Thesis for the Degree of Master of Science

Correlation between respiratory burst activity and hepatic health of black rockfish (*Sebastes schlegeli*)

by

ABDELLAOUI Najib

Department of Aquatic Life Medicine

The Graduate School

Pukyong National University

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조피볼락(*Sebastes schlegeli*) 호흡 폭발 활성과 간장의 건강도와의 관계

Advisor: Prof. Min Do HUH

by

ABDELLAOUI Najib

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ABDELLAOUI Najib

Approved by :

(Chairman) Professor Park Soo Il

(Member) Professor Jeong Hyun Do

(Member) Professor Min Do HUH

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Correlation between respiratory burst activity and hepatic health of black rockfish (*Sebastes schlegeli*)

Najib Abdellaoui

Department of Aquatic Life Medicine, The Graduate School,

Pukyong National University

간장의 정상적인 기능은 어류의 면역상태와 밀접한 관련이 있다. 간장의 조직학적 구조는 생리기능과 밀접한 관련이 있다. 생리학적으로 완전한 상태는 면역학적으로 완전하다고 이해되어야 한다. 이러한 점에서 기존의 원인체 중심 면역파라메타는 개념에서 재검토 되어야 한다. 간장의 조직학적 변화는 진정한 파라메타로 생각되는 것이 당연하다. 따라서 본 연구는 기존의 고전적인 면역파라메타중의 하나인 호흡폭발반응(respiratory burst)과 간장의 조직학적 건강도 사이 상관성을 조사하고자 하였다. 실험어류는 한국 남동부 해안 통영지역의 양식장에서 채집한 외관상으로 건강한 조피볼락(59±2.27g)이다. 어체는 nitroblue tetrazolium 감소 테스트를 사용하여 호흡폭발을 측정하기 위해 두신을 제외한 여러 다른 기관을 광학현미경 관찰용 표본 제작을 일련의 표본제작과정을 통해 제작하였고 HE염색을 하였다. 현미경 관찰 상으로는 10%는 간 비

대, 12% fatty change type A, 24%는 fatty change type B, 44% fatty change C, 10% 간 위축을 보였다. 기타 조직장기에는 특기할 병리학적 변화가 없었다. 비대한 간은 hepatosomatic 지수(HSI)와 면역지수(대식세포의 호흡 Burst)는 간장의 조직소견에 따른 차이를 보이지 않았다. 따라서 간장의 건강도와 호흡폭발과의 상관성은 인정되지 않았다.

핵심어: 조직병리학, 조피볼락, 호흡폭발, NBT

1. Introduction

Regardless of a huge development in aquaculture industry of South Korea, there have been disease problems (Han *et al.*, 2011) that leads to a high mortality among rockfish plus stress from aquaculture practices (handling, low water quality, high rearing densities, over feeding) causing reduced growth and performance (Barton & Dwyer, 1997, Wedemeyer, 1997) and leads to reduced immune capacity and increases the disease susceptibility (Fevolden *et al.*, 1994, Pickering, 1989).

Until now many scientists have tried to overcome the negative effects of diseases on aquaculture industry. But there have been no effective measures to prevent economic loss from diseases (Shoemaker *et al.*, 2009, McPhearson *et al.*, 1991). As we all know, specialists have insisted pathological agent-oriented measures such as the use of antibiotics, vaccines, anthelmintic agents in addition to surveillance of water quality. Especially fish diseases are mainly composed of infective diseases by bacteria, virus, fungi and parasites. In aquaculture fish of South Korea we mainly have edwardsiellosis, streptococcosis and vibriosis as bacterial diseases, and iridoviral and

rhabdoviral diseases, and scuticociliatosis as a parasite disease (Jung & Oh, 2000, Kim *et al.*, 2004, Oh *et al.*, 2006). All pathological agents causing diseases in fish and shellfish are opportunistic.

In this aspect, if fish have sufficient immunity there will be no infective diseases in relation to disease susceptibility. The still non-solved disease problems strongly suggest that we scientists do not understand the full and exact meaning of immunity. Originally immunology is from physiology, that is, a branch of physiology. In other words physiological integrity directly means immunological integrity. Immune exerts by concerted physiological functions of various tissues and organs rather than classically accepted immune related cells (B/T cells, NK cells) and organs such as spleen, thymus, bone marrow, and various kinds of lymphoid tissues. Therefore the meaning of immunity should be strictly reviewed and corrected.

