsA supplementation at 4 weeks notably affected the decrease in the accumulation, compared to the 100 mg/kg AsA.

In the muscle Pb accumulation, there was no considerable accumulation except for the group supplementing 100 mg/kg AsA for 240 mg/kg Pb exposure. After 4 weeks of the dietary lead exposure depending on AsA supplementation, the profile of tissue Pb accumulation was kidney > liver > spleen > intestine > gill > muscle.

Fig. 3-1. Pb accumulation of rockfish, *Sebastes schlegelii* combinedly exposed to the different concentration of dietary lead and ascorbic acid for 4 weeks. Values with different superscript are significantly different in 2 and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.2. Growth performance

The growth performance of *S. schlegelii* by the dietary Pb exposure depending on the AsA supplementation is demonstrated in Fig. 3-2. The daily length gain of *S. schlegelii* was notably reduced over Pb 120 mg/kg with AsA 100 and 200 mg/kg and in the concentration of Pb 240 mg/kg with AsA 400 mg/kg after 2 weeks. After 4 weeks, a considerable decrease in the daily length gain was observed over Pb 120 mg/kg exposure. In the daily weight gain, a notable increase was observed over Pb 120 mg/kg exposure after 2 and 4 weeks. The condition factor was significantly decreased over Pb 120 mg/kg with AsA 100 mg/kg after 2 weeks, and a notable decrease was observed in the concentration of Pb 240 mg/kg after 4 weeks. In the hepatosomatic index of *S. schlegelii*, a substantial decrease was observed in the concentration of Pb 240 mg/kg with AsA 100 and 200 mg/kg. After 4 weeks, the hepatosomatic index was considerably decreased in the concentration of Pb 240 mg/kg.

Fig. 3-2. Daily length, daily weight gain, condition factor, and hepatosomatic index factor of rockfish, *Sebastes schlegelii* combinedly exposed to the different concentration of dietary lead and ascorbic acid for 4 weeks. Values with different superscript are significantly different in 2 and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.3. Hematological parameters

The RBC count, hematocrit value, and hemoglobin concentration of *S. schlegelii* exposed to the dietary Pb exposure depending on the AsA supplementation are demonstrated in Fig. 3-3. In the RBC count of *S. schlegelii*, a significant decrease in the RBC count was observed over Pb 120 mg/kg after 2 and 4 weeks. The hematocrit of *S. schlegelii* was notably reduced over Pb 120 mg/kg with AsA 100 and 200 mg/kg and in the concentration of Pb 240 mg/kg with AsA 400 mg/kg after 2 weeks. After 4 weeks, a substantial decrease in the hematocrit was observed over Pb 120 mg/kg. In the hemoglobin of *S. schlegelii*, the concentration was significantly decreased over Pb 120 mg/kg after 2 weeks. After 4 weeks, a considerable decrease in the hemoglobin was observed over Pb 120 mg/kg with AsA 100 and 200 mg/kg and in the concentration of Pb 240 mg/kg with AsA 400 mg/kg.

The alterations in plasma components of *S. schlegelii* by the dietary Pb exposure depending on the AsA supplementation are shown in Fig. 3-4. In the inorganic components such as calcium and magnesium, the calcium was reduced over Pb 120 mg/kg with AsA 100 mg/kg after 2 and 4 weeks, but there was no significant change in the group supplementing AsA 200 and 400 mg/kg. The magnesium was considerably decreased in the concentration of Pb 240 mg/kg with all AsA supplementation groups. In the organic components such as glucose, cholesterol, and total protein, the

glucose and cholesterol were notably increased by the dietary Pb exposure, whereas there was no alteration in total protein. In the enzyme components, the GOT and GPT of *S. schlegelii* were considerably increased, but there was no change in ALP. In the GOT of *S. schlegelii*, a significant increase was observed over Pb 120 mg/kg with AsA 100 mg/kg and in the concentration of Pb 240 mg/kg with AsA 200 and 400 mg/kg after 2 and 4 weeks. The GPT of *S. schlegelii* was considerably increased by the dietary Pb exposure over 120 mg/kg after 2 and 4 weeks.

Fig. 3-3. Changes of RBC count, Hematocrit and Hemoglobin in rockfish, *Sebastes schlegelii* combinedly exposed to the different concentration of dietary lead and ascorbic acid for 4 weeks. Values with different superscript are significantly different in 2 and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

Fig. 3-4. Changes of plasma parameters in rockfish, *Sebastes schlegelii* combinedly exposed to the different concentration of dietary lead and ascorbic acid for 4 weeks. Values with different superscript are significantly different in 2 and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.4. Antioxidant system

Antioxidant response analysis (SOD, GST, and GSH) in liver and gill tissues of *S. schlegelii* by the dietary Pb exposure depending on the AsA supplementation is demonstrated in Fig. 3-5, 3-6, 3-7. The SOD activity of liver in *S. schlegelii* was significantly increased over Pb 120 mg/kg exposure with AsA 100 mg/kg and in the concentration of Pb 240 mg/kg with AsA 200 and 400 mg/kg after 2 week. A considerable increase in the SOD activity of liver was observed over Pb 120 exposure after 4 weeks. The SOD activity of gill was also notably increased over Pb 120 mg/kg exposure with AsA 100 mg/kg and in the concentration of Pb 240 mg/kg with AsA 200 and 400 mg/kg after 2 week. After 4weeks, the SOD activity of gill was substantially increased over Pb 120 exposure.

