Thesis for the Degree of Master of Science

Combined effects of water temperature and arsenic on hematological parameters, antioxidants and heat shock protein of starry flounder, *Platichthys stellatus*.

by

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강도다리의 혈액, 항산화 효소 및 열 충격 단백질에 미치는 비소와 온도의 복합적 영향

Advisor: Prof. Ju-Chan Kang

by

Jae-Min Han

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요　약

비소 노출과 온도차이에 의한 독성 평가를 위해, 비소 0, 150, 300, 600㎍/L의 농도와 12℃와 18℃온도로 4주 간 강도다리, *Platichthys stellatus* (전장15.9 ± 0.4 cm, 체중 62.2 ± 4.2 g)를 노출시켰다. 비소 노출에 따른 생체 축적은 간과 신장에서 유사하게 높은 축적이 나타났다. 성장인자는 600㎍/L이상에서 현저한 감소가 나타났다.

혈액학적성상은 전반적으로 감소하는 경향을 보였으며, RBC count, Hematocrit, Hemoglobin은 600㎍/L에서 유의한 감소가 있었으며, Ht와 Hb는 18℃가 약간 더 감소 하였다. 혈장 무기 성분인 calcium (Ca)와 magnesium (Mg)의 농도는 유의적인 변화가 나타나지 않았다. 혈장 유기 성분인 total protein은 4주차 600㎍/L농도의 18℃에서 뚜렷한 감소를 나타냈고, glucose는 600㎍/L에서 뚜렷한 증가가 나타났다. 혈장 효소 성분인 GOT, GPT는 4주차 600㎍/L농도에서 상당한 증가를 나타냈다.

항산화 효소는 간과 아가미를 이용하여 측정하였고, SOD는 2주차 600㎍/L농도의 18℃와 4주차 600㎍/L에서 상당히 증가 했으며 18℃가 12℃보다 값이 높았다. CAT는 또한 2주차 600㎍/L농도의 18℃와 4주차 600㎍/L농도에서 상당히 증가 했으나 온도에 대한 관계는 나타나지 않았다. GST는 비소의 농도에 따라 증가를 하였고 600㎍/L농도에서 유의하게 증가했다. 반대로 GSH는 600㎍/L농도에서 뚜렷한 감소가 나타났으며, 18℃가 더 감소 했다.

스트레스 지표인 열 충격 단백질은 간과 아기미를 이용하여 측정했고, HSP 70은 2주차의 600㎍/L농도와 4주차의 300㎍/L 농도이상에서 유의한 증가를 보였으며, HSP 90도 2주차의 600㎍/L농도와 4주차의 300㎍/L농도이상에서 유의한 증가를 보였고 12℃와 비교하여18℃에서 그 값이 조금 더 높았다.

결론적으로, 비소의 노출에 의해 생체축적을 유발하였으며, 혈액학적 성상, 항산화 반응 및 열 충격 단백질 반응에 변화를 나타내었다. 적정 사육 온도내인 12℃와 18℃ 경우는 차이가 있지만 그 큰 차이가 아닌 것으로 보아 온도 그 자체의 영향보다는 높은 온도에서의 호흡률과 신진대사율의 증가로 인한 스트레스가 영향을 미친 것으로 보인다.

I. Introduction

Arsenic, a metalloid element, is a useful factor in human life. It used in commercial applications, such as metal smelters and chemical manufacturing, also used in agriculture, like herbicide and pesticide. These anthropogenic activities spread arsenic to the aquatic environment (Schlenk et al., 1997). In addition, arsenic is released into the aquatic system not only by anthropogenic activities but also by geothermal activity and leaching of rocks (Singh and Banerjee, 2008). For this reason, arsenic is a ubiquitous in the aquatic environment. However, arsenic is a potentially toxic trace element (Canivet et al., 2001). Due to the indiscriminate use of arsenic, arsenic is widespread in the aquatic environment, and has led to high concentrations of As in aquatic creatures. The arsenic released is a concern because aquatic organisms are biologically available and result in toxic effects (Pedlar et al., 2002). A high concentration of arsenic is fatal for most organisms, and also chronic intake of low concentration of arsenic are associated with increased risk of cancers, damage to organ, diabetes and cardiovascular disease (Bears et al., 2006). In the aquatic environment, arsenic is present in arsenite (As^{3+}) or arsenate (As^{5+}) form, and the arsenite (As^{3+}) is considered more toxic than the arsenate (As^{5+}) (Liao et al., 2003). The reason is that arsenite (As^{3+}) in the cell binds to the protein thiol group due to its high affinity for thiol-contatining molecules such as GSH and cysteine whereas arsenate (As^{5+}) competitively interferes with the phosphorylation by the similarity of structure and nature with phosphates (Kim and Kang, 2015).

The influence of arsenic toxicity on the aquatic environment is concerned with physicochemical characteristics of water such as temperature, pH, salinity, and hardness. Among the many characteristics, water temperature has many effects on life activities of fish such as reproduction, and osmotic pressure control (Min and Kang, 2014). In addition, metabolic activity and growth of fish depends on the surrounding water temperature because they are poikilothermic animals. And water temperature also influences respiration because it changes oxygen supply according to its change (Besson et al., 2016). Therefore, changes in water temperature make it difficult to adapt to the environment, and will affect direct stress on survival (Chang et al., 2001).

In addition, bioaccumulation due to heavy metals released into ecosystems is also associated with toxicity to many species (Pourang, 1995), and bioaccumulation of heavy metals is widely used to evaluate the health of aquatic ecosystems because it can alter the physiological activities and biochemical parameters in tissues (Vinodhini and Narayanan, 2008).

Growth factor represents a variety of responses by environmental and physiological variables that affect fish. These variables include toxicity, temperature, and stress etc, and several variables, not just one of these variables, have a complex impact on growth (Beyers et al., 1999).

Physiological responses of the toxicant can help predict important sublethal effects using analyses of biochemistry,

hematology, and histopathology. Kavitha et al. (2010) reported that the hematological induces such as blood parameter, inorganic substances, organic substances, and enzyme activity are used to assess the toxic stress. Also Manik et al. (2013) mentioned that blood parameters are used as indicators of metals in the aquatic environment and are considered to be important in diagnosing the structural functional status of fish exposed to toxic substances.

The toxic appearance of arsenic is due to the reactive oxygen species (ROS) caused by the imbalance between pro-oxidants and antioxidant homeostasis, and ROS have been shown to damage many cellular components such as DNA, proteins and lipids (Samuel et al., 2005). To the risk of ROS, cells contain a number of antioxidants to maintain normal ROS. Antioxidants include superoxide dismutase (SOD), catalase acticity (CAT), Reduced glutathione (GSH), and Glutathione S-transferase (GST), which are the main mechanism of ROS defense in fish such as mammals (Kim and Kang, 2015). SOD catalyzes the conversion of reactive superoxide anion to hydrogen peroxide (H_2O_2), and CAT detoxifies hydrogen peroxide (H_2O_2) with oxygen (O_2) and water (H_2O). GSH decreases both lipid hydro peroxides and hydrogen peroxide (H_2O_2), and GST is associated with the combination and elimination of xenobiotics (Kim et al., 2015). Therefore, the antioxidant analysis such as SOD, CAT, GSH, and GST can be regarded as indicators of arsenic toxicity.

Exposure of biological and abiotic stressors may cause general physiological changes in the fish. These changes increase the stress hormones concentration of the neuroendocrine system and can induce a family of proteins known as heat shock proteins (HSP) (Basu et al., 2001). HSPs are a group of proteins that are expressed in response to a wide range of stressors and are also called stress proteins. In general, HSP is a protein expressed by heat and cold shock, but the expression of HSP is affected by various fish cells and tissues, in response to heat and cold shock as well as abiotic stressors such as environmental contaminants (Iwama et al., 1998).