From this concept there have been no criteria to evaluate over all immune of living organisms including fish.

In this perspective, most of the studies were focusing on using the classical immunological parameters like respiratory burst, lysozyme activity, and phagocytic index (Abreu *et al.*, 2009, Marcogliese *et al.*,

2001, Kim *et al.*, 1999, Nya & Austin, 2009). Traditionally the histopathological assessments have only focused on identification of micro morphological lesions for disease diagnosis (McHugh *et al.*, 2011, Teh *et al.*, 1997, Marchand *et al.*, 2009) or studying the effects of feeding in organs structure (Ferri *et al.*, 2011) but there was not an essay to use the histology as tool for evaluating the fish health based in the structural integrity of organs.

As we know histology deals with relationship between structure and function. So normal composition of histological structures directly means normal function or physiological state. Reversely abnormal structure means abnormal physiology which again can be connected to abnormal immune function (Vinay Kumar, 2012, Takashima, 1995).

Even if fish are externally normal and not diseased, their hepatic histological findings are uneven. In the midst of those findings hypertrophy, mild to severe vacuolation by accumulation of fat and atrophy are constant histological deviations. The liver could be considered to be responsible for above 90% of host immune because of a variety of physiological function (Jobling, 2012, Takashima,

1995) .It could be easily thought that histological alterations of the parenchyma are directly related to functional alterations which can be connected to immune state.

In other words it could be said that the liver plays a crucial role to keep homeostatic level to be normal.

As already known under farming conditions, fish where diets generally contain higher levels of lipid, it is usually lighter in color than in the equivalent wild specimen. In previous results from this laboratory experimental overfeeding changes morphological characteristics of fish liver grossly and microscopically (Lee, 2008, Fasil.T.W, 2013).

In this study, by using the histopathological parameters based on liver condition and using the immunological parameters deploying the burst respiratory, we will try to make correlation between normal immunological state and seemingly healthy fish. Recently a unique health evaluation system has been developed and introduced in this laboratory, healthiness is graded according to 5 levels (grade A, B, C, D, and E in order of healthiness, grade A is the best and Grade E is

the worst). Our health evaluation system is based on morphological basis (anatomical and histological examination).

Traditionally histopathological findings have been mainly used simply to diagnose specific or non-specific diseases. But we tried to apply these histopathological findings as healthiness parameters for “seemingly healthy” fish individuals and groups from the aspect of structure-function relationship. Structural integrity can be connected to functional or physiological integrity and again immunological integrity as well.

Immunological integrity could not permit any kinds of pathologic agents and undesirable environmental factors around to invade and threaten the survival of an organism. Healthiness means immunologic state; in this respect, I wanted to know whether the correlation between some classical immunological parameters and the histological health of liver would exist or not.

2. Materials and methods

2.1. Fish source

The fish of *Sebastes schlegeli,* with a mean weight of 59±2.27g, were provided from a farm in Tongyeong area, and transported using plastic aquarium, which were aired and whose temperature were maintained by adding ice bags to the aquarium to avoid stress.

2.2. Aquarium set up

A recirculating system which comprises two 170 liter glass aquariums was used for the experiment. External biofilters were also used to recycle the water. Each aquarium was fitted to two external biofilters.

Water temperature was monitored by submerged thermometers. Twin water proof compact pH meter and dissolved oxygen meter were used to measure pH and dissolved oxygen. The fish were kept for one week in each sampling.

2.3. Histology assay

2.4. Dissection

Benzocaine was used to euthanize the fish before dissection. Body and length measurement were taken prior to dissection.

The head kidney was separated and put it in ice until use in respiratory burst assay. Individual livers were weighed during dissection and fixed in Bouin solution with the other organs (stomach, spleen, intestine, and gill).

2.4. Fixation and re-fixation

Subsequently after dissection, organ tissue sample were fixed in Bouin solution. After 24 hours sample were cut into suitable pieces with sharp blade and put in labeled cassettes after being categorized as organ and individuals. Then, these samples were re-fixed in 10% buffered formalin solution.