In the GST activity of liver in *S. schlegelii*, a considerable increase was observed over Pb 120 mg/kg exposure with AsA 100 mg/kg and in the concentration of Pb 240 mg/kg with AsA 200 and 400 mg/kg after 2 week. After 4 weeks, the GST activity of liver was notably increased over Pb 120 mg/kg exposure. The GST activity of gill was substantially elevated over Pb 120 mg/kg exposure with AsA 100 mg/kg and in concentration of Pb 240 mg/kg with AsA 200 and 400 mg/kg after 2 weeks. After 4 weeks, a significant increase in the GST activity of gill was observed over 120 mg/kg with AsA 100 and 200 mg/kg and in

the concentration of Pb 240 mg/kg with AsA 400 mg/kg.

The GSH level of liver in *S. schlegelii* was notably reduced by the Pb exposure over 120 mg/kg after 2 and 4 weeks. In the GSH level of gill, a substantial decrease was observed over Pb 120 mg/kg with AsA 100 and 200 mg/kg and in the concentration of Pb 240 mg/kg with AsA 400 mg/kg after 2 weeks. After 4 weeks, the GSH level of gill was significantly decreased by the Pb exposure over 120 mg/kg.

The antioxidant responses such as SOD activity, GST activity, and GSH level were affected by the concentrations of Pb exposure, the high level of AsA supplementation alleviated the alterations in the antioxidant indicators by the Pb exposure.

Fig. 3-5. SOD activity of rockfish, *Sebastes schlegelii* combinedly exposed to the different concentration of dietary lead and ascorbic acid for 4 weeks. Values with different superscript are significantly different in 2 and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

Fig. 3-6. GST activity of rockfish, *Sebastes schlegelii* combinedly exposed to the different concentration of dietary lead and ascorbic acid for 4 weeks. Values with different superscript are significantly different in 2 and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

Fig. 3-7. GSH level of rockfish, *Sebastes schlegelii* combinedly exposed to the different concentration of dietary lead and ascorbic acid for 4 weeks. Values with different superscript are significantly different in 2 and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.5. Inhibition of AChE activity

The inhibition of AChE activity of *S. schlegelii* by the dietary Pb exposure depending on the AsA supplementation is demonstrated in Fig. 3-8. The AChE activity of brain in *S. schlegelii* was significantly inhibited over Pb 120 mg/kg with AsA 100 mg/kg and in the concentration of Pb 240 mg/kg with AsA 200 and 400 mg/kg after 2 weeks. After 4 weeks, a notable inhibition of brain AChE was observed over Pb 120 mg/kg regardless of the level of AsA supplementation after 4 weeks. In the AChE of muscle in *S. schlegelii* after 2 weeks, a substantial decrease was observed in the concentration of Pb 240 mg/kg with AsA 100 and 200 mg/kg, but there was no significant alteration in the group supplementing AsA 400 mg/kg. After 4 weeks, a notable inhibition of AChE in muscle was observed in the concentration of Pb 240 mg/kg.

Fig. 3-8. AChE activity of rockfish, *Sebastes schlegelii* combinedly exposed to the different concentration of dietary lead and ascorbic acid for 4 weeks. Values with different superscript are significantly different in 2 and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

3.6. Specific gene expression

The relative MT and HSP 70 mRNA gene expression of *S. schlegelii* by the dietary Pb exposure depending on the AsA supplementation is shown in Fig. 3-9. The MT gene expression was considerably increased over Pb 120 mg/kg exposure regardless of the level of AsA supplementation after 2 and 4 weeks. But, the MT increase in the low level of AsA supplementation was more significant than those in the high level of AsA supplementation.

A substantial increase in relative HSP 70 mRNA was observed by the dietary Pb exposure. But, there was no alteration depending on the levels of AsA supplementation.

Fig. 3-9. Relative MT and HSP 70 mRNA of rockfish, *Sebastes schlegelii* exposed to the different concentration of dietary lead for 4 weeks. Values with different superscript are significantly different in 2 and 4 weeks ($P < 0.05$) as determined by Duncan's multiple range test.

4. Discussion

4.1. Bioaccumulation

Bioaccumulation in aquatic animals by the heavy metal exposure in aquatic environment is affected by various factors such as uptake, elimination, and exposure concentration (Sivaperumal et al., 2007). The accumulation in the specific tissues of aquatic animals exposed to metals can be highly toxic when the reactions such as excretory, metabolic, storage and detoxification mechanisms do not respond to the metal uptake (Kalay and Canli, 2000). In this study, the dietary Pb accumulation in the kidney of rockfish, *S. schlegelii* depending on the AsA supplementation was substantially elevated by the dietary Pb exposure, which is the highest accumulation among the tissues. Romeo et al. (2000) also demonstrated that kidney was the major tissue in the sea bass *Dicentrarchus labrax* exposed copper. Similarly with the kidney tissue of *S. schlegelii*, a considerable accumulation in the liver of *S. schlegelii* was observed by the dietary Pb exposure. Vinodhini and Narayanan (2008) also reported a notable accumulation in liver of common carp, *Cyprinus carpio* exposed to metals such as lead, nickel, and cadmium, which induced severe liver damage. Kidney and liver represent major target organs appropriate for histopathological examination to evaluate damages to tissues and cells (Oliveira Ribeiro et al., 2006), which are vulnerable tissues

for the prolonged metal exposure both through waterborne and dietary pathways (Olsvik et al., 2000). The kidney and liver in marine fish are main organs in the accumulation under the exposure to toxicants due to those functions in detoxification mechanisms (Thophon et al., 2003). The dietary Pb exposure depending on the AsA supplementation caused a considerable accumulation in the spleen of *S. schlegelii*. Ciardullo et al. (2008) also observed a substantial accumulation in the spleen of rainbow trout, *Oncorhyncus mykiss* exposed to mercury. A significant accumulation in spleen results from its high metabolic activity, which induces a higher accumulation in spleen than other tissues (Shukla et al., 2007).