Starry flounder, *P. stellatus*, is a major commercial fishery on the coast of North America. It lives mainly in the East Sea in Korea and widely in the entire North Pacific Ocean. Starry flounder is found mainly in the coastal area, and is a euryhaline fish that is observed in freshwater and brackish water zone (Lim et al., 2007). It is a varieties suitable for diversification and competitiveness of aquaculture species as a substitute for flounder which occupies most of the marine aquaculture industry in Korea (Oh et al., 2009) In addition, starry flounder is resistant to low water temperature and is appropriate for the environment in Korea where frequent inflow of fresh water is frequent. The optimal temperature of the starry flounder is 13 ~ 18℃, so starry flounder does not intake food at high water temperatures above 20℃, and its resistance becomes weak. However, because starry flounder is strong against low temperature, it can feed and grow at 5℃. Since the late 1990s, starry flounder has been studied in terms of pollution and toxicity, but there are no many studies on starry flounder (National Institute of Fisheries Science, 2009).

Although 18℃ enters the optimum water temperature, it is considered to be a somewhat higher temperature in cold water

species such as flounder, and information on the physiological changes due to pollutant exposure is needed. Therefore, the purpose of this study was to assess the effect of arsenic on the accumulation, growth rate, blood parameters, antioxidants and heat shock proteins of the starry flounder at minimum temperature (12℃) and maximum temperature (18℃) within the optimal temperature range of starry flounder.

II. Materials and Methods

2.1. Experimental fish and conditions

Juvenile *P. stellatus* were obtained from a local fish farm in Gijang, Korea. The fish were domesticated to adapt to the laboratory environment for two weeks. For temperature acclimation, the temperature was set at two sections (12℃, 18℃) and the temperature was maintained using Electronic thermostats (MS701-H, Mink, Korea). The water temperature control was also used with an electronic thermostats, temperature was raised by 1℃ per day to reach a final temperature of 12℃ and 18℃. During the acclimation period, the fish were fed 3% of body weight once per two days and water was changed every day. After acclimatization, 96 fishes (body length, 15.9 ± 0.4 cm; bodyweight, 62.2 ± 4.2 g) were randomly selected for the experiment.

The arsenic experiment was performed with waterborne, and the exposure solution was sodium arsenite (Sigma, St. Louis, MO, USA). Waterborne As exposure took place in 40 L aquaria containing 12 fish per treatment group. The concentrations of arsenic were divided into 0, 150, 300 and 600μg per L using sodium arsenite solution diluted in distilled water. The aquaria totally changed the water once every two days and kept the same concentration in each aquaria. The total exposure period was 4 weeks, and sampling was performed at 2 weeks and 4 weeks. The fishes were anesthetized with buffered 3-aminobenzoic acid ethyl ester methane sulfonate (Sigma Chemical, St. Louis, MO) and sampled the internal organs and blood.

2.2. Bioaccumulation

The tissue samples of liver, kidney, spleen and gill of *P. stellatus* were performed with freeze-dried to mea-sure dry weight of the samples. The freeze-drying samples were digested by wet digestion method. The dried samples were digested in 65% (v/v) HNO3, and re-dried at 120◦C. 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 um membrane filter (Advantecmfs, Ins.) under pressure for analysis. For determination of total arsenic 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 arsenic concentrations were determined by external calibration. ICP multi-element standard solution VI (Merck) was used for standard curve. The arsenic bioaccumulation in tissue samples was expressed㎍/g dry wt.

2.3. Growth performance

The weight and length of the starry flounder were measured immediately before the start of the experiment and at 2 and 4 weeks after the start of the experiment. Daily length gain, daily weight gain, condition factor, and feed efficiency were calculated. These values were calculated using the following formula.

Daily length gain = (final length weight - initial length weight) / day

Daily weight gain = (final length weight - initial length weight) / day

Condition factor (%) = [weight (g) / length3 (cm)]x100

Feed efficiency = live weight gain / dry feed given

2.4. Hematological analysis

Blood samples were collected from the caudal vein of fish using a heparinized disposable syringe to prevent clotting. The total red blood cell (RBC) count, hematocrit (Ht), and hemoglobin (Hb) were analyzed immediately after blood collection. The RBC counts were counted using an optical microscope with a hemo-cytometer (Improved Neubauer, Germany) after 400 times dilution with Hendrick dilution solution.

The Ht values were obtained by collecting blood from microhaematocrit capillary tubes and centrifuging at 12,000 rpm for 5 minutes at 4℃ in microhematocrit centrifugation (Model; 01501, HAWKSLEY AND SONS Ltd., England). Then, Ht values were measured using a reader (Micro-Haematocrit reader, HAWKSLEY AND SONS Ltd., England).

The Hb concentrations were measured by the Cyan-methemoglobin technique using a clinical kit (Asan Pharm. Co., Ltd.).

2.5. Serum

The collected blood was centrifuged at 3,000g for 5 minutes at 4℃ to separate the serum. The separated serum samples were analyzed for changes in inorganic substances, organic substances and enzyme activity using clinical kit (Asan Pharm. Co.,Ltd.).

The inorganic substances assay included calcium and magnesium. Calcium was analyzed by the o-cresolphthalein-complexon technique and magnesium was analyzed by the xylidyl blue technique.

The organic substances assay included glucose and total protein. Glucose was analyzed by GOD/POD technique and total protein was analyzed by biuret technique.

The enzyme activity assay included glutamic oxalate transaminase (GOT) and glutamic pyruvate transaminase (GPT). GOT and GPT were analyzed by Kind-king technique using clinic al kit.

2.6. Antioxidant analysis

To analysis antioxidant responses, liver and gill were washed with PBS buffer (0.1M PBS, pH 7.4) and excised and then homogenized with ice-cold homogenization buffer 10 times volume using Teflon-glass homogenizer (099CK4424, Glass-Col, Germany). This homogenate was centrifuged at 10,000g for 30 minutes at 4℃ and the obtained supernatant was used for the experiment. All supernatants were stored at -80℃ (MUF-U53V, SANYO Electric Co. Ltd., Japan) until analysis.

Superoxide dismutase (SOD) activity was assayed using a SOD assay kit (Dojindo Molecular Technologies, Inc.) at a 50% inhibitor ratio for WST-1 reduction. Each sample was diluted 5 times (25, 125, 625 times) in 0.1 M PBS and the concentration corresponding to the 50% inhibitor rate was confirmed. One unit of SOD is indicated by the amount of enzyme contained in 20 μL of the sample solution that inhibits the reduction reaction of WST-1 and superoxide anions by 50%. SOD activity was measured at 450 nm and expressed as unit/ mg protein.

The catalase (CAT) activity was assayed using the OxiSelect TM Catalase Assay Kit (Cell biolabs, Inc.). The Catalase in the sample decomposes H2O2 into water (H2O) and oxygen (O2), and the remaining H2O2 without decomposition reacts with the reagent. This means that if the CAT activity is strong, the color reaction is weak, while if the CAT activity is low, the color reaction is strong. CAT activity was measured at 520 nm using a spectrophotometer and expressed as unit / mg protein.

Reduced glutathione (GSH) was analyzed with reference to the method of Beutler et al. (1963). The reagents used are DTNB solution, Phosphate solution and Precipitation solution. After mixing the sample and the reagent, the mixture was developed in a dark place for 15 minutes and measured at 412 nm using a spectrophotometer. After mixing the sample and the reagent, the mixture was developed in a dark place for 15 minutes and measured at 412 nm using a spectrophotometer. The units were expressed as nmol GSH / mg protein and measured with reduced glutathione standard curve using L-Glutathione reduced, Minimum 98% (Sigma Aldrich Co., Korea).

