JFMSE, 29(6), pp. 1758~1767, 2017.
수산해양교육연구, 제29권 제6호, 통권90호, 2017.
www.ksfme.or.kr
http://dx.doi.org/10.13000/JFMSE.2017.29.6.1758

Hematological Analysis and Non-specific Immune Responses of Emaciated Olive Flounder, *Paralichthys olivaceus* in Korea

Seung-Min KIM* · Lyu-Jin JUN* · Da-Won LEE* · Hyun-Kyung PARK* · Jong-Sung KIM** · Joon-Bum JEONG†

(*†Jeju National University · **Dalhousie University)

우리나라 여윔증상 넙치의 혈액학적 분석 및 비특이적 면역반응

김승민* · 전려진* · 이다원* · 박현경* · 김종성** · 정준범†

(*†제주대학교 · **댈하우지대학교)

Abstract

In this study, a research was conducted on hematological analyses and comparisons of the changes in non-specific immune response with regards to emaciated and non-emaciated fishes that are causing severe damages to olive flounder aquafarms in Jeju. First, the researcher selected a sample of raised olive flounder that exhibited typical symptoms of emaciation in three aquafarms in Jeju for experimental use, and then selected healthy-looking olive flounder from one aquafarm that had no past record of emaciation disease, and checked for possibilities of infection through PCR. Averagely, in the hematological analysis shows that haematocrit (Ht), glutamic oxaloacetic transaminase (GOT), and glucose (GLU), emaciated fish groups (Farm A, B, and C) displayed higher experimental values than control group, and in the glutamic pyruvic transaminase (GPT), total cholesterol (Tchol), and total protein (TP), exhibited lower experimental values than control group. As a result of measuring lysozyme, nitro-blue-tetrazolium (NBT) and myeloperoxidase (MPO), which are markers for non-specific immune responses, lower experimental values were observed in the three experiments. Thus, it can be concluded that a high mortality rate was exhibited due to stress and internal organ damages that resulted from the destruction of the primary defense mechanism that protects fishes from getting infected by emaciation agents.

Key words : Emaciation, Olive flounder, Hematological analyses, Non-specific immune

Ⅰ. Introduction

Fish farming in the country began to develop in the 1980's and is now being done in many areas of the coast. Among others, farming of olive flounder accounted for the largest share and was distributed widely in the marine fish farming such that its economic value has been steadily increasing. Most especially, Jeju island has been producing about 50 % of the national production of

† Corresponding author : 064-754-3426; jeongjb@jejunu.ac.kr
※ 이 논문은 2015년도 제주대학교 교원성과지원사업에 의하여 연구되었음.

olive flounder farming. According to the National Statistical Office data, 73,108,000 tons of farming production in 2013 was observed from a more than 2000% increase as compared to the 1,065,000 tons in 2003, whereas 3,155,000 tons of fishery production in 2013 increased by 26 % against the 2,487,000 tons in 2003 NFRDI (2013). However, diseases by virus, bacteria, fungi and parasites are rampant in the fishery industry, including olive flounder farming, and represent one of the biggest problems in the fish farming industry. Notably, after the year 2007, an unknown disease occurred among the olive flounder within the olive flounder (20cm in body length) cultured in Jeju island. The mortality rate of the olive flounders was steadily increasing within 1-3 weeks after some symptoms appeared. Affected fish showed darkening body color, decrease in body weight and hemorrhages on the body surface (Kim et al., 2015). According to a research by Kim et al., (2015), An experiment was carried out to compare the etiological agents, *Enteromyxum leei* in Japan and Korea. Polymerase chain reaction (PCR) experiments were conducted with the primer set of *Enteromyxum leei* (1,589 bp) to test the flatfishes of Jeju island. The results showed that the etiologic agent was not identical to that of Japan, as a negative reaction was observed. Another PCR experiment was conducted using degenerate primers from a sequence of mucus sporozoite, which is registered in the GenBank database (NCBI, USA). The DNA of olive flounders that showed symptoms of emaciation were split and used in the experiment; the results showed a positive reaction. Through DNA sequencing, it was shown that the sequence of olive flounders was identical to that of mucus sporozoite, but it was determined to be a novel species that was not yet registered in GenBank, according to Kim et al. (2015).

In most fish farms, cultured fish experience a lot of stress due to high-density farming and deteriorating water quality (Bonga, 1997). These stresses play a role in reducing the productivity of cultured fish. It has been reported that most fishes that endure these stressors experience a reduction in their productivity through changes in their internal metabolism and physiological states (Wardie, 1981). They also suffer a drop in their resistance to diseases due to a reduction in immune function (Pickering, 1992), and showed a change in internal metabolism and hematological composition (Barton and Iwama, 1991; Ryan, 1995; Park et al., 1999; Chang et al., 2006).