Generally, the uptake routes of toxic substances affect the tissue accumulation of toxicants in aquatic animals; via digestive organs by feeding or gill tissues by dissolved form. Considering the uptake of toxic substances in aquatic animals generally occurs by two major routes, the intestine and gill should be critical tissues for the metal accumulation in aquatic animals (Kim and Kang, 2014). Considering the dietary Pb exposure to *S. schlegelii*, the higher accumulation in the intestine was observed than those of gill tissue. The considerable accumulation in intestine of *S. schlegelii* may result from the direct uptake by dietary source. Gill tissue is also a major tissue in the accumulation for the metal exposure, but the route through dietary source is not significant compared to the waterborne pathway (Ciardullo et al., 2008). In

the muscle tissue, no significant accumulation was observed by the dietary Pb exposure except for the Pb 240 mg/kg exposure with AsA 100 mg/kg supplementation. Alves and Wood (2006) reported the lowest accumulation in muscle of rainbow trout, *Oncorhynchus mykiss* was observed by the dietary Pb exposure. Tulasi et al. (1992) also reported the metabolically active tissues such as kidney, liver, and gill of *Anabas testudineus* (Bloch) exposed to the Pb were significantly higher than other tissues like the muscle. The relative accumulation of *S. schlegelii* exposed to the dietary Pb depending on the AsA supplementation was: kidney > liver > spleen > intestine > gill > muscle. The dietary Pb exposure to *S. schlegelii* caused a significant accumulation in specific tissues. Given that the tissue-specific accumulation can be a sensitive indicator for the metal exposure in aquatic toxicology (Carriquiriborde and Ronco, 2008), the dietary Pb exposure to *S. schlegelii* considerably affected the experimental fish by the accumulation in specific tissues.

In this study, the levels of AsA supplementation as well as the concentrations of the Pb exposure notably influenced the accumulation in all tissues of *S. schlegelii*. The effect of AsA on the inhibition of the Pb absorption has been clarified by various studies, and AsA elevates the availability of iron by decreasing ferric iron to ferrous iron in the duodenum, which is a mechanism to compete with lead for intestinal absorption (Patrick, 2006). Kumar et al. (2009) reported the much lower Cd concentration of

liver and kidney in catfish, *Clarias batrachus* exposed to cadmium with AsA supplementation was observed than those of only cadmium exposure, which was caused by the functions of AsA to excrete metal in the bile by catalyzing its synthesis of glutathione and compete for the sulphydryl binding sites on metallothioneins. Dawson et al. (1999) reported that the AsA supplementation in male smokers effectively lowered the blood lead levels in the smokers. The AsA supplementation also significantly lowered the lead levels of rats exposed to the dietary lead (Dalley et al., 1990). Although many studies have reported the lead levels in tissues for the Pb exposure is effectively reduced by the AsA supplementation, there has been no report for the teleost under the Pb exposure. This study demonstrates that the AsA supplementation can also significantly lower the lead accumulation in tissues in teleost.

4.2. Growth performance

The fish growth is influenced by various external factors such as temperature, nutrient, and toxicants. Among, the various factors, the toxicant exposure are a critical component inhibiting the growth. The growth performance of the *S. schlegelii* was significantly decreased by the dietary Pb exposure, which may be due to the energy change from the growth and development to the detoxification. Friedmann et al. (1996) indicated the metal

accumulation in aquatic animals can cause retarded growth performance. The hepatosomatic index in aquatic animals is generally considered as a critical indicator to assess the toxic effects under the metal exposure (Datta et al., 2007). The dietary Pb exposure caused the decrease in the hepatosomatic index of *S. schlegelii*. The AsA supplementation was effective to attenuate the growth reduction in *S. schlegelii* by the dietary Pb exposure.

4.3. Hematological parameters

Hematology has been widely used to evaluate the health status of animals exposed to environmental toxicants, and many authors reported hematological alterations in fish exposed to various stress-induced substances (Mattsson et al., 2001; Affonso et al., 2002; Carvalho and Fernandes, 2006). In this study, the dietary Pb exposure to *S. schlegelli* induced a significant Pb accumulation in the blood, and the high AsA supplementation was effective to attenuate the accumulation levels by the Pb exposure. Fish blood can be a notable accumulation section, because the absorbed metal is transported to the liver through the bloodstream to be metabolized and excreted by generating the metal-binding proteins such as metallothioneins. Mazon and Fernandes (1999) reported a considerable copper accumulation in the blood of prochilodontidae, *Prochilodus scrofa* exposed to copper, which was caused by the excessive metal exposure. Ascorbic acid is one of the most

critical nutrients, which is effective to attenuate the Pb accumulation by reducing ferric iron to ferrous iron to compete with lead for absorption (Patrick, 2006). The hematological parameters of *S. schlegelli* such as RBC count, Ht value, and Hb concentration was markedly decreased by the dietary Pb exposure. Many studies reported the decrease in RBC count, Ht value, and Hb concentration in fish exposed to various toxicants (Benifey and Biron, 2000). The toxicity exposure such as heavy metals commonly induces the lysing of erythrocytes in aquatic animals, which leads to the depiction in hemoglobin and hematocrit values in addition to the deformed erythrocytes and anemia (Maheswaran et al., 2008). Toplan et al. (2004) indicate that the lead that has a high affinity with RBC increases the osmotic and mechanic susceptibility of RBC with the reduced deformability and shortened life span, and the high concentration lead in blood impair the heme synthesis, which leads to the inhibition of the hemoglobin synthesis and anemia by the lead poisoning.