2.5. Tissue processing

All tissues were passed through a series of solvents before finally being embedded fully with paraffin wax. Tissues were washed and dehydrated through ethyl alcoholic grades (70%, 80%, 90%, 95%, 100%, 100%, and 100%) and cleaned in xylene. Cleaned tissues were embedded with paraffin wax at 58-62˚C.

2.6.Sectioning

A portion of embedded blocks of each tissue sample were sliced into 5μm sections using a rotary type microtome. The sectioned ribbon was floated on warm bath (50 ˚C) to flatten the section. The sections were carefully collected on to a glass slide and allowed to dry fully before proceeding Hematoxylin and Eosin staining.

2.7. Staining

Hematoxylin and Eosin staining method was followed to stain tissue sections through routine process.

2.8. Mounting

The stained samples were mounted with Canada balsam for permanent preservation.

2.9. Photography

Histological examination of each slide was done using light microscope (Olympus optical, Japan). Images of the prepared slides were taken using the DP2-BSW (Olympus, Japan) software.

2.10. Respiratory burst assay

2.11. Isolation of head kidney macrophage

The isolation of head kidney macrophages were established as described by Secombes (Secombes, 1990). Briefly the head kidney were aseptically

removed from the black rockfish and minced through a 100 μm nylon mesh with 3 ml of Minimal Essential Media (MEM, Sigma) containing 2% of FBS (fetal bovine serum), one % penicillin and streptomycin, and 0.2% heparin. A discontinuous gradient was made up of 3ml of 34% of Percoll (Sigma) which was on the top of 3 ml of 51% Percoll. The resultant suspension from the minced kidney was laid on the top of the discontinuous gradients and centrifuged at 600g, 30 min and 4°C in centrifuge equipped with a swing-out rotator. The cells banding in the 34%-51% surface were collected using a syringe and put it in a microtube for centrifugation (600g, 4°C, and 10 min). The supernatant was removed and the pellet was washed twice using 1 ml of 2% FBS by centrifugation at 600g, 4°C, 10 min.

After the second washing, one ml of 0, 1% FBS was added to the pellet to resuspend the cells. The viability and counting of cells were confirmed by taking 20 μl of solution, and put it in 96 well cell cultures and adding 20 μl of tryphan blue 1%. The count was done by using the hematocytomter. After the count, aliquots of the cell suspension, containing 10^6 cell/ ml, were plated in 96 well cell cultures; by putting 100 μl with 6 duplicates for each sample. The well was incubated 20°C for 2 hours. Afterwards, the well was

washed two times using MEM solution containing 5 % FBS and 0.2 % heparin.

2.12. Preparation of Zymosan

Twenty mg of zymosan (Sigma) was mixed with 1ml of FBS and incubated in 20 ˚C for 30 min. The opsonized zymosan was separated by centrifugation at 3000rpm for 10 min. The supernatant was removed and the pellet was washed two times using MEM medium (3000 rpm, 10 min at 4°C). After preparing a solution of nitroblue tetrazolium 1mg/ml of MEM, 10 ml of NBT solution was added to the pellet of zymosan.

2.13. NBT assay

The detection of superoxide anion formation by the reduction of nitroblue tetrazolium was performed as described by (Pick *et al.*, 1981), with modification by (Chung & Secombes, 1988). Briefly, the macrophages monolayers in 96-well culture were incubated with 1m/ml of NBT in culture medium, with or without stimuli (zymosan). The 96-well was incubated in 25°C for 30 min. After that the supernatant was removed and washed with 100 µl of 5% FBS 2 times. The macrophages were fixed by adding 100µl of 100% methanol and

incubated for 10 min at room temperature. After that the well were washed using 70% methanol (100µl) two times and let be dry for 2 hours or overnight. The reduced formazan within the macrophages was dissolved in 120 µl of 2M KOH and 140 µl of dimethylsulphoxide (DMSO) and read in Multiskan spectrophotometer at 620 nm using KOH/DMSO as a blank.

2.14. Hepatosomatic index (HSI) determination

The HSI was determinate (Htun-han, 1978) as:

[Liver weight (g)/body weight (g)] x100

2.15. Data analysis

Histopathological description of morphological alterations, immunologic data and statistical results were used to present the research findings. One way ANOVA and Duncan`s multiple range test (Duncan, 1955) were used on SPSS version 16 (SPSS Inc. Chicago, USA) to detect the significant differences among the samples.