The hematological composition of fish is commonly utilized as a useful means to investigate the level of a fish's physiology as its constituents are altered by toxins. Moreover, the serum of fishes can be used to determine the levels of different physiological conditions and internal immune functions (Davis and Parker, 1990; Sharma et al., 1976; Munck et al., 1984).

Non-specific defenses of fishes are known to be their first line of defense against pathogenic infections. It is reported as well that their resistance to pathogens can be enhanced by the activation of their immune system (Anderson and Siwicki 1994). As such, the activities of phagocytes (J∅rgensen et al., 1993a), macrophages (Kajita et al. 1991), and lysozymes (Engstad et al., 1992; J∅rgensen et al., 1993b) in the fish are utilized as indicators for diseases, and are used as a method to observe its immune system (Chen and Ains-worth, 1992).

In this study, we performed experiments to determine indicators of emaciation in fishes through hematological analyses and differences in the non-specific immune responses between infected and

uninfected olive flounder.

Ⅱ. Materials and methods

1. Fish selection

Farming olive flounders with emaciation symptoms, such as the darkening of the body color, the emaciation of the abdomen and the loss of weight (length 19.8±2 cm, weight 70±5 g) from 3 farms in Jeju island were selected and used as the experimental group, while healthy olive flounders (length 20±3 cm, weight 90±10 g) from 1 farm without any history of emaciation disease were selected and used as the control group. All fish were packaged in oxygen-filled bags and sent to the laboratory. The blood of these fish were collected, and an MS-222 anesthetic was administered following the ethical guideline of animal testing. Following to the method presented by Kim et al. (2015), three olive flounders were tested in each experiment. The kidneys of each fish were removed, and checked by PCR using *E. leei*-specific primer sets.

2. DNA extraction

To isolate DNA from tissue samples, DNeasy® Blood & Tissue Kit (Qiagen Hiden, Germany) was used with reference to Kim et al. (2015). First, 180 μL ATL buffer and 20 μL proteinase K were added to the kidney samples, and the solution was left until completely dissolved at 56 ℃. After the reaction, 200 μL AL buffer and 200 μL ethanol (100 %) were added. The mixed solution was then placed in a spin column and centrifuged for 1 minute at a speed of 6,000 G, after which the column was placed in a new tube. The column was washed with 500 μL AW1 buffer and 500 μL AW2 buffer, and DNA was segregated by adding 50 μL AE buffer. Segregated DNA was stored in -80 ℃ until the next step.

3. PCR

The primers used for Polymersase chain reaction (PCR) were suggested by Kim et al. (2015) <Table 1>. PCR reactions contained 0.4 μM of each primer, 1 X Ex-Taq reaction buffer, 200 M of each dNTP, 0.5 U of Ex-Taq DNA polymerase and 1ul of the respective template DNA. The reaction mix was topped up to 20 μL with distilled water. The conditions for one reaction were as follows: pre-denaturation at 95 ℃ for 3 minutes, denaturation for 30 seconds, annealing at 55 ℃ for 30 seconds, and then extending for 30 seconds at 72 ℃. This reaction was repeated for 35 cycles, after which a post-extension at 72 ℃ was conducted for 7 minutes. The PCR amplification product was put in 1 × TAE buffer and electrophoresed after the addition of 0.5 μg/mL ethidium bromide (EtBr) to the 1 % agarose gel as an intercalating agent. The size of the product was then detected using a ultraviolet light.

<Table 1> PCR primers used in this study

Primer	Oligonucleotide sequences (5'-3')	Expected sizes	Reference
EM-F	CAACCGCAATGTGTTTACTC	812bp	Kim et al. (2015)
EM-R	CCAAACAACCTGCCACAATG		