The calcium and magnesium of inorganic components in plasma are major indicators to assess metal toxicity due to its functions of ion regulator for homeostasis (Kim and Kang, 2014). The dietary Pb exposure caused a considerable decrease in calcium and magnesium of *S. shlegelii*. Rogers et al. (2005) reported the ionoregulatory toxicity such as the disruption of $Na^+$and Cl^- balance of the rainbow trout, *Oncorhynchus mykiss* by the

Pb-induced toxicity. In the organic components, the glucose and cholesterol of *S. schlegelii* were substantially increased by the dietary Pb exposure, whereas there was no alteration in total protein. In case of the blood glucose levels, it is commonly increased by the carbohydrate metabolism elevation under the toxicant exposure stress such as heavy metals (Hontela et al., 1996), and the elevation in blood glucose is considered as a general secondary response to the stress in fish, which can be a sensitive indicator for environmental stress (Sepici-Dincel et al., 2009). Cicik and Engin (2005) reported a notable elevation in the glucose of common carp, *Cyprinus carpio* exposed to cadmium, which may be closely associated with the decrease in glycogen reserves in muscle and liver tissues. Cholesterol is a critical structural component of membranes and precursor of all steroid hormones. Given that heavy metals are well known to inversely affect the cell structure, the increase in cholesterol may be a sensitive indicator for metallic induced environmental stress (Oner et al., 2008). The cholesterol in the plasma of *S. schlegelii* was notably increased by the dietary Pb exposure. Firat et al. (2011) also reported a considerable increase in the cholesterol of Nile tilapia, *Oreochromis niloticus* exposed to copper and lead, which may increase due to liver and kidney failure inducing the release of cholesterol into the blood. Total plasma protein is synthesized in the liver, which is commonly used as a critical indicator to evaluate liver impairment (Yang and Chen, 2003). However, there

was no toxic effect on the total protein in the *S. schlegelii* exposed to the dietary Pb. Alterations in enzyme activities in aquatic organism such as GOT, GPT, and ALP have been used to indicate the tissue damage by toxicant stress under metal exposure (Lavanya et al., 2011). The GOT and GPT in the plasma of *S. schlegelii* were noticeably increased by the dietary Pb exposure, and the increases in plasma GOT and GPT activities are in agreement with the findings of many authors who found that heavy metals induced a significant increase in GOT and GPT activity, which may be attributed to tissue damage, particularly liver (Zikic et al., 2001; Levesque et al., 2002; Mekkawy et al., 2011). In the plasma ALP of *S. schlegelii*, there was no alteration. In this study, the dietary Pb exposure induced a significant alteration in the hematological parameters and plasma components of *S. schlegelii*. The AsA supplementation was highly effective to attenuate the alterations by the dietary Pb exposure. The changes of the hematological parameters such as RBC count, Ht value, and hemoglobin concentration in addition to the plasma components such as calcium, glucose, GOT, and GPT for the dietary Pb exposure were effectively moderated by the AsA supplementation. Mekkawy et al. (2011) reported that the vitamin E supplementation to Nile tilapia, *Oreochromis niloticus* was effective to attenuate the cadmium-induced toxicity in the hematological parameters. Hounkpatin et al. (2012) reported the protective effects of vitamin C on hematological parameters by

the cadmium, mercury, and combined two heavy metals. Dietary AsA supplementation also helps decrease in the proportion of glycated insulin in circulation, which alleviates insulin resistance leading to insulin-stimulated glucose uptake by peripheral tissues (Abdel-Wahab et al., 2002).

4.4. Antioxidant system

In the aquatic environment, the exposure to toxicants in aquatic animals stimulates the reactive oxygen species (ROS) production, and the increase in the ROS production leads to the oxidative damage, which is connected to pollutant-mediated mechanism of toxicity in fish liver (Ferreira et al., 2005). Activities of antioxidant systems and free radical scavengers are closely associated with physiological or pathological status, and oxidative stress occurs when the ROS production exceeds the antioxidant defense capacity to cope with the oxidants. Among the mechanisms to cope with the oxidative stress by the ROS production, superoxide dismutase (SOD) is a first defense mechanism against oxygen toxicity by transferring superoxide radicals to H_2O_2 (Kim and Kang, 2015). In this study, the SOD activity of liver and gill in *S. schlegelii* was significantly elevated by the dietary Pb exposure, which is a protective mechanism to minimize the oxidative damage by the dietary Pb exposure. Farombi et al. (2007) reported a significant increase in the SOD

activity of African cat fish, *Clarias gariepinus* exposed to heavy metals such as Pb, Zn, Cu, Cd, and As. A considerable induction in the SOD activity of marine dinoflagellate, *Gonyaulax polyedra* was observed by the metal exposure such as Hg, Cd, Pb, and Cu (Okamoto and Colepicolo, 1998). As a biomarker of exposure to xenobiotics, glutathione S-transferase (GST) is a critical enzyme against oxidative stress, which is involved in detoxification for various xenobiotics in phase II metabolism by conjugation with glutathione of the liver in fish (Lee et al., 2005). Basha and Rani (2003) reported a notable increase in the GST activity of tilapia, *Oreochromis mossambicus* exposed to cadmium. The GST activity of liver and gill in *S. schlegelii* was notably increased by the dietary Pb exposure, which is a defense mechanism to protect them against ROS damage. Glutathione is mainly present in cells, which acts as a defense for oxidizing molecules and potentially toxic xenobiotics like metals (Ali et al., 2004). It also functions in the proteins and DNA synthesis and signal transduction in addition to the free radical scavenging as an essential cofactor of many enzymes (Atli and Canli, 2008). A significant increase or decrease in the levels of GSH has been shown in fish exposed to metals by their organ-specific responses (Sayeed et al., 2003; Ali et al., 2004). The dietary Pb exposure to *S. schlegelii* induced a substantial reduction in the GSH level of liver and gill tissues in *S. schlegelii*. Pandey et al. (2008) also reported a notable decrease in the GSH of spotted snakehead, *Channa punctate*