Glutathione-S-transferase(GST) activity was assayed according to the method of modified Habig et al. (1974). 0.2 M phosphate buffer (pH 6.5), 10Mm GSH, distilled water, smaple and 10mM CDNB were mixed and the mixture was mixed in order. The change in absorbance was measured at room temperature for 5 minutes at an interval of 30 seconds using a spectrophotometer at 450 nm. The section with the highest change in absorbance was used and expressed in nmol / min / mg protein.

2.7. Heat shock protein analysis

To analysis heat shock protein, liver was washed with PBS buffer (0.1M PBS, pH 7.4) and excised and then homogenized with ice-cold homogenization buffer 10 times volume using Teflon-glass homogenizer (099CK4424, Glass-Col, Germany). This homogenate was centrifuged at 10,000g for 30 minutes at 4℃ and the obtained supernatant was used for the experiment. All supernatants were stored at -80℃ (MUF-U53V, SANYO Electric Co. Ltd., Japan) until analysis. Heat shock protein 70 & 90 were assayed using Heat Shock Protein 70 & 90 ELISA Kit (MyBioSource, Inc.) The ELISA kit is based on the principle of antigen-antibody binding. The antibody of Heat Shock Protein 70 originated from Perca flavescens and the antibody of Heat Shock Protein 90 originated from Dicentrarchus labrax. Add the standard solution, sample, and sample diluent to the microelisa stripplates embedded in the kit. Add the microelisa stripplate sample that is contained in the kit, add HRP conjugate reagent and incubate for 1 hour. After 1 hour, it is wash and then add Chromogen Solution and develop color in the dark for 15 minutes. After 15 minutes, the stop solution is added to stop the reaction. HSP 70 & 90 were measured at 450nm using a spectrophotometer and expressed as pg/ml.

III. Results

3.1. Bioaccumulation

Arsenic accumulations in liver, gill, spleen and kidney of *P. stellatus* were demonstrated in figure 1. The As accumulation value in the liver after 2 weeks was significantly increase from the concentration of 300μg/L. The accumulation value of 600μg/L increased more than the value of 300μg/L and the value of 18℃ was higher than the value of 12℃. At 4 weeks, there was a significant increase from the concentration of 150μg/L at 18℃, and regardless of temperature, it increased in earnest at the concentration of 300μg/L and was higher at the concentration of 600μg/L. In the gill after 2 weeks and 4 weeks, there were also considerably increase from the concentration of 300μg/L. The accumulation value of 600μg/L increased more than the value of 300μg/L and the value of 18℃ was higher than the value of 12℃. The As accumulation values in spleen at both 2 weeks and 4 weeks were notably increase from the concentration of 300μg/L, and the highest accumulation value was indicated at the concentration of 600μg/L. There was a slight difference between the two temperatures, but it was not a notable difference. In the kidney, the substantial increase was observed from the concentration of 300μg/L at all period. At 2 weeks, the accumulation value of 600μg/L increased more than the value of 300μg/L and the value of 18℃ was higher than the value of 12℃. There was a substantial difference between the two temperatures at 4 weeks, and the 18℃ value was higher.

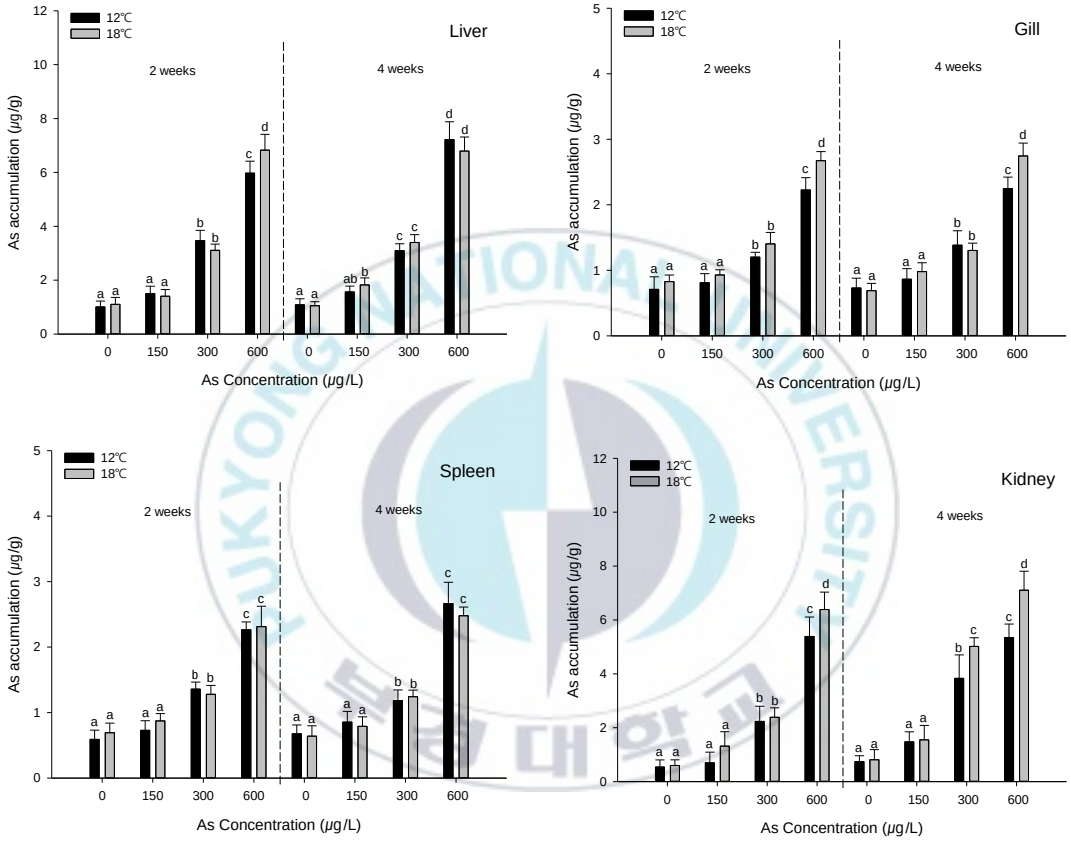

Fig. 1. As accumulation of starry flounder, *Platichthys stellatus* exposed to the different arsenic concentrations and water temperature. Values with different superscript are significantly different in 2 and 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

3.2. Growth Performance

The growth rates of *P. stellatus* are demonstrated in figure 2. The daily length gain was considerably decreased at the concentration of 600㎍/L at 12℃ after 2 weeks and at the concentration of 600㎍/L at 12℃ and 18℃ after 4 weeks. In daily weight gain, it was observed to the totally same tendency as the result of the daily length gain. A significant decline in condition factor was indicated at the concentration of 600㎍/L of all temperature and all period. The feed efficiency was notably declined at the concentration of 600㎍/L at 12℃ after 2 weeks and at the concentration of 600㎍/L at 12℃ and 18℃ after 4 weeks.