3. Results

3.1. Gross Liver Observation

All of fish were normal without any lesion and no abnormality was observed. The livers appeared to be normal (Fig. 1.A) with a yellow color. No external abnormality was observed (Fig. 1.B).

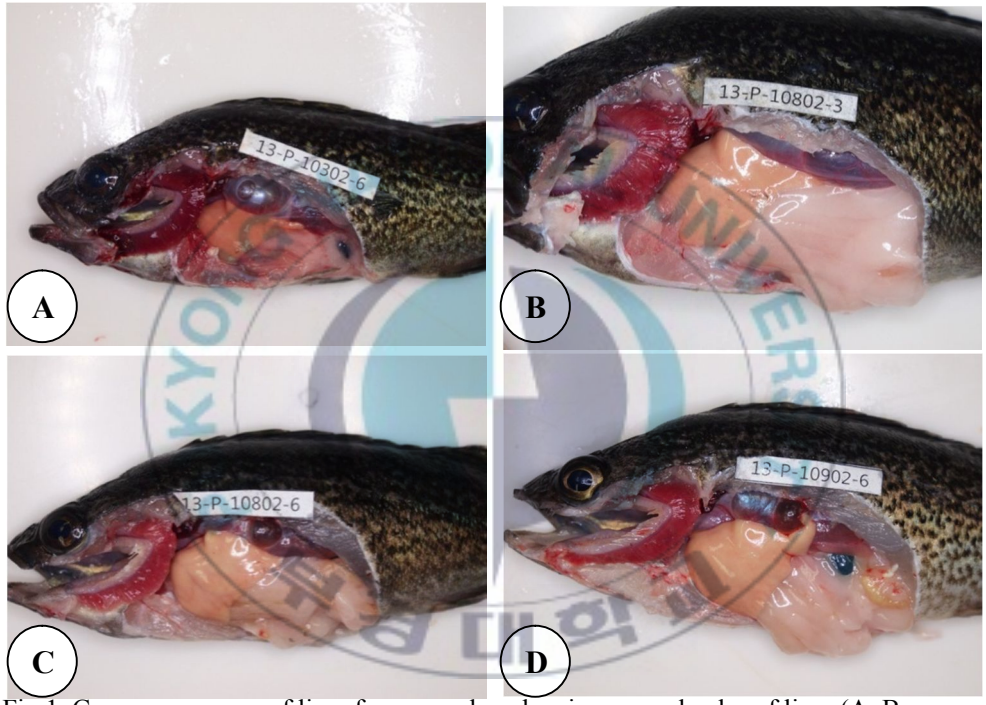

Fig.1. Gross appearance of liver from samples, showing normal color of liver (A, B, C, and D).

3.2. Microscopic Liver Observation

The liver microscopic aspect shows very large sized hepatocytes with nuclei dislocated to the cells border indicating a hypertrophy of the liver (Fig.2A).

Moreover, circular lipid vacuoles were also present together with large sized hepatocytes indicating a fatty liver change with different degree (Fig.2.B, C, and D).

In some liver samples, the hepatocytes have a highly decreased volume and severe hepatocytes vacuolization indicating a severe fatty liver change.

In some samples, the microscopic examination shows shrinking of cytoplasm of the hepatocytes with indicating an atrophy of the liver (Fig.3, D).