exposed to multiple metals. GSH forms metal complexes with various metals via its thiolate sulfur atom, and the decrease in the GSH of *C. punctate* means that the exposure can be vulnerable to the experimental fish. Ahmad et al. (2005) also reported a substantial decrease in the GSH levels of European eel, *Anguilla anguilla* L. exposed to Cu, which was associated with the elevated use of GSH to protect them from its oxidative stress by preventing the redox cycling and free radicals regeneration.

As a major antioxidant agent, AsA inhibits the chain reactions of the metal-induced free radicals or scavenges the free radicals before they reach the cell targets in the specific organs (Antunes et al., 2000). Padayatty et al. (2003) suggest AsA can be an effective method to reduce ROS such as O_2^-, H_2O_2, OH^-, and singlet oxygen. The AsA supplementation prevents the autooxidation chain reaction, which is associated with a mechanism to ameliorate the toxic effects of oxygen radicals (Sayeed et al., 2003). In this study, the levels of AsA supplementation to *S. schlegelii* significantly attenuated the antioxidant responses such as SOD activity, GST activity, and GSH level by the dietary Pb exposure. Similarly, it was observed that the lead exposed workers showed the higher SOD activity, and the vitamin C and E supplementation was effective to reduce the increased SOD activity in the lead exposed workers, which was accompanied by the reduction in total antioxidant capacity by the vitamins supplementation (Rendon-Ramirez et al., 2014).

Monteiro et al. (2009) suggested an antioxidant such as selenium was effective to decrease in the elevated SOD and GST activity by the organophosphate insecticide exposure in matrinxa, *Brycon cephalus*. Donpunha et al. (2011) reported the Cd exposure to mice induced the depleted GSH by inhibiting SH-dependent enzymes, and the AsA supplementation attenuated the reduction by the Cd exposure, which is connected to the ability of AsA to restrain the formation of oxidants and maintain GSH and redox balance.

4.5. Inhibition of AChE activity

The oxidative damage by the high dose and chronic lead exposure was closely connected to the neurotoxicity in vertebrates, and the toxic effects on central nervous system such as behavioral abnormalities and impaired cognitive functions have been considered as a major lead toxicity (Hsu and Guo, 2002). The lead exposure induces the neural alterations of fish such as locomotor activity patterns and sensorimotor responses (Rice et al., 2011). Among various neurotransmitters, acetylcholinesterase (AChE) is one of the most important substances to catalyze the breakdown of acetylcholine and other choline esters for the maintenance of the proper ACh level (Richetti et al., 2011). Generally, toxicants inhibit the AChE activity in aquatic animals as neurotoxicity that induces immoderate ACh accumulation, nerve

system disruption, and even death, and many authors reported the inhibition of AChE activity in aquatic animals by the metal exposure (Tsangaris et al., 2007; Attig et al., 2010). Jebali et al. (2006) also reported a substantial inhibition of AChE in the amberjack, *Seriola dumerilli* exposed to cadmium. In this study, the dietary Pb exposure to *S. schlegelii* induced a significant inhibition of AChE. The AsA supplementation was effective to alleviate the AChE inhibition by the dietary Pb exposure, which may due to the reduced neurotoxicity by the lower oxidative damage.

4.6. Specific gene expression

Metallothionein (MT) has been considered as a sensitive and reliable biomarker in aquatic organisms for heavy metal contamination due to its important function to maintain proper redox potentials, and it acts as the homeostasis of essential metals and binding non-essential metals for removal (Wu et al., 1999; Atli and Canli, 2003). Olsson et al. (1996) suggested that the cadmium exposure to rainbow trout, *Oncorhynchus mykiss* caused the accumulation in kidney and liver, which induced the significant increase in metallothionein levels correlated with the cadmium accumulation. Atli and Canli (2008) observed a considerable MT increase in the liver of Nile tilapia, *Oreochromis niloticus* exposed to Cd. Yologlu and Ozmen (2015) also reported

a considerable increase in the MT levels of tadpoles, *Xenopus laevis* exposed to metal mixture, which was induced by the protective role of MT to prevent the cell damage by binding the metal ions. Considering the metal exposure stimulates MT synthesis (Atli and Canli, 2003), a notable MT gene expression of *S. schlegelii* was induced by the dietary Pb exposure. Given that the AsA reduces intestinal absorption of lead by increasing the availability of iron for competing with lead (Patrick, 2006), the AsA supplementation to *S. schlegelii* also notably alleviated the MT gene expression by the dietary Pb exposure.

The lead accumulation in aquatic animals causes the stress by affecting physiological systems in addition to the toxic symptoms such as cognitive dysfunction, neurological damage, and oxidative stress (Rabitto et al., 2005; Patrick, 2006). The dietary Pb exposure also induced a significant increase in heat shock protein 70 (HSP 70) of *S. schlegelii*. Rajeshkumar and Munuswamy (2011) reported a considerable expression in HSP 70 of milk fish, *Chanos chanos* in the polluted sites with heavy metals, which may be caused by a protective effect against stress-induced injury for heavy metal exposure. The levels of AsA supplementation did not affect the HSP 70 values of *S. schlegelii*, whereas the dietary Pb exposure induced a substantial expression in HSP 70. Biomarkers have been used as sensitive indicators to monitor toxic effects of pollution in marine environment. Given that toxicants generally cause stress in biota of the ecosystem, the stress responses such

as HSP 70 can be valuable and reliable indicators of cellular stress in organisms (Sherman and Goldberg, 2001).