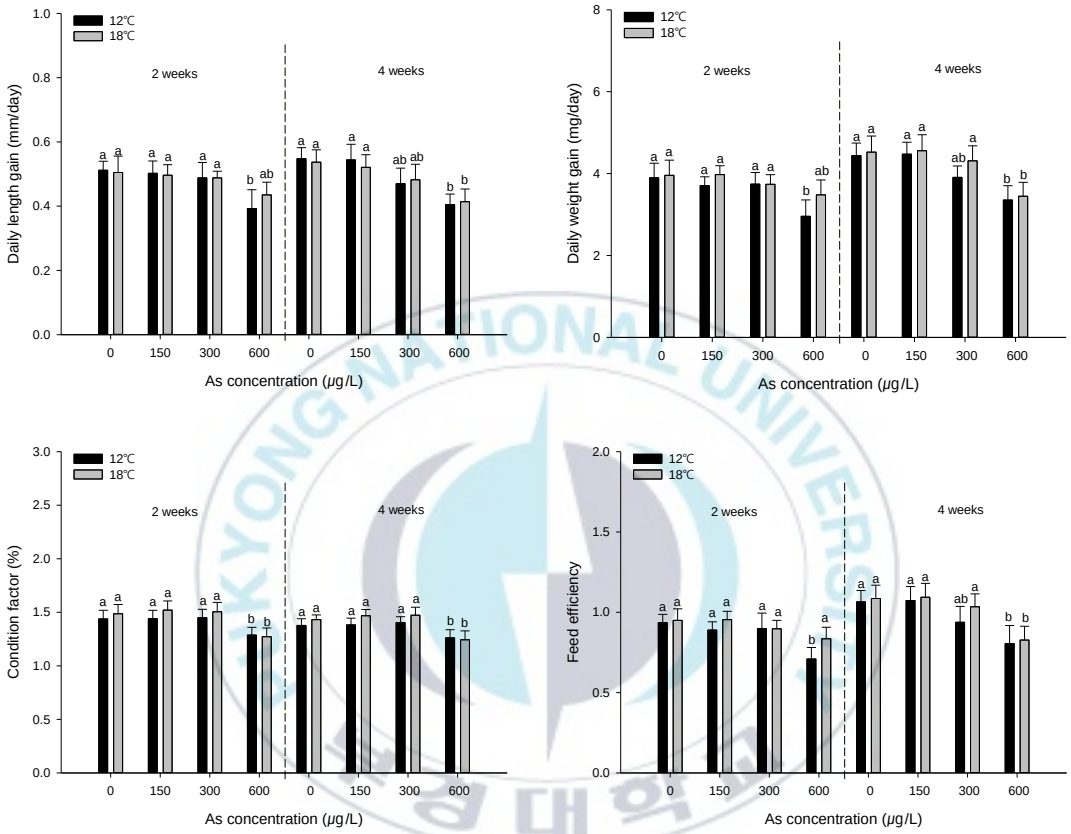

Fig. 2. Daily length gain, daily weight gain, condition factor, and feed efficiency of starry flounder, *Platichthys stellatus* exposed to the different arsenic concentrations and water temperature. Values with different superscript are significantly different in 2 and 4 weeks (P<0.05) as determined by Duncan's multiple range test.

3.3. Hematological parameters

Change in RBC counts, hematocrit (Ht) and hemoglobin (Hb) concentrations of *P. stellatus* are demonstrated in figure 3. The RBC counts were significantly decreased at the concentration of 600㎍/L at 18℃ after 2 weeks, and significantly decreased at the concentration of 600㎍/L at 12℃ and 18℃ after 4 weeks. The Ht was significantly decreased at the concentration of 600㎍/L at 18℃ after both 2 and 4 weeks. The Hb was a noticeable decline at the concentration of 600㎍/L at 18℃ after 2 weeks, and was a noticeable decline at the concentration of over 300㎍/L at 12℃ and 18℃ after 4 weeks.

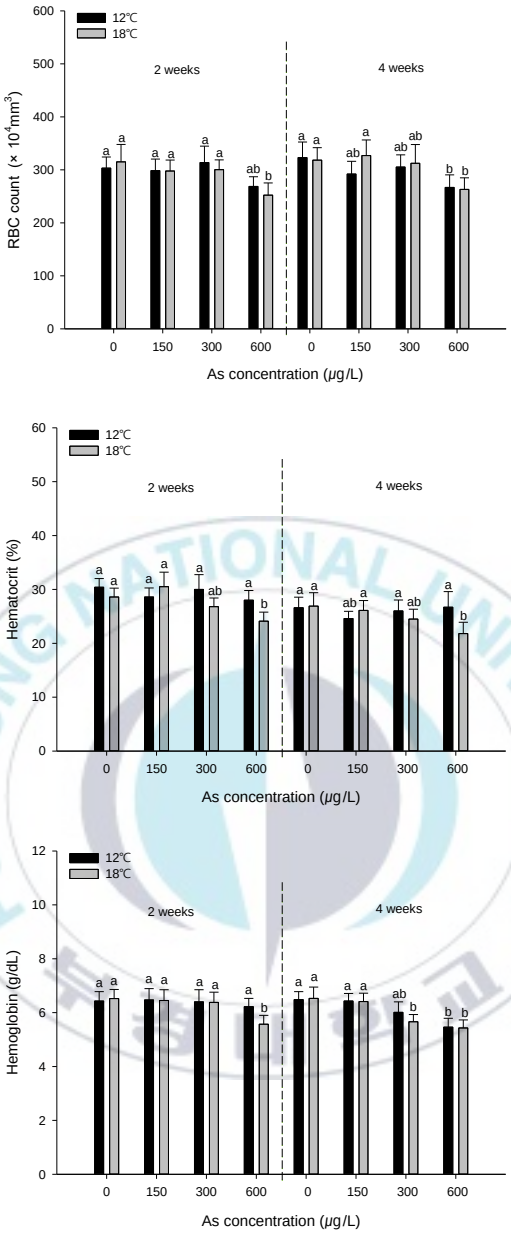

Fig. 3. Changes of RBC count, Hematocrit, and Hemoglobin in starry flounder, *Platichthys stellatus* exposed to the different arsenic concentrations and water temperature. Values with different superscript are significantly different in 2 and 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

3.4. Serum

The serum inorganic substances of *P. stellatus* are demonstrated in Table 1. and analyzed for calcium and magnesium. The calcium and magnesium did not change in all of the sections. After 4 weeks, calcium and magnesium were slightly reduced with increasing arsenic concentration, but not remarkable. The serum organic substances of *P. stellatus* are demonstrated in Table 2. and analyzed for total protein and glucose. Total protein was notable decreased only at the concentration of 600μg/L at 18℃. Glucose was notable increased at the concentration of 600μg/L at both 12 ℃ and 18 ℃ after 2 weeks. At 4 weeks, there was a notable increase at the concentration of 600μg/L at 12 ℃ and a notable increase at the concentration of over 300μg/L at 18 ℃. The serum enzyme activity of *P. stellatus* are demonstrated in Table 3. and analyzed for GOT and GPT. GOT and GPT were not shown any considerably change compared with the control group of each temperature range after 2 weeks, whereas, after 4 weeks, It seems to increase overall and a considerably increase was shown at the concentration of 600μg/L.

Table. 1. Changes of serum inorganic substances in starry flounder, *Platichthys stellatus* exposed to the different sodium arsenate concentration and water temperature.

Parameters	Period (weeks)	Temperature (℃)	Arsenic concentration (μg/L)			
			0	150	300	600
Calcium (mg/dL)	2	12℃	9.97±1.15 [a]	10.58±1.06 [a]	10.79±0.88 [a]	10.16±1.21 [a]
		18℃	10.14±0.72 [a]	9.74±0.70 [a]	9.92±0.82 [a]	9.05±0.64 [a]
	4	12℃	8.96±0.95 [a]	9.63±0.92 [a]	10.14±0.91 [a]	9.40±0.98 [a]
		18℃	9.85±0.70 [a]	10.19±0.80 [a]	9.58±0.85 [a]	9.01±0.75 [a]
Magnesium (mg/dL)	2	12℃	2.38±0.17 [a]	2.42±0.15 [a]	2.41±0.20 [a]	2.33±0.14 [a]
		18℃	2.41±0.24 [a]	2.42±0.21 [a]	2.38±0.16 [a]	2.27±0.20 [a]
	4	12℃	2.37±0.19 [a]	2.46±0.18 [a]	2.34±0.19 [a]	2.35±0.25 [a]
		18℃	2.33±0.17 [a]	2.21±0.16 [a]	2.25±0.18 [a]	2.19±0.19 [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.