4. Hematological analyses

Blood was collected from the caudal vein without anesthesia using a heparin treatment syringe, and within 30 seconds, the hematocrit (Ht) value was measured using the microhematocrit

method. The samples were centrifuged at 3,000 × G for 10 minutes at 4 ℃. And then the supernatants were collected in separate 1.5 mL microtubes for each farm, for use in hematological analyses, lysozyme, and nitro-blue-tetrazolium (NBT) assays, and to measure the activation of myeloperoxidase (MPO). All experiments were performed in triplicates, and the obtained plasma was stored in -80 ℃ until further analysis. Biochemical changes in the blood were measured by using the hematological chemistry analyzer (Express plus system, Bayer, USA). Six metrics were used, such as glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), glucose (GLU), total cholesterol (Tchol), total protein (TP), while Ht. serum lysozyme activity was modified as described by Ellis (1988) and Obach et al. (1993). Briefly, 10 μL of individual serum was mixed with 200 μL of a *Micrococcus lysodeikticus* (Sigma, USA) suspension at 0.2 mg ml-1 in 0.05 M sodium phosphate buffer (pH 6.2). The reaction was performed at 25 ℃, and the absorbance was measured at 530 nm after 1 and 6 minutes in an ELISA plate reader. One unit of lysozyme activity was defined as the amount of enzyme that produces a decrease in absorbance of 0.001 min-1 ml-1 serum. MPO activity was measured according to Quade and Roth (1997), with the modification by Kumari and Sahoo (2005). Briefly, serum (20 μL) was diluted with HBSS (Hanks balanced salt solution without Ca2+ or Mg2+, Sigma, USA) and 5mM H2O2 was added. The color change reaction was stopped after 2 minutes by adding 35 μL of 4 M sulfuric acid. Finally, the OD was read at 450 nm. The oxidative radical production by phagocytes during respiratory burst was measured by the NBT Assay (Sigma, USA) as described by Anderson and Siwicki (1995) with modifications by Kumari and Sahoo (2005). Briefly, blood and 0.2 % NBT were mixed in equal proportions (1:1), incubated for 30 minutes at room temperature, 50 μL of the mixture was dispensed into glass tubes. Then, 1 mL of dimethylformamide (Sigma, USA) was added, and the mixture was centrifuged at 2,000 × G for 5 minutes. Finally, the optical density of the supernatant was measured at 540 nm. Dimethylformamide was used as the blank.

5. Statistical analyses

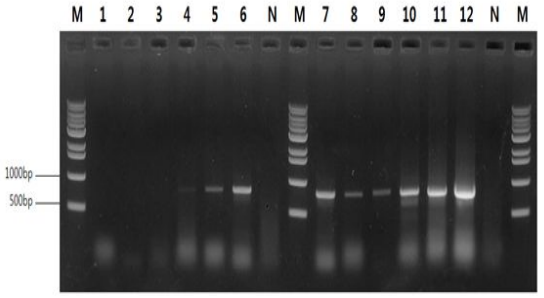
Multiple comparisons of the significant ANOVA were performed by Duncan's multiple comparisons test. A P Value < 0.05 was considered as statistically significant. All data were analyzed with the help of the statistical package program SPSS 11.0 (SPSSInc.,USA).

Ⅲ. Results

1. Diagnosis of emaciation disease

In this study, hematological analyses and measurement of non-specific immune responses for infected (with emaciation disease) and uninfected fish were performed in order to establish a diagnostic indicator for emaciation disease which is frequently occurring in the farms of Jeju island. First, we collected 9 olive flounders showing emaciation symptoms from 3 farms (3 fish from each), and 3 uninfected olive flounders (based from external appearance) from another farm. We collected their blood samples, enucleated a part of their kidneys, and checked for signs of emaciation through a PCR test. The first 3 farms were used as the experimental group because positive results were observed, whereas the latter farm was used as

the control group since the experiments came out negative [Fig. 1].