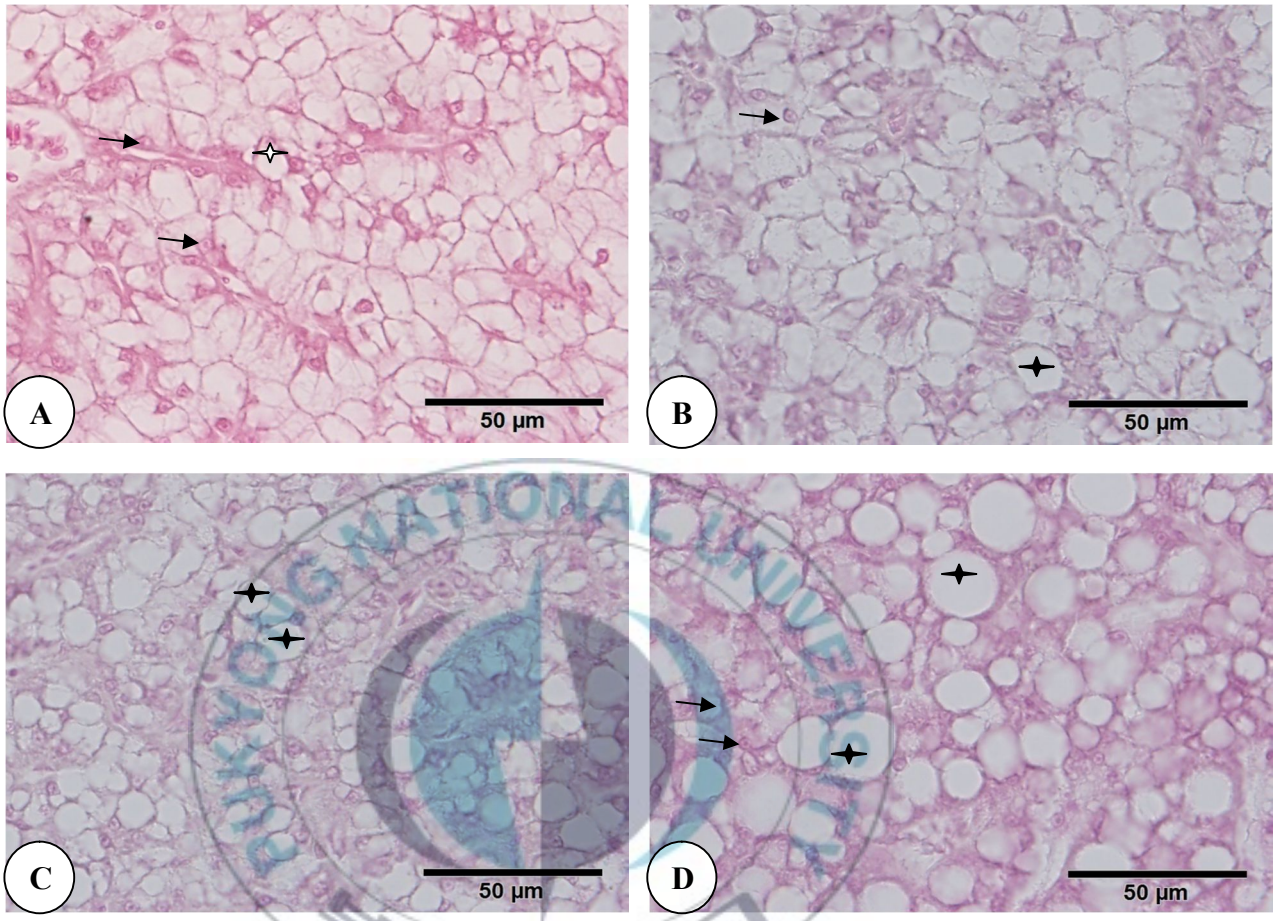

Fig.2. Liver microscopic structure with different status: (**A**) hypertrophic liver with dilatation of cytoplasm (white star)**,** (**B**) fatty change type A with vacuoles (black star) and dislocated nuclei (arrows), (**C**) fatty change type B with numerous vacuolization (black star), and (**D**) fatty change type C with lipids vacuoles (black star) and atrophic cell (arrows). (**H&E**, X400, Scale bars -50 μm).