Table. 2. Changes of serum organic substances in starry flounder, *Platichthys stellatus* exposed to the different sodium arsenate concentration and water temperature.

Parameters	Period (weeks)	Temperature (℃)	Arsenic concentration (μg/L)			
			0	150	300	600
Total protein (mg/ml)	2	12℃	2.59±0.16[a]	2.55±0.15[a]	2.52±0.14[a]	2.51±0.17[a]
		18℃	2.52±0.13[a]	2.47±0.14[a]	2.49±0.16[a]	2.43±0.12[a]
	4	12℃	2.54±0.18[a]	2.58±0.23[a]	2.54±0.17[a]	2.48±0.12[a]
		18℃	2.55±0.16[a]	2.41±0.15[ab]	2.36±0.19[ab]	2.14±0.22[b]
Glucose (mg/ml)	2	12℃	92.71±7.80[a]	95.49±8.60[a]	96.36±7.59[a]	111.31±5.61[b]
		18℃	90.16±8.57[a]	92.78±10.14[a]	96.66±5.58[a]	113.46±6.52[b]
	4	12℃	88.58±5.79[a]	92.69±8.46[a]	96.47±8.27[a]	114.11±9.16[b]
		18℃	90.63±8.09[a]	102.62±10.14[ab]	113.11±9.97[b]	116.14±9.00[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.

Table. 3. Changes of serum enzyme activity in starry flounder, *Platichthys stellatus* exposed to the different sodium arsenate concentration and water temperature.

Parameters	Period (weeks)	Temperature (℃)	Arsenic concentration (μg/L)			
			0	150	300	600
GOT (karmen/ml)	2	12℃	23.28±2.59[a]	22.58±1.76[a]	24.27±2.13[a]	25.51±2.69[a]
		18℃	23.03±2.28[a]	23.66±1.44[a]	25.14±1.79[a]	24.64±1.59[a]
	4	12℃	23.28±1.96[a]	23.37±2.12[a]	24.66±1.52[a]	27.04±2.39[ab]
		18℃	23.22±1.53[a]	23.36±1.72[a]	26.96±2.22[a]	28.73±2.26[b]
GPT (karmen/ml)	2	12℃	12.39±1.35[a]	15.50±1.93[a]	16.65±1.82[a]	15.60±0.94[a]
		18℃	16.20±1.33[a]	16.00±0.65[a]	16.56±0.87[a]	17.45±1.49[a]
	4	12℃	16.25±1.56[a]	16.94±2.02[a]	19.54±1.81[ab]	18.99±1.49[ab]
		18℃	16.41±1.87[a]	17.15±1.89[a]	19.81±1.90[ab]	21.07±2.27[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.

3.5. Antioxidant

Antioxidant responses in liver and gill of *P. stellatus* are shown in figure 4-5. Superoxide dismutase(SOD) activity showed a significant increase after 4 weeks compared with 2 weeks in the liver and gill. After 2 weeks, SOD activities were increased only at the concentration of 600μg/L at 18℃ in liver, whereas after 4 weeks, both 12℃ and 18℃ increased at the concentration of over 300μg/L, and at the concentration of 600μg/L at 18℃, a larger increase was observed. SOD activities of the gills showed a similar tendency to SOD activities of the liver, a high increase was observed at the concentration of above 300μg/L at 18℃ after 4 weeks. Catalase(CAT) activity confirmed analogous to change of SOD activity. After 2 weeks, CAT activities were considerably increased in liver and gill at only the concentration of 600μg/L at 18 ℃, and considerably increased in each organ at the concentration of 600μg/L at all temperature after 4 weeks. For reduced glutathione(GSH), there were notably declined in liver at the concentration of above 300μg/L at 18℃ after 2 weeks, and after 4 weeks, there were notably declined at the concentration of 600μg/L at all temperature. Furthermore, the GSH of the gills were notably declined at the concentration of 600μg/L at 18℃ after 2 weeks and 4 weeks. Glutathione-S-transferase(GST) of liver showed a substantially increased at the concentration of 600μg/L at 18℃ after 2 weeks and at all temperature after 4 weeks. As with the GST of liver, the GST of the gills were also substantially increased at the concentration of 600μg/L at 18℃ after 2 weeks and at all temperature after 4 weeks.

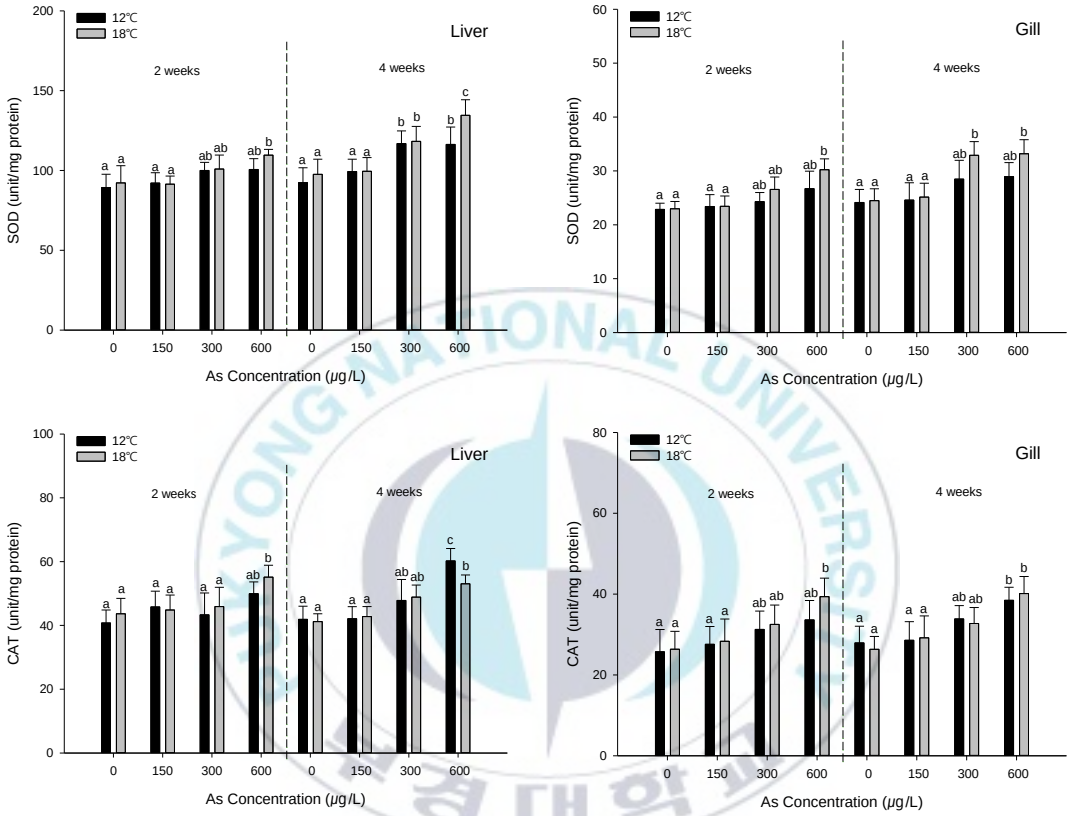

Fig. 4. Antioxidant system analysis (SOD and CAT) of starry flounder, *Platichthys stellatus* exposed to the different arsenic concentrations and water temperature. Values with different superscript are significantly different in 2 and 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