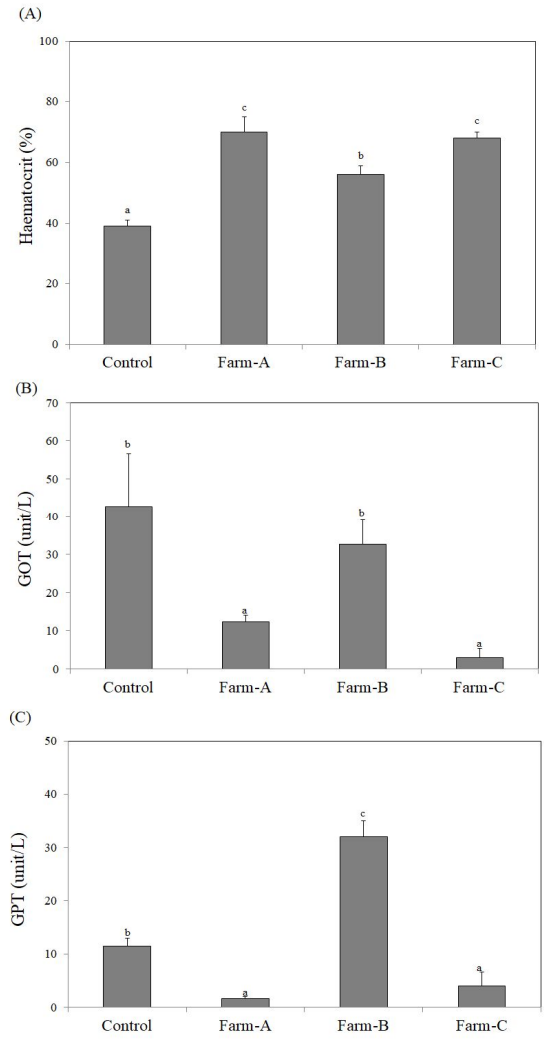


[Fig. 1] Results of PCR using EM-F/R primers on extracted kidney DNA of olive flounder. Lane1 to lane3, control; lane 4 to lane 6, Farm-A; lane 7 to lane 9, Farm-B, lane 10 to lane 12, Farm-C; M, 1 kb DNA ladder (Bioneer USA); N, negative control.

2. Hematological examination

The hematocrit (Ht) results of the study confirmed that farms A (70±5 %), B (56±3 %), and C (68±2 %) from the experimental group had higher values when compared to the control group (39±2 %), where farm A was significantly the highest [Fig. 2A]. The emaciated fish were also observed to have lower levels of GPT and GOT: GPT (control: 42.6±14 U/L,; farm-A: 12.3±1.7 U/L,; farm-B: 32.6±6.4 U/L,; farm-C: 3±2.4 U/L), GOT (control: 11.5±1.5 U/L,; farm-A: 1.6±0.4 U/L,; farm-B: 32±3 U/L,; farm-C: 4±2.6 U/L), with the exception of farm B. All levels were in the general range [Fig. 2B & 2C].

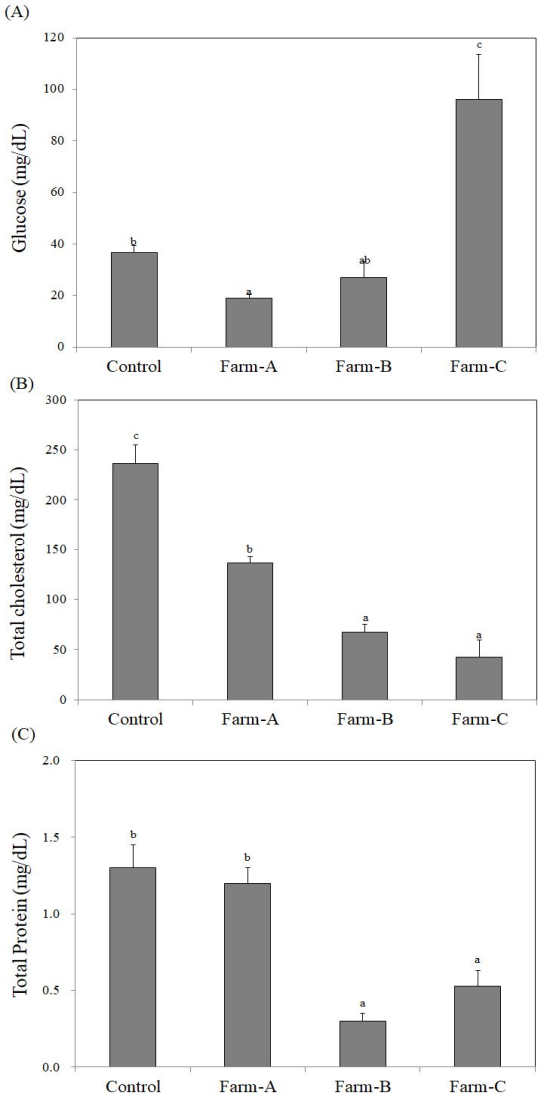
To accurately measure the concentration of the glucose, the research staff surveyed the feed supply times (form 7 a.m. to 7:30 a.m. and from 4:30 p.m. to 5:00 p.m.) ahead of time, and conducted the research sampling at 2 p.m. For the Glucose results, farms A (19±1.8 g/dL) and B (27±6.08 g/dL) had lower values than the control group



[Fig. 2] Analysis of haematocrit (A), GPT (B), GOT (C) in the serum of olive flounder (*Paralichthys olivaceus*). *The mean values in the column bearing same superscript do not vary significantly ($p<0.05$).