Overall discussion

Lead exposure commonly causes lead bioaccumulation in specific tissues of aquatic animals, causing toxic effects. In the dietary lead exposure experiment, the dietary lead induced a significant accumulation in specific tissues of rockfish. The highest lead accumulation was observed in the kidney of *S. schlegelii*, and the relative accumulation of dietary lead in specific tissues was kidney > liver > spleen > intestine > gill > muscle. The high accumulation in the kidney and liver is because of the high reaction tendency of oxygen in the carboxylate group, nitrogen in the amino group, and sulfur in the mercapto group of the metallothionein protein during metabolic detoxification processes, as reported by several authors (Hogstrand and Howx, 1991; Al-Yousuf et al., 2000; Ashraf, 2005; Vutukuru et al., 2007). Generally, higher metabolic activity causes higher accumulation in tissues, because the tissues function in amino acid and energy metabolism, blood composition, and detoxification of toxicant (Reynders et al., 2006). The high accumulation in the kidney, liver, and spleen of *S. schlegelii* was induced by the high metabolic activity. In the aquatic environment, the absorption of metals in fish occurs through the gills from ionized metals in the water and via ingestion from their diets (Van Der Putte and Part, 1982). Intestinal lead accumulation in *S. schlegelii* from dietary lead exposure was higher than gill tissue accumulation, which may

be due to the dietary exposure pathway. In muscle lead accumulation in *S. schlegelii*, there was a significant correlation between increased accumulation and increased exposures. However, the muscle accumulation level was not considerable compared to other tissues, which may be due to the lower metabolic activity in the muscle. Research on bioaccumulation patterns in fish tissue from metal exposure can be used as a reliable and sensitive indicator to understand toxicological processes.

Heavy metal exposure inhibits fish growth, and growth rate is an effective indicator to evaluate metal toxicity (Forrester et al., 2003). The growth performance of *S. schlegelii* was notably inhibited by dietary lead exposure. It is widely supported that toxicant exposure induces the growth performance in aquatic animals, because the allocation of energy for growth and reproduction is transferred to tissue repair. Hematological parameters such as RBC count, hematocrit, and hemoglobin can suggest a physiological response to toxic substances (Dethloff et al., 2001), and the indicators can effectively evaluate the physiological status of fish under metal exposure stress (Vutukuru, 2003). The RBC count, hematocrit, and hemoglobin of *S. schlegelii* were significantly decreased by dietary lead exposure. Lead exposure decreases oxygen-binding capacity, resulting in cell deformation and damage through swelling (Witeska and Kosciuk, 2003). The plasma homeostasis of *S.*

schlegelii was negatively affected by dietary lead exposure, leading to significant variations to calcium, magnesium, glucose, cholesterol, total protein, GOT, and GPT.

Toxicant exposure leading to necrosis and cell degeneration in aquatic animals induces lipid peroxidation that causes free radicals to attack biological structures. ROS induced by toxic substances causes oxidative damage to the organs of aquatic animals (Taglian et al., 2004). The SOD activity of *S. schlegelii* was significantly increased by dietary lead exposure. SOD activity increases through the catalytic activity of scavenging superoxide radicals to produce H_2O_2 (Doherty et al., 2010). The GST activity of *S. schlegelii* was also considerably increased by dietary lead exposure, by the detoxification of metal-induced toxicity from dietary lead exposure. The GSH levels in *S. schlegelii* were considerably decreased by dietary lead exposure. Oxidative stress causes GSH to bind exclusively to the sulfhydryl group, which interferes with its antioxidant activity (Stohs and Bagghi, 1995; Gurer and Ercal, 2000). Significant changes in the antioxidant responses demonstrated that dietary lead exposure to *S. schlegelii* substantially affected oxidative stress. As dietary lead exposure is closely related to neurotoxicity because of its risk of CNS impairment by decreasing cognitive and neurobehavioral functions (She et al., 2009), AChE activity in *S. schlegelii* was significantly inhibited. Specific gene expression of MT and HSP-70 was stimulated by dietary lead exposure. The MT gene expression of

S. schlegelii may be due to the lead accumulation in the tissues, because MT functions as a metal-binding protein during detoxification processes (Ay et al., 1999). The HSP 70 gene expression of *S. schlegelii* was also considerably stimulated by the dietary lead exposure. Because HSP 70 in fish is induced by stress responses, it is a good indicator of environmental toxicity. The innate immune system protects aquatic animals from invading pathogens by recognizing foreign microorganisms during the initial stages of infection (Wang and Wang, 2013). IgM and lysozyme activity of *S. schlegelii* were significantly stimulated by dietary lead exposure, indicating that the lead exposure stimulates the immune responses of *S. schlegelii*.