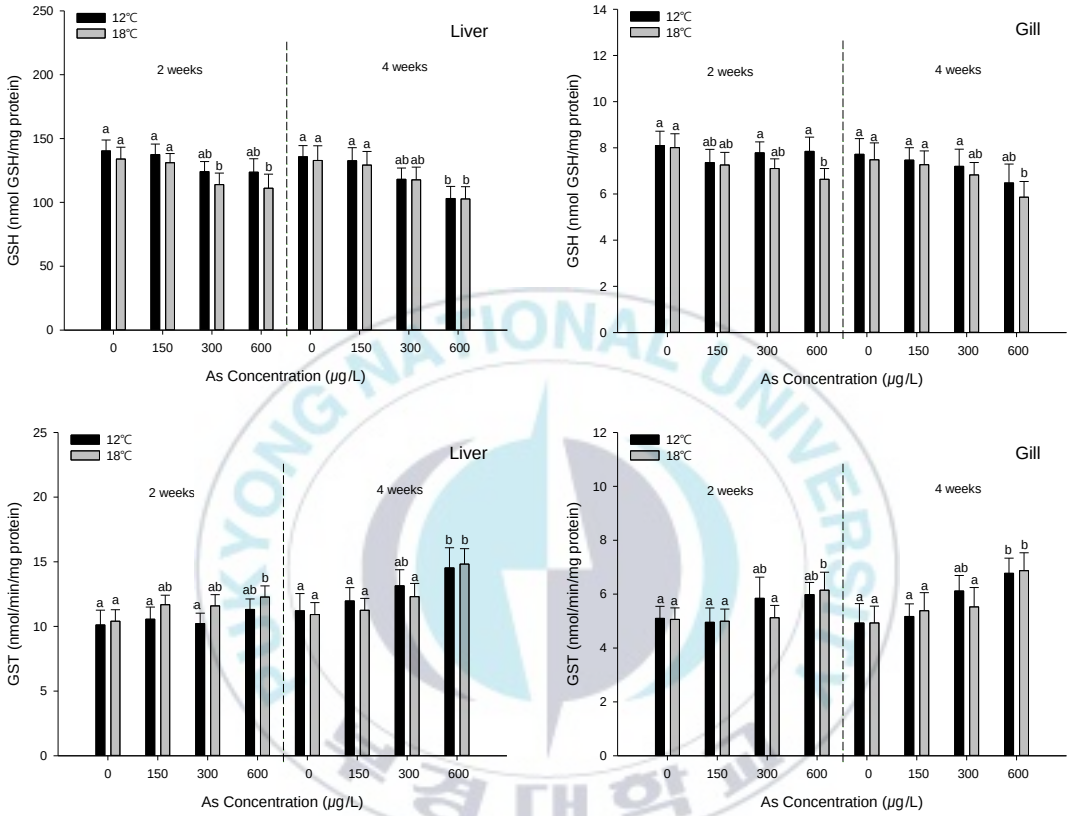

Fig. 5. Antioxidant system analysis (GSH and GST) of starry flounder, *Platichthys stellatus* exposed to the different arsenic concentrations and water temperature. Values with different superscript are significantly different in 2 and 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

3.6. Heat shock protein

Heat shock protein in liver and gill of *P. stellatus* are shown in figure 5. Analysis of heat shock protein was performed with heat shock protein (HSP 70) and heat shock protein (HSP 90). HSP 70 in the liver was significantly increased at concentration of 600μg/L at 18℃ after 2 weeks. After 4 weeks, HSP 70 in the liver was significantly increased at concentration of 300μg/L at 18℃ and at concentration of 600μg/L at 12℃ and 18℃. HSP 70 in the gill was significantly increased at concentration of 600μg/L at 18℃ after 2 weeks. After 4 weeks, HSP 70 in the gill was significantly increased at concentration of over 150μg/L at 18℃. HSP 90 in the liver was considerably increased at concentration of 600μg/L at 18℃ after 2 weeks and at concentration over 300μg/L at 18℃ after 4 weeks. HSP 90 in the gill showed very similar results to HSP 90 in liver, and a considerable increase was observed at the concentration of 600μg/L at 12℃ after 4 weeks.

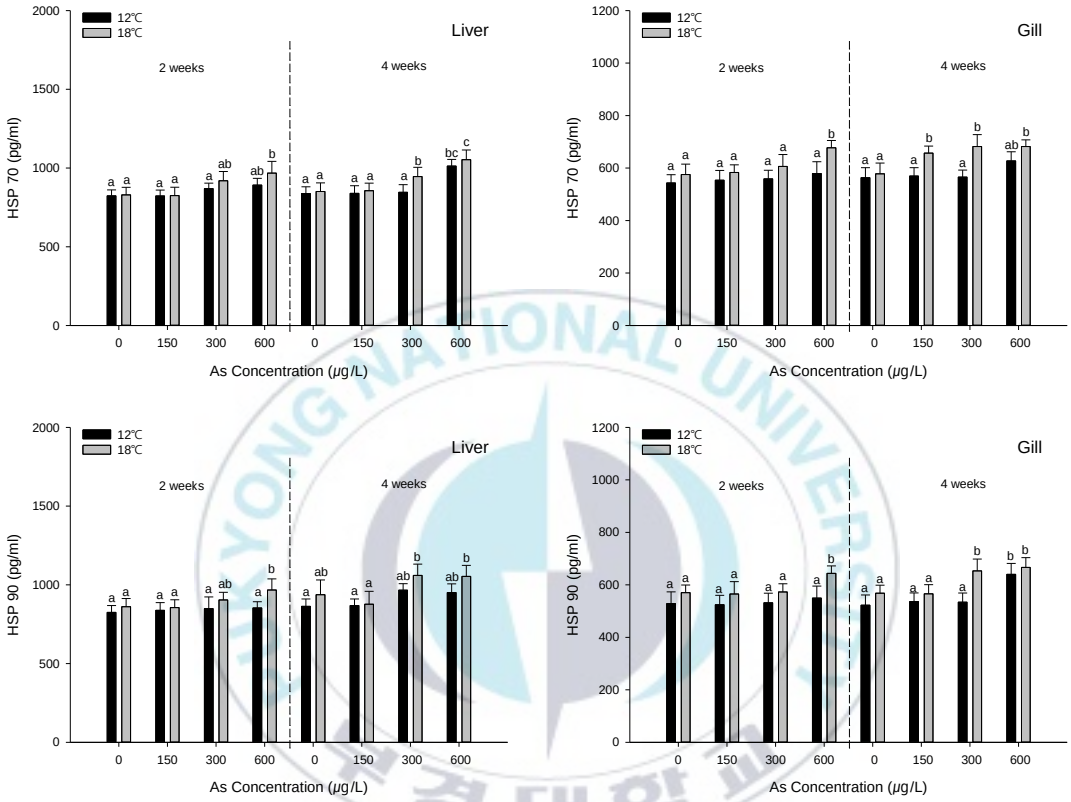

Fig. 6. Heat shock protein (HSP 70 and HSP 90) of starry flounder, *Platichthys stellatus* exposed to the different arsenic concentrations and water temperature. Values with different superscript are significantly different in 2 and 4 weeks ($P<0.05$) as determined by Duncan's multiple range test.

IV. Discussion

Metallic materials that enter the aquatic environment accumulate in aquatic animal tissue. Aquatic animals metabolize to release this metal substances, but they can be toxic if not removed during metabolism (Farombi et al., 2007). The accumulation of metals such as arsenic affects a variety of physiological systems, including fish growth, reproduction, immune function and enzyme activity (Datta et al., 2009). Changes in water temperature are able to affect fish metabolism, and water temperatures outside the appropriate temperature range have a detrimental effect on fish (Bagnyukova et al., 2007). In particular, rising water temperature accelerates oxygen consumption and metabolic rate, and can cause stress and immunity degradation (Lushchak and Tetyana, 2006). In this study, physiological and biochemical changes of starry flounder were analyzed by arsenic and temperature.