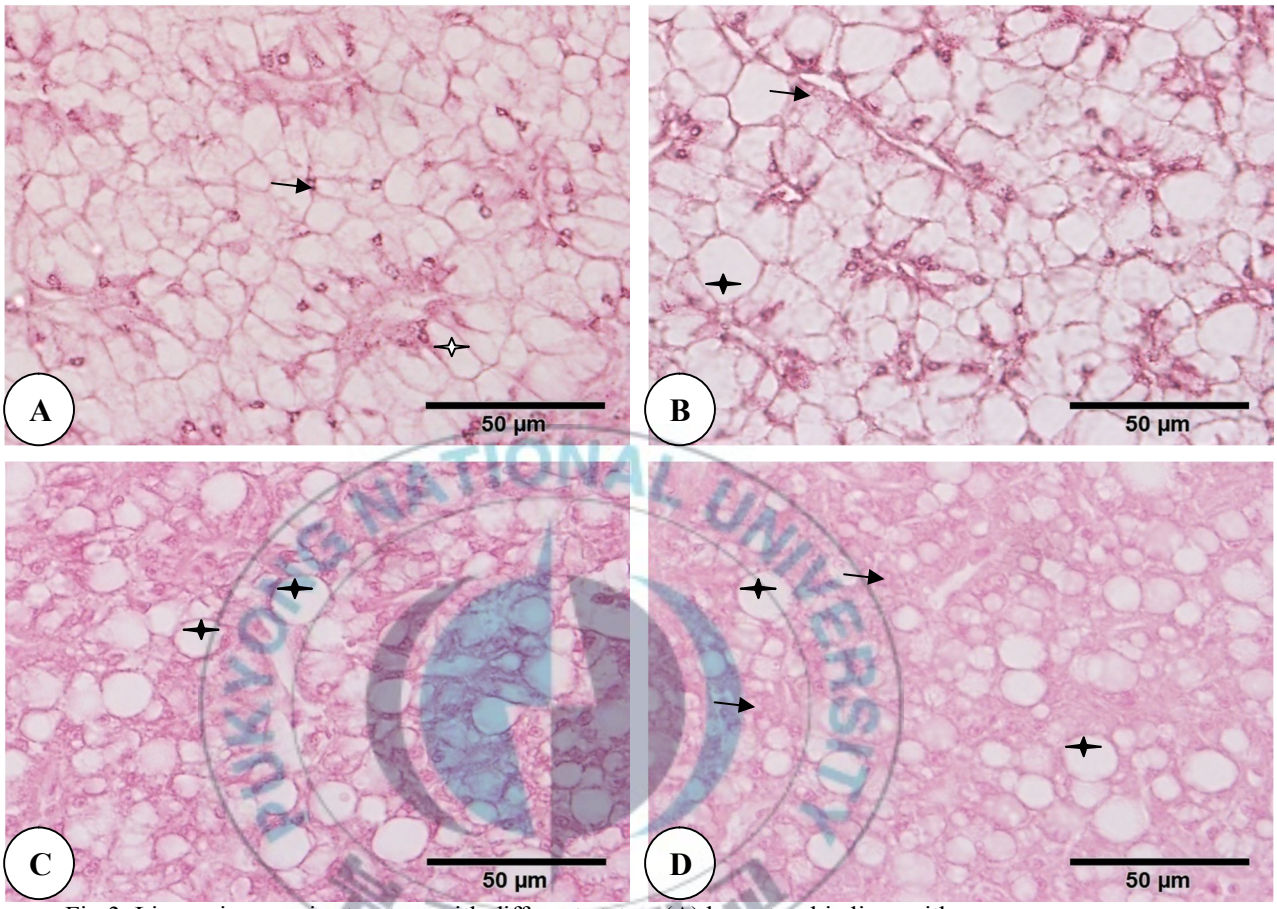

Fig.3. Liver microscopic structure with different status: (**A**) hypertrophic liver with dilatation of cytoplasm (white star), (**B**) fatty change type A with vacuoles (black star) and dislocated nuclei (arrows), (**C**) fatty change type B with numerous vacuolization (black star), and (**D**) fatty change type C with lipids vacuoles (black star) and atrophic cell (arrows). (**H&E**, X400, Scale bars -50 µm).

3.3. Liver status

Microscopic observation of the liver permitted to classify the status of the liver in different categories depending in the size of hepatocytes, number of vacuoles, and shape of nucleus (table 1).

Table1. Percentage of different liver status.

Liver status	Number of samples	Percentage (%)
hypertrophy	5	10
Fatty change A	6	12
Fatty change B	12	24
Fatty change C	22	44
Atrophy	5	10
Total	50	100