(36.6±2.8 g/dL), while farm C (96±17.5 g/dL) had a significantly higher value than control [Fig. 3A]. Total cholesterol was significantly lower in all experimental groups (farm A: 136±6.9 g/dL; farm B: 67.3±6.9 g/dL; farm C: 42.6±16.5 g/dL) than in control (236.6±18.4 g/dL) [Fig. 3B]. Total protein was also lower in the all experimental groups (farm

A: 1.2±0.1 g/dL; farm B: 0.3±0.05 g/dL; farm C: 0.53±0.1 g/dL) than control (1.3±0.15 g/dL), but the difference was not significant [Fig. 3C].



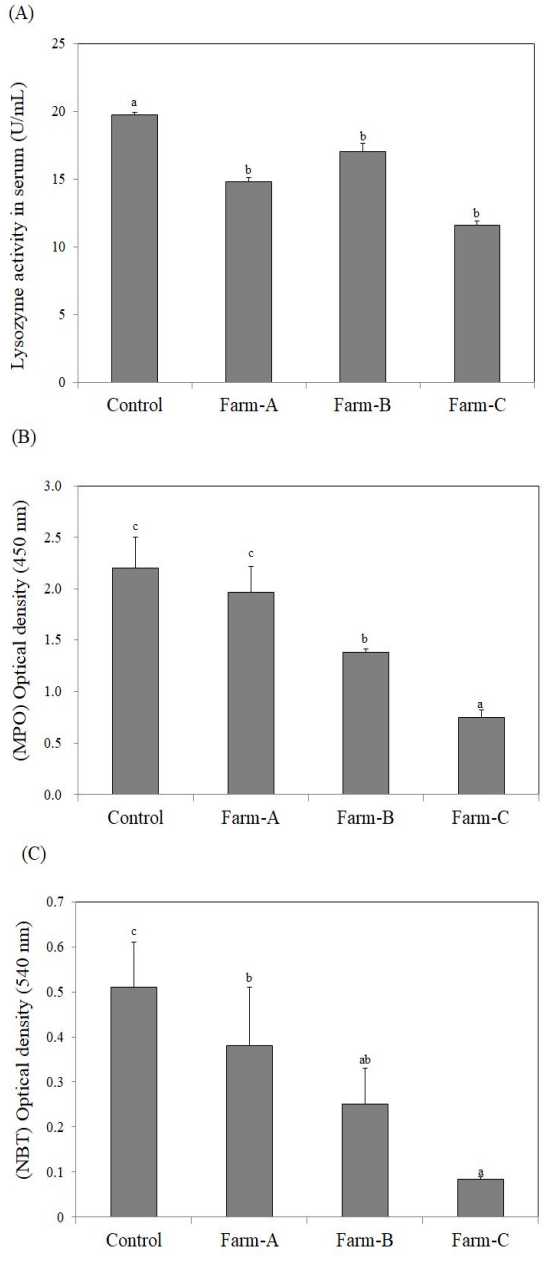
[Fig. 3] Analysis of glucose (A), total cholesterol (B), total protein (C) in the serum of olive flounder (Paralichthys *olivaceus*). *The mean values in the column bearing same superscript do not vary significantly (p<0.05).

3. Non-specific immune response

The lysozyme activity, as measured from the serum, of all experimental groups (farm A: 14.8±0.3 U/mL; farm B: 17±0.6 U/mL; farm C: 11.6±0.3 U/mL), were lower than the control group (19.8±0.2 U/mL), with farm C differing significantly [Fig. 4A]. In addition, the results for MPO activity, which acts on infection and inflammation during non-specific immune responses, showed lower values as well in all experimental groups (absorbance 450 nm, farm A: 1.97±0.25; farm B: 1.38±0.03; farm C: 0.75±0.07) when compared to the control (absorbance 450 nm, control: 2.2±0.3). Here, farms B and C were observed to have significantly lower values [Fig. 4B]. The measurement of NBT activity, which plays a role in cell-mediated immunity, revealed that all experimental groups (absorbance 540 nm, farm A: 0.38±0.13; farm B: 0.25±0.08; farm C: 0.085±0.004) had lower levels than control (absorbance 540 nm, 0.51±0.1), with farms B and C differing significantly [Fig. 4C].

Ⅳ. Discussion

In general, the variations in Ht value is used as an index of stress and physiological activity when fishes are exposed to diseases (Chang et al., 1999). In saltwater fish, it is reported that stress increases Ht levels, and results in acidification of the blood, reduction of melt (Kim et al., 2010). The results of our experiments showed that the Ht values in the experimental group is higher than the control [Fig. 2A]. This is consistent with the reports of Choi et al. (2012), and is the expected result as well since the invasion of pathogens is known to disturb the physiological activities of fish. Moreover, there was



[Fig. 4] Analysis of lysozyme activity (A), myeloperoxidase activity (B), nitroblue tetrazolium (C) in the serum of olive flounder (Paralichthys *olivaceus*). *The mean values in the column bearing same superscript do not vary significantly (p<0.05).

no other disease in the experimental group and the control group.