Toxic substances such as heavy metals or metalloids in the aquatic environment may enter into the fish body from the body surface, gill and digestive tract and accumulate. During this accumulation process, fish gills and digestive tract are damaged. After accumulation, pathological anomalies have been shown frequently to occur in the liver and kidneys, resulting in tremendous stress (Amundsen et al., 1997). In this study, high As accumulation was observed in liver and kidney tissues. Abdel-Baki et al. (2011) reported that heavy metals accumulated in the liver at the highest concentrations, and accumulate at high concentrations in the kidneys. Kim and Kang (2015) also reported that the highest As accumulation was observed in the liver tissue. The reason for the high content of heavy metals in liver compared to other organs is that it acts as a primary organ for storage and detoxification (Malik et al., 2010). The kidneys play an important role in the excretion of trace metal ions, so it also usually have to a high accumulation of metal toxicity such as arsenic (Akan et al., 2012). The higher the heavy metal concentration, the more influence on the detoxification mechanism of the kidneys, thereby delaying metal removal and increasing the accumulation (Gupta and Srivastava, 2006). In this study, As accumulation was also observed in gills and spleen. The gill is the first organ that passes through the body when heavy metal enters the body through respiration. For this reason, a large amount of metal is adsorbed on the gill surface, and the possibility of metal accumulation may be relatively high (Eneji et al., 2011). In addition, the gills have quite thin epithelium, and metals can pass through thin epithelium and accumulate. Therefore, it is suitable as a target organ showing the index of pollution (Farombi et al., 2007). Liang et al. (1999) reported that accumulation of Arsenic was confirmed in spleen with liver and kidney and Ribeiro et al.(2005) reported that spleen in fish is involved in the immune system with the production and replacement of blood cells, resulting in the accumulation of metals.

Growth is an expression of dietary intake, such as energy metabolism. When fishes are exposed to toxicity, feed intake

rate and growth rate decreases (Farkas et al., 2002). In addition, the toxic effects of heavy metals in fish can reduce the metabolic rate of fish and consequently reduce growth (Hayat et al., 2007). The growth factors in this study showed a decrease at the highest concentration of the experimental concentrations and most of the high temperature values were higher than the low temperature values, but it was not significant. In most cases there is a negative relationship between heavy metal concentrations and fish weights (Woodward et al., 1994). According to a study by Hussain et al. (2010), the chronic and high concentrations of heavy metal toxicity are associated with a decrease in growth and mortality.

The hematological characteristics of fish are used to monitor environmental pollution in aquatic ecosystems, and arsenic can lead to changes in hematologic characteristics (Kavitha et al., 2010). Haematological parameters such as RBC, WBC, Ht and Hb are often used to assess health status of fish (Carvalho and Femandes, 2006). In this study, hematologic parameters such as RBC counts, hematocrit (Ht) and hemoglobin (Hb) tended to decrease overall. The striking decrease in RBC count was observed at the highest of concentration arsenic regardless of temperature. In the case of Ht, there was change at 12℃ and 18℃ high concentration section. Hemoglobin was significantly decreased at the highest concentration of arsenic and high temperature. Arsenic exposure affects blood cells and lymphocytes because arsenic toxicity is associated with bone marrow damage (Ferrario et al., 2008). Such hematopoietic tissue damage may result in insufficient erythropoiesis and low concentration of hematocrit and hemoglobin. In addition, arsenic-induced anemia due to hemolysis of intravascular erythrocytes may also occur (Cockell et al., 1991).

The serum inorganic substances, calcium and magnesium were slightly decreased at high concentration after 4 weeks, but there was no significant decrease. Serum calcium is maintained at a certain level and related to various enzymatic actions. When exposed to metallic substances, plasma calcium concentration decreases in a short period of time, but gradually recover to a certain level over time (Pratap et al 1989). Calcium in this experiment was not significant but decreased overall. Therefore, the serum calcium level decreased in the short term and finally recovered. Magnesium, a serum inorganic substances, is also presumed to be a mechanism like calcium.

The serum organic substances, total protein was a notable decrease only at high concentration after 4 weeks, but glucose increased with rising concentrations of arsenic in all periods and noticeably increased at higher concentrations. Total protein is a biological parameter important for understanding health status and metabolism by toxic stress. Decreased plasma protein can be a cause of protein synthesis disorder and appears to be the result of arsenic accumulation in the liver. In addition, arsenic is also accumulated in the kidney, which affects glucose concentration changes (Lavanya et al., 2011). The formation of metal complexes changes the metabolism of cells and affects carbohydrate metabolism such as glucose, glycogen, and lactate. Among these, glucose is frequently used as an indicator of environmental stress. Elevated blood glucose levels may be due to gluconeogenesis to fulfill increased metabolic demands by arsenic (Kavitha et al., 2010)

Liver function tests have been used as an index of liver function changes to arsenic exposure, and plasma enzyme (GOT, GPT) analysis is one of the liver function tests (Abdel-Hameid, 2009). In this study, the serum enzyme activity such as GOT and GPT showed a considerable increase at high concentration after 4 weeks irrespective of temperature. Abdel-Hameid. (2009) reported substantial increases in GOT and GPT of Nile Catfish, Clarias gariepinus exposed to arsenic, and elevated levels of these parameters may reflect liver damage due to arsenic toxicity This means exposure to metal toxicity, such as arsenic, can lead to elevated plasma enzymes as a whole, and significant increases in high concentrations of arsenic suggest that liver regeneration may proceed to restore GOT and GPT levels when exposure to low concentrations of arsenic (Roy and Shelley, 2006).

Exposure of metal toxic substances such as arsenic increases the formation of ROS and is associated with protein oxidative damage resulting in structural changes and functional inactivation of proteins (Samuel et al., 2005). In addition, heat stress due to temperature causes a physiological disorder and has a direct effect on metabolism (Verlecar et al., 2007), especially at high temperature, fish activity rises and higher metabolic rates, so the rate of ROS formation increases as a result of increased cell respiration (Abele and Puntarulo, 2004). Increased ROS formation causes oxidative stress if it exceeds the acceptable range (Kim and Kang, 2015), and oxidative stress caused by ROS greatly affects the organ of aerobic organisms, including fish (Martinez-Álvarez et al., 2005). Fish liver, which is the organ used in the experiment, plays an important role in the ingestion, accumulation and excretion of toxic substances, and for this reason is the main target organ of arsenic (Kavitha et al., 2010). Another organ, gill is a tissue in direct contact with water and serves as a respiratory organ of fish. Since the gill is a thin epithelium, it absorbs not only oxygen but also metal ions, and metal substances can accumulate (Farombi et al., 2007). The defense system to prevent the effects of ROS is found in aquatic animals such as fish and is called antioxidant activity (Aruljothi and Samipillai, 2014). ROS can be detoxified by an enzyme defense system including the activity of superoxide dismutase (SOD), catalase, reduced glutathione (GSH) and glutathione S-transferase (GST) (Figueiredo-Fernandes et al., 2006). Therefore, this experiment showed antioxidative effects on the liver and gills of fish by the ammonia exposure depending on water temperature.