Meanwhile, AsA supplementation resulted in a significant increase the daily growth gain, body weight gain, and specific growth rate, and decrease in feed conversion ratio of *S. schlegelii*. These results indicated that adequate exogenous AsA supplementation is required to maintain normal growth and increase feed efficiency. Optimum AsA supplementation is different for fish species, size, metabolic rate, and experimental conditions. In the present study, the optimum AsA supplementation level was over 200 mg/kg AsA. Hematological parameters demonstrate the physiological effects of dietary AsA supplementation (Sandnes et al., 1990), and can effectively indicate the physiological condition of fish including stress, metabolic abnormalities, reproductive dysfunctions, and diseases (Fazio et al., 2012). The hematological parameters such

as the RBC count, Ht value, and Hb concentration were notably increased by the dietary AsA supplementation. Many authors reported that there is a positive relation between the AsA supplementation and hematological parameters in fish, caused by hematopoiesis. AsA supplementation to *S. schlegelii* caused a considerable increase in the AChE activity, as it catalyzes the detoxification process.

The opposite results between lead exposure and AsA supplementation were observed. In hematological parameters, the lead exposure induced significant decreases in RBC count, hematocrit, and hemoglobin, whereas the AsA supplementation induced notable increases in these parameters. The results indicated that the dietary Pb exposure of *S. schlegelii* induced a heme synthesis inhibition, anemia, hemo-dilution, and red blood cell fragility, whereas the AsA supplementation induced the hematopoiesis. In growth factors, the lead exposure caused a notable decrease in growth, while the AsA supplementation induced a significant increase in growth. The results demonstrated that the dietary Pb exposure induced the allocation of energy for growth to the use to compensate tissue repair, whereas the AsA supplementation is required to maintain normal growth in addition to the increase in feed efficiency. In AChE activity, the lead exposure caused a notable inhibition in AChE, while the AsA supplementation induced a significant increase in AChE. The results indicated the dietary Pb exposure is closely associated

with neurotoxicity due to its risk for impairment in the central nervous system, while the AsA supplementation is effective to increase AChE activity due to its neurotransmitter synthesis function.

The most critical point in the present study was to assess AsA detoxification on the toxicity caused by dietary lead exposure. AsA supplementation has many protective functions and detoxification processes resulting from metal exposure. In the present study, the high AsA supplementation to *S. schlegelii* significantly alleviated lead accumulation in the tissues from dietary lead exposure. The effect of AsA on the inhibition of lead absorption has been clarified by various studies; it enhances the availability of iron by decreasing ferric iron to ferrous iron in the duodenum, which competes with lead for intestinal absorption (Patrick, 2006). As there has only been limited research on the effects of AsA on heavy metal bioaccumulation in fishes, these results present a standard in AsA levels inhibit lead accumulation from dietary lead exposure. The high levels of AsA supplementation in *S. schlegelii* effectively attenuated the growth inhibition from dietary lead exposure through detoxification. The AsA supplementation also effectively attenuated alterations in the hematological parameters and the plasma components from dietary lead exposure. Many authors reported the protective effects of AsA on the hematological parameters and blood components by the metal exposure (Mekkawy et al., 2011; Hounkpatin et al.,

2012). The AsA supplementation decreases the glycated insulin concentration in circulation, which alleviates insulin resistance leading to insulin-stimulated glucose uptake by peripheral tissues (Abdel-Wahab et al., 2002). As a critical antioxidant agent, AsA inhibits the chain reactions of the metal-induced free radicals or scavenges the free radicals before they reach the cell targets in the specific tissues (Antunes et al., 2000), and effectively reduces ROS such as O_2^-, H_2O_2, OH^-, and singlet oxygen. In the present study, the high levels of the AsA supplementation to *S. schlegelii* significantly alleviated alterations in the antioxidant responses including SOD activity, GST activity, and GSH level from dietary lead exposure. AChE inhibition was also alleviated by high AsA levels, which may be due to the reduced neurotoxicity from reduced oxidative damage. The high levels of the AsA supplementation were effectively attenuated an increase MT, but did not affect the HSP 70 value of *S. schlegelii*. Considering the MT is closely related to the metal-binding protein for detoxification, the effect of the AsA supplementation on the MT gene is due to the lower lead accumulation in the tissues of *S. schlegelii*.

The comprehensive results in the present study indicated that dietary lead exposure to *S. schlegelii* induced significant alterations in various factors indicating extensive toxicity, and the high levels of AsA supplementation effectively attenuated these toxic responses.

The results of the present study provide a valuable and novel approach to assess the positive effects of AsA on heavy metal bioaccumulation in fishes.

Although there are many difficulties, an integrated study regarding the combined effects should be considered. Moreover, additional research using other metals, physiological active substances, and fish species should be conducted in the future.

Summary

Chapter I: Juvenile rockfish *Sebastes schlegelii* (mean length 14.2±1.9 cm, and mean weight 57.3±5.2 g) were exposed for 4 weeks with the different levels of dietary lead (Pb^{2+}) at 0, 30, 60, 120 and 240mg/L. The highest Pb accumulation was observed in the kidney tissue by the dietary lead exposure. The growth rate and hepatosomatic index were considerably inhibited over 120 mg/kg. The hematological parameters such as red blood cell (RBC) counts, hematocrit (Ht) value, and hemoglobin (Hb) concentration were significantly decreased over 60 mg/kg Pb concentration. In the inorganic components, the values of calcium and magnesium in plasma were significantly decreased. The glucose and cholesterol values were notably increased, whereas total protein was decreased. The enzyme components, glutamic oxalate transaminase (GOT) and glutamic pyruvate transaminase (GPT), were significantly elevated by the dietary lead exposure, but no change was observed in alkaline phosphatase (ALP). In antioxidant responses, The superoxide dismutase (SOD) and glutathione S-transferase (GST) activity of liver and gill were significantly increased in response to the high concentrations of dietary Pb exposure, whereas a considerable decrease was observed in the glutathione (GSH) levels of liver and gill. In neurotoxicity, AChE activity was markedly inhibited in brain and muscle tissues by dietary Pb exposure. Metallothionein (MT) gene

in liver was significant stimulated by the exposure to dietary Pb. The results indicate that dietary Pb exposure can induce significant alterations in antioxidant responses, AChE activity and MT gene expression. The heat shock protein 70 of *S. schlegelii* was also notably elevated over 60 mg/kg for 4 weeks. In the immune response, the immunoglobulin M of *S. schlegelii* was considerably increased over 120 mg/kg for 4 weeks. A significant increase was observed in lysozyme activity. The plasma lysozyme activity of *S. schlegelii* was elevated over 120 mg/kg after 2 weeks and 60 mg/kg after 4 weeks, and kidney lysozyme activity was also increased at 240 mg/kg after 2 weeks and over 120 mg/kg after 4 weeks.