The reports on GPT and GOT state that disturbance in liver function can occur if the physiological conditions of the fish is not good and serum concentrations are increased (Gordon, 1968; Casillas and Ames, 1985; Rao et al. 1990). The results of our experiment showed a lower value in both GPT and GOT, except for farm B's GOT, but all of them were in the general range. A presumption was made that there is no direct correlation between the pathogen of emactiation and the liver tissue, and that it is necessary to normalize the values in order to compare between fishes. This is because GPT and GOT are basic requirements for blood tests in people and are impacted with volatility according to the environmental conditions [Fig. 2 B&C]. In addition, GPT and GOT are known to be affected by the change of environment within the farm (Changes in water temperature, low oxygen, pH, ammonia, heavy metals) (Pan et al., 2003). The results of the research showed that the variation in values of olive flounders affected by emaciation in each fish farm could be due to the differences in environment.

The stress process of fish can be divided into three stages of reactions (Wedeyer and McLeay, 1981). Glucose levels of the blood is known to induce lower immunity, this 3 rd stress reaction is the representative index of the 2 nd stress reaction, which occurs through the secreted endocrine system, and is a result of the endocrine system activity from the 1 st reaction (Wedemeyer et al., 1990). According to the result of our glucose study, the values in farms A and B were lower than the control group, but the values from farm C were higher than control [Fig. 3A] (Kwon and Jung,

2012). Fish exposed no disease shows resistance as well as various responses such as increasing breath, expansion of blood vessels, increasing erythropoiesis, and decrease in glucogenesis. In this study, low concentration levels of farm-A and farm-B are early inflammatory symptoms attributed to the resistance against diseases.

It has been reported that blood parameters of the fish are routinely used in health and stress analyses, as cholesterol changes in accordance with deficiency in essential nutrients contained in their feed (Garrido et al. 1990). According to the results from this study's cholesterol measurement, all experimental groups had lower cholesterol levels than the control [Fig. 3B]. It seemed that total cholesterol levels decreased because of a transient deficiency in cholesterol, which in turn resulted from decreased feed intake quantity due to infection from emaciation.

The concentration of the total protein in the serum can be reduced by contaminants, which can be caused by malabsorption as a result of intestinal canal loss (Mater et al., 1985; Yamawaki et al., 1986). Falling and destruction of the intestinal tissue are normally shown in fishes with emaciation disease in the country (Kim et al., 2012). The results of the total protein experiment showed lower values in all experimental groups when compared to the control. Among these, farms A and B were observed to have distinctly lower values. [Fig. 3C]. These results, as what was included in the report of Kim et al. (2015), deduce that the absorption capacity of fishes deteriorated due to loss of their gastrointestinal tract.

Lysozyme is an enzyme that is widely distributed in nature, and plays a major role in the defense mechanisms of the higher animals as it is involved in opsonic, anti-viral, bacterial, and anti-cancer activities, etc. (Jolles and Jolles. 1984). NBT increases the active oxygen by hydrolyzing invaded bacteria, viruses, and parasites (reactive oxygen species, ROS), and serves as the first defense system for external invasion as these immune cells constitute 60~70% of those involved in sterility and phagocytosis. MPO is reported to be the enzyme acting on the infectious and inflammatory responses, and kills the invading antigens that are toxic to the host (Palic et al., 2005).

The results of the lysozyme activity as measured from the serum of fish demonstrated that all experimental groups had lower values than the control [Fig. 4A]. Lower values in the experimental groups were observed as well for MPO activity [Fig. 4B]. In addition, lysozyme and MPO had the same results as those of NBT activity that plays a role in cellular immunity [Fig. 4C]. From these results, the innate immunity of fish can be resisted well by pathogens, which are known to grow and survive in the bloodstream and tissues of its host. These pathogens can resist death by macrophages, and are protected from the host by growing inside these macrophages. This first line of defense seems to be destroyed by the disease in which a disturbance in the immunological balance is caused by the penetration of the pathogens of emaciation.

In addition, the levels of non-specific immunity of each fish farm that had infections of emaciation were observed. The results showed low levels of immunity of farms A, B, and C, in decreasing orders of immunity level. These results are consistent with those from the PCR, where a bright band was observed and was therefore assumed to be a resulting value in relation to the degree of infection. However, more research will have to be conducted in order to confirm these results.