Superoxide dismutase (SOD) is one of the most important antioxidant enzymes and decomposes superoxide anion into hydrogen peroxide and oxygen molecule (Kim and Kang, 2015). In this study, the SOD activities of liver and gill in *P. stellatus* were significantly increased at high concentration of arsenic and high temperature. Excess H2O2 was accumulated in the tissue during arsenic exposure, and SOD was found to decode H2O2 (Bhattacharya et al., 2007). Bagnyukova et al. (2007) suggested that SOD activity on arsenic exposure of goldfish was significantly increased at longer exposure times. Abele et al. (1998) measured the production of ROS by temperature experiments on Nacella concinna and reported that activation of the antioxidant system was evident at elevated temperatures. Besides, additional evidence for antioxidant

reactions at high temperatures was provided by SOD and CAT analyzes.

The catalase (CAT) activities of liver and gill in *P. stellatus* increased considerably at high concentration of arsenic and high temperature, similar to SOD activities. CAT is another antioxidant enzyme that acts to decompose hydrogen peroxide into water and oxygen molecules (Adeyemi et al., 2015). This means CAT is an effective inhibitor when H2O2 accumulates in tissues. And it is present in the peroxisome of almost all living cells (Arulijothi and Samipillai, 2014). In the study by Waheed et al. (2013) among the antioxidants, CAT has the highest activity against exposure to arsenic in fish. And arsenic, known as an antioxidant inducer, reported that it acts as an exogenous factor that increases the activity of antioxidants. According to experiments by Vinagre et al. (2012), the CAT activity of seabass was increased about 7 times in the range of temperature higher than the optimal temperature, which means that thermal stress is generated at high temperature and CAT activity is very sensitive to thermal stress.

Glutathione (GSH) is an antioxidant that prevents cell damage from ROS. GSH is converted to glutathione disulfide (GSSG) by glutathione peroxidase in the metabolic process, but this GSSG circulates back to the reduced glutathione by the glutathione reductase (Roy and Bhattacharya, 2006) In general, when the intracellular oxidative stress increases, the concentration of GSSG increases, which may be thought to be due to the oxidation of GSH. This means that GSH is reduced by oxidative stress (Adeyemi et al., 2015). The principle of GSH analysis is to measure the absorbance of '2-Nitro-5thiobenzoic acid' produced by the reaction of DTNB (5,5'-dithiobis-2-nitrobenzoic acid) with GSH, and in this study the GSH of liver and gill in *P. stellatus* was notably decreased at high concentration of arsenic and high temperature. Bhattacharya and Bhattacharya (2007) reported that arsenic exposure to catfish promotes GPx activity, leading to an increase in GSSG and a decrease in GHS. And the metabolites of arsenic can act as potent inhibitors of GR activity (Styblo et al., 2001).

GSH has another function that it can be excreted in combination with xenobiotics. This combination of GSH and toxic substances is possible by the action of the glutathione S-transferases (GST) enzyme. Thus, GST is mainly involved in the detoxification of xenobiotics, and when exposed to xenobiotics, the gene is overexpressed (Rhee et al., 2007). In other words, when the oxidative stress reactants are elevated, the defense system in the body is activated and the GST is increased, and as a result, the GSH is reduced by the combining reaction (Figueiredo-Fernandes et al., 2006). In this study, the GST of liver and gill in *P. stellatus* were substantially increased at high concentration of arsenic and high temperature. Greani et al. (2017) reported that GST is significantly induced in trout liver during chronic exposure to arsenic. Verlecar et al. (2007) suggested that heat stress produced ROS, and that GST increased activity during heat stress was activated in the gills.

Cells induce stress protein synthesis in response to a variety of chemical and physical stressors. Several heavy metal

ions are stress protein inducers, and heat shock proteins can also be induced. Likewise, arsenic also induces heat shock proteins (Ochi, 1997). In this study, the HSP70, 90 of liver in *P. stellatus* were substantially increased at high concentration of arsenic and 18 ℃. Rajeshkumar and Munuswamy (2011) reported that high expression of HSP70 was observed in fish liver in contaminated areas and that the increase of HSP70 was a protective effect against stress-induced damage. Basu et al. (2001) reported an increase in heat shock protein expression in an environment 5 to 10℃ higher than higher than normal environmental conditions. Clarke and Nadine (1990) studied the relationship between ambient temperature and metabolic rate, suggesting that metabolic rate increases as temperature rises. Anesitis et al. (2007) also reported that high temperatures caused stress as a result of increased metabolic rates and increased oxygen supply mechanisms to meet oxygen-dependent oxygen demand.

Increased metabolism is another stressor, and can quickly increase the synthesis of HSP (Iwama et al., 1999). Therefore, the conclusion of this study is that metabolism has a greater effect on hsp than temperature because there is a change in hsp within the optimal temperature range.

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감사의 글

가장 먼저 이 논문을 완성할 수 있도록 열정으로 지도해주시고 사랑으로 키워주신 존경하는 강주찬 지도교수님께 감사의 마음을 전해 드립니다. 복학 후 아무것도 모를 때, 학부 2학년 때 들어와 석사 졸업까지 근 5년간 교수님의 품에서 정말 많은 것들을 받으며 배우며 드디어 논문을 마무리 지어 마음이 후련하지만, 한편으로는 교수님의 품을 벗어난다고 생각하니 아쉬운 마음이 들기도합니다. 교수님과 함께한 실험실생활을 밑거름으로 사회에 나가서 훌륭한 사람이 되겠습니다.

실험실에 처음으로 왔을 때 수권환경학실험실에 먼저 오라고 권유해주시고, 실험실에서 정말 많은 가르침과 도움을 주신 김준환선배님께 감사를 드리고, 실험실에서 아버지같이 가장 많을 일을 하시고 바쁜 와중에도 실험실원들을 돌보시고 고생하신 든든한 박희주선배님께 깊은 감사를 드립니다. 홀로 실험실 석사를 할 때, 석사로 들어와 실험뿐만 아니라 심적으로 의지할 수 있는 멋진 친구가 되어준 혜동 형과 내가 실수하거나 모자란 부분이 있을 때 바로 잡아주고 지금은 기장에서 열심히 실험실일을 하고 있는 혁찬이에게도 고마운 마음을 전합니다. 그리고 늦게 들어와 누구보다 열심히 석사생활을 하고 있는 태준 형과 수연 누나에게 실험실에서는 낮은 자세로 열심히 배우고 밖에서는 인생선배로써의 모습을 보여주어 너무 감사하고 존경의 마음을 전합니다. 이제 석사과정을 밟는 영빈, 상목, 희수는 열심히 하되 즐거운 마음으로 임하고 행복한 석사과정이 되길 바랍니다. 학부생인 재호, 현지, 지은이에게 부족한 나를 너무너무 잘 따라 주어서 고맙고, 많은 것을 나누지 못 해 아쉽고 미안하게 생각합니다.

석사와 같이 학교생활을 마무리하며, 내가 학교생활을 잘 할 수 있게 옆에서 날 지켜주며, 도와주고 같이 고생했던 석사 동기인 경식이와 항상 내편이 되어주고 잘 할 수 있도록 뒤에서 많이 지지 해준 고마운 기태, 학생회를 하며 많이 가르쳐야 했던 나에게 오히려 더 가르침을 주고 좋은 동생이 되어 주었던 재민, 종욱, 현재, 현주, 현지(지수) 외 14학번 후배들에게 무한한 감사를 드립니다. 그리고 내가 정말 사랑하고 보고싶은 영지와 민성이에게 정말 감사하고 미안하다는 마음을 전합니다. 마지막으로 너무나 존경스럽고 따라가고 싶은 나의 롤 모델인 아버지와 따뜻한 배려와 모나지 않게 저를 키워주신 어머니에게 너무나 감사하고 은혜로 보답하고 싶습니다. 많은 분들을 일일이 다 적지 못 한점 너그럽게 용서해주시고, 모든 사람들 항상 건강하고 행복한 일만 가득했으면 좋겠습니다.