Chapter II: Juvenile rockfish *Sebastes schlegelii* (mean length 13.6±1.4 cm, and mean weight 53.6±4.2g) were fed twice daily with diets containing varing levels of ascorbic acid (0, 50, 100, 200, and 400 mg/kg) for 4 weeks. Significant increases in daily weight and length occurred in fish fed greater than 50 mg/kg ascorbic acid. The major hematological findings were significant increase in the red blood cell count, hematocrit value, and hemoglobin level in *S. schlegelii* fed diets over 100 mg/kg ascorbic acid supplement. The dietary ascorbic acid supplement caused a considerable increase in the glucose and total protein, whereas a notable decrease was observed in GOT and GPT. However, there was no alteration in calcium and magnesium.

Acetylcholinesterase (AChE) was measured to evaluate the effects of the dietary ascorbic acid supplement on neurotransmission. The AChE activity of fish fed diets containing ascorbic acid was considerably increased over 200 mg/kg ascorbic acid.

Chapter III: Juvenile rockfish *Sebastes schlegelii* (mean length 11.3±1.2 cm, and mean weight 32.5±4.1 g) were exposed for 4 weeks with the different levels of dietary lead (Pb^{2+}) at 0, 120 and 240 mg/L and ascorbic acids (AsA) at 100, 200 and 400 mg/L. The exposure concentration and period of Pb induced significant amount of it the specific tissues of *S. schlegelii*, and the levels of ascorbic acids affected the decrease in the accumulation in the specific tissues. The highest Pb accumulation was observed in the kidney tissue by the dietary Pb exposure. The high AsA supplementation caused the reduced Pb accumulation in the tissues. The growth performance of *S. schlegelii* was considerably decreased. In hematological parameters, the RBC count, hematocrit, and hemoglobin were significantly increased, and the notable alterations in plasma components such as calcium, magnesium, glucose, cholesterol, GOT, and GPT were observed. But, there was no change in total protein and ALP. The dietary Pb exposure affected the hematological parameters and growth performance of *S. schlegelii*, and the high concentration of AsA supplementation was substantially effective to attenuate the toxic effects of the Pb

exposure. The SOD activity, GST activity, and GSH level of liver and gill were assessed as oxidative stress indicators against the dietary Pb exposure depending on AsA supplementation. The SOD and GST activity of liver and gill were considerably elevated by the dietary Pb exposure. In contrast, the GSH level of liver and gill was significantly reduced by the Pb exposure. The high levels of AsA supplementation attenuated the increase in the SOD and GST activity and decrease in the GSH level. In neurotoxicity, AChE activity was substantially inhibited in brain and muscle tissues by the Pb exposure. The AsA supplementation also affected the attenuation of the AChE inhibition by the Pb exposure. Metallothionein (MT) gene in liver was notably stimulated by the Pb exposure, and the AsA supplementation attenuated the increase in MT gene expression. The HSP 70 was significantly increased by the dietary Pb exposure, but there was no effect in the HSP 70 according to the ascorbic acids.

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부족한 저의 박사 논문이 좀 더 나은 논문으로 거듭날 수 있게, 지도 편달해주신 김기홍 교수님, 김도형 교수님, 김현우 교수님, 황운기 선임연구사님, 강주찬 지도교수님께 진심으로 감사의 인사를 드립니다. 부족한 부분을 말씀해 주시고, 박사 논문은 평생 함께 따라간다는 진심어린 조언으로 부족함을 채울 수 있는 기회를 주셔서 감사합니다. 바쁘신 와중에도 기꺼이 논문 심사에 참가해주셔서 깊은 감사를 드립니다.

대학원 과정 많은 가르침을 주신 정현도 교수님, 정준기 교수님, 김기홍 교수님, 허민도 교수님, 김도형 교수님께 깊은 감사의 마음을 드립니다. 연구 과제를 수행하며, 많은 가르침과 도움을 주신 황운기 선배님과 구자근 선배님에게 깊은 감사를 드립니다. 그리고 부족한 제게 항상 역할 모델이 되어주시는 지정훈 선배님께도 감사의 인사드립니다. 멀리서도 조언을 아끼지 않으시는 김상규 선배님과 이영주 선배님에게도 감사의 마음을 전합니다. 실험실 생활을 하며, 항상 곁에서 조언과 힘이 되어준 민은영 선배님에게 감사의 마음을 전하며, 이제 곧 실험실에서 박사과정을 본격적으로 수행할 박희주 선배님께도 건승하시길 바라는 마음을 전합니다. 언제나 바쁜 일정에도 채집과 실험을 함께한 신후, 인기, 경욱, 보미에게도 동료로써 고마운 마음을 전하며, 수질 분석과 실험실

에서 최선을 다하는 태준, 재민, 정훈, 혁찬이에게도 고마운 마음을 전합니다.

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