References

Anderson, D. P. & Siwicki A. K.(1994). Duration of protection against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion. The Progressive Fish-Culturist 56, 258~261.

Anderson, D. P. & Siwicki, A. K.(1995). Basic haematology and serology for fish health programs. Fish Health Section, Asian Fisheries Society, 185~202.

Barton, B. A. & Iwama, G. K.(1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annual Review of Fish Diseases 1, 3~26.

Bonga, S. E.(1997). The stress response in fish. Physiological reviews 77, 591~625.

Chang, Y. J. & Hur, J. W.(1999). Physiological responses of grey mullet (*Mugil cephalus*) and nile tilapia (*Oreochromis niloticus*) by rapid changes in salinity of rearing water. Korean Journal of Fisheries and Aquatic Sciences 32, 310~316.

Chang, C. Y. · Min, B. H. · Kim, N. N. Cho, S. H. & Chang, Y. J.(2006). Expression of HSP90, HSP70 mRNA and changes of plasma cortisol and glucose during water temperature rising in freshwater adapted back porgy, *Acanthopagrus schlegli*. Journal of Aquaculture 19, 315~322.

Choi, H. S. · Jun, L. J · Kim, S. M. · Jeong, H. D. · Kim, Y. K. · Lim, H. · Yeo, I. k. & Jeong, J. B.(2012). Clinical features of fish with pathogens isolated from emaciated olive flounder *Paralichthys oliveaceus*. Journal of fish pathology 25, 67~76.

Casillas, E. & Ames, W.(1985). Serum chemistry of diseased English sole, *Parophrys vetulus* Girard, from polluted areas of Puget Sound, Washington. Journal of Fish Diseases 8, 437~449.

Chen, D. & Ainsworth, A. J.(1992). Glucan administration potenitiates immune defense mechanism of channel catfish *Ictalurus punatatus* Rafinesque. Journal of Fish Diseases 15, 295~304.

Davis, K. B. & Parker, N. C.(1990). Physiological stress in striped bass: Effect of acclimation temperature. Aquaculture 91, 349~358.

Ellis, A. E.(1988). Fish Vaccination. London : Academic Press. 255.

Engstad, R. E, · Robertson, B. & Frivold, E.(1992). Yeast glucan induce increase in activity of lysozyme and complement-mediated haemolytic activity in Atlantic salmon blood. Fish & Shellfish Immunology 2, 287~297.

Garrido, M. A. · Perez, P. · Titus, J. A. · Valadayo, M. J. · Winkler, D. F. · Barbieri, S. A. · Wunderlich, J. R. & Segal, D. M.(1990). Targeted cytotoxic cells in human peripheral blood lymphocytes. The Journal of Immunology 144, 2891~2898.

Gordon, R. B.(1968). Distribution of transaminases (*Aminotransferases*) in the tissue of the Pacific Targeted cytotoxic cells in human peripheral salmon (*Oncorhynchus*) with emphasis on the properties and diagnostic use of glutamic oxaloacetic transaminase. Journal of the Fisheries Board of Canada 25, 1247~1268.

Jolles, P. & Jolles, J.(1984). What's new in lysozyme research? Always a model system, todays as yesterday. Molecular and cellular biochemistry 63, 165~189.

J∅rgensen, J. B. · Lunde, H. & Robertsen, B.(1993a). Peritoneal and head kidney cell response to intraperitoneally injected yeast glucan in Atlantic salmon Salmo salar. Journal of Fish Diseases 16, 313~325.

J∅rgensen, N. · Glwercman, · A & Muller, J.(1993b). Immunohistochemical markers of carcinoma of the testis also expressed in normal infantile germ cells. Histopathology 4, 373~378.

Kajita, Y. · Sakai, M. · Kobayashi, M. & Kawaushi, H.(1991). Enhancement of non-specific cytotoxic activity of leucocytes in rainbow trout *Oncorhynchus mykiss* injected with growth hormone. Fish & Shellfish Immunology 2,155~157.

Kim, J. W. · Cho, M. Y. · Park, G. H. · Won, K.M. · Choi, H. S. · Kim, M. S. & Park, M. A.(2010). Statistical data on infectious diseases of cultured olive flounder *Paralichthys olivaceus* form 2005 to 2007. Journal of fish pathology 23, 369~377.

Kim, Y. K. · Jeong, J. B. · Lee, M. K. · Park, S. I. · Park, M. A. · Choe, M. K. & Yeo, I. K.(2011). Pathophysiology of olive flounder *Paralichthys*

olivaceus suffering from emaciation. Journal of fish pathology 24, 11~18.

Kim, S. M. · Jun, L. J. · Park, M. A. · Jeong, H. D. & Jeong, J. B.(2015). Characterization of the myxosporean parasite isolated from emaciated olive flounders *Paralichthys oliveceus* on Jeju island. Korean Journal of Fisheries and Aquatic Sciences 48, 337~345.

Kumari, J. & Sahoo, P. K.(2005). Seasonal variation in the innate immune parameters of the Asian catfish *Clarias batrachus*. Aquaculture 252, 121~127.

Kwon, M. G & Jung, S. H.(2012). Comparative study of pathogenicity following single or coinfection with *Edwardsiella tarda* and *Streptococcus iniae* in olive flounder, *Paralichthys olivaceus*. Journal of Fisheries and Marine Sciences Education 24, 591~601.

Mater, K. S. · Mayer, F. L. & Witt A.(1985). Waste transformer oil and PCB toxicity to rainbow trout. Transactions of the American. Transactions of the American Fisheries Society 114, 869~886.

Munck, A. Guyre, P. M. & Holbrook, J.(1984). Physiological fucntions of glucocorticoids in stress and their relationship to pharmacological action. Endocrinol reviews 5, 5~34.

National Fisheries Research and Development Institute (NFRDI).(2013). Statistical yearbook of marine fisheries.

Obach, A. · Quentel, C. & Bandin, L. F.(1993). Effects of alpha-tocopherol and dietary oxidized fish oil on the immune response of sea bass *Dicentrarchus labrax*. Dis Aquat Org 15, 175~185.

Palic, D. · Andreasen, C. B. · Menzel, B. W. & Roth, J. A.(2005). A rapid, direct assay to measure degranulation of primary granules in neutrophils from kidney of fathead minnow (*Pimephales promelas* Rafiesque, 1820). Fish & Shellfish Immunology 19, 217~227.

Pan, C. H. · Chien, Y. H. & Hunter, B.(2003). The resistance to ammonia stress of *Panaeus monodon* Fabricius juvenile fed diets supplemented with astaxanthin. Journal of Experimental Marine Biology and Ecology 297, 107~118.

Park, M. Y, Chang, Y. J. & Kang, D. Y.(1999). Physiological response of the cultured olive flounder (*Paralichthys olivaceus*) to the sharp changes of water temperature. Aquaculture 12, 221~228.

Pickering, A. D.(1992). Rainbow trout husbandry: management of the stress response. Aquaculture 100, 125~139.

Quade, M. J. & Roth, J. A.(1997). A repid direct assay to measure degranulation of bovine neutrophil primary granules. Veterinary Immunology and Immunopathology 58, 239~248.

Rao, P. P. · Joseph, K. V. & Rao, K. J.(1990). Histopathological and biochemical change in the liver of a fresh water fish exposed to heptachlor. Journal of Nature Conservation 2, 33~137.

Ryan, S. N.(1995). The effect of chronic heat stress on cortisol levels in the Antartic fish *Pagothenia borchgrevinki* Experientia. 51, 768~774.

Sharma, K. K. · Sharma, A. L. · Dwivedi, K. K. & Sharma, P. K.(1976). Effect of raw and boiled garlic on blood cholesterol in butter fat lipidemia. Indian journal of nutrition and dietetics 13, 7~11.

Wardle, C. S.(1981). Physiological stress in captive fish. Aquaculture systems/edited by AD Hawkins 403~414.

Wedmeyer, G. & McLeay, D. J.(1981). Methods of determine the tolerance of fishes to environmental stressors in stress in fish. Academic press 247~275.

Wedmeyer, G. A. · Barton, B. A. & McLeay, D. J.(1990). Stress and acclimation. Fisheries Soc. 451~489.

Yamawaki K, Hashimoto W, Fujii K, Koyama, J. · Ikeda, Y. & Ozaki, H.(1986). Hemochemical changes in carp exposed to low cadmium concentrations. Bulletin of the Japanese Society of Scientific Fisheries 52, 459~466.

Yamawaki, K. · Hashimoto, W. · Fujii, K. · Koyama, J. · Ikeda, Y. & Ozaki, H.(1986). Hemochemical changes in carp exposed to low cadmium concentrations. Bulletin of the Japanese Society of Scientific Fisheries 52, 459~466.

• Received : 16 August, 2017
• Revised : 04 September, 2017
• Accepted : 27 October, 2017