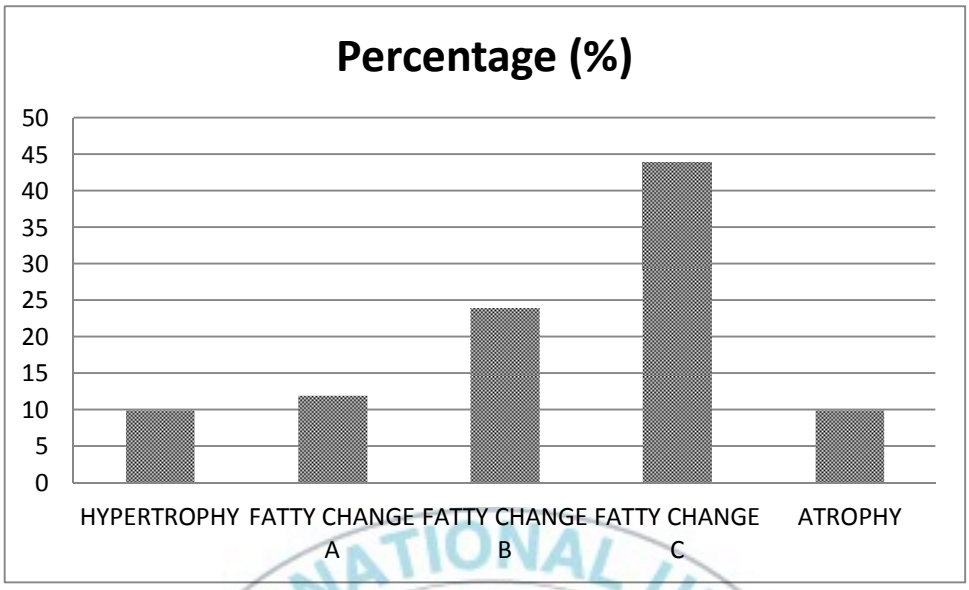

Fig.4. Percentage of different liver status.

3.4. Hepatosomatic Index (HSI)

The hepatosomatic index increased with the increase of lipid accumulation in the liver (Fig.5). However the hepatosomatic index for the atrophic liver category was higher than the category including fatty change C. It could be explained by the small number in this last category. The hepatosomatic index of the different liver status was statically similar with no significant difference ($p>0.05$) (Fig. 5)

Table2.Average values of hepatosomatic index.

Liver status	Number of samples	HSI± SE
hypertrophy	5	2.69±0.25
Fatty change A	6	3.44±0.63
Fatty change B	12	4.29±0.96
Fatty change C	22	2.58±0.39
Atrophy	5	2.83±1.25
Total	50	3.13±0.32

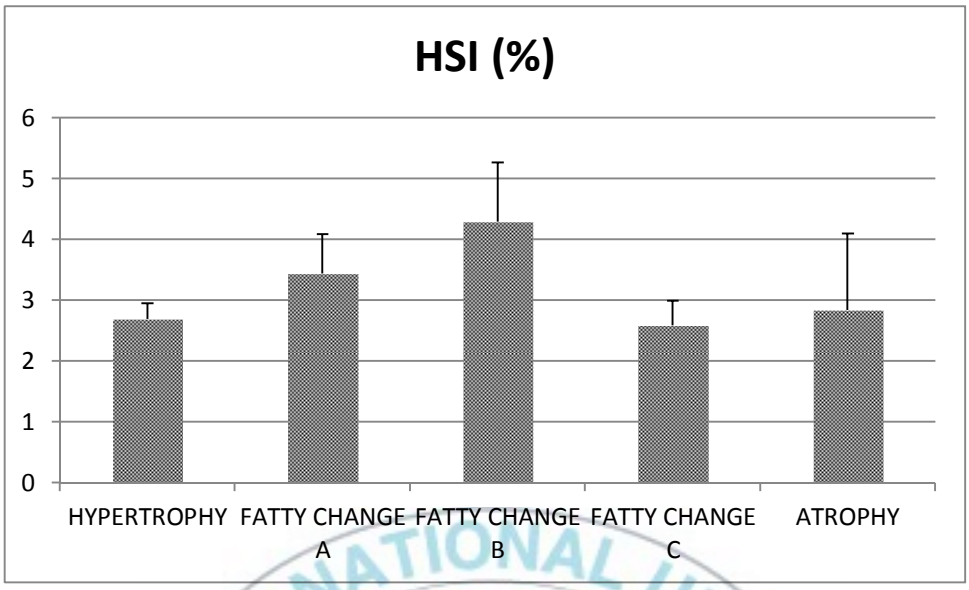

Fig.5. Hepatosomatic index (HSI) of all groups (P>0.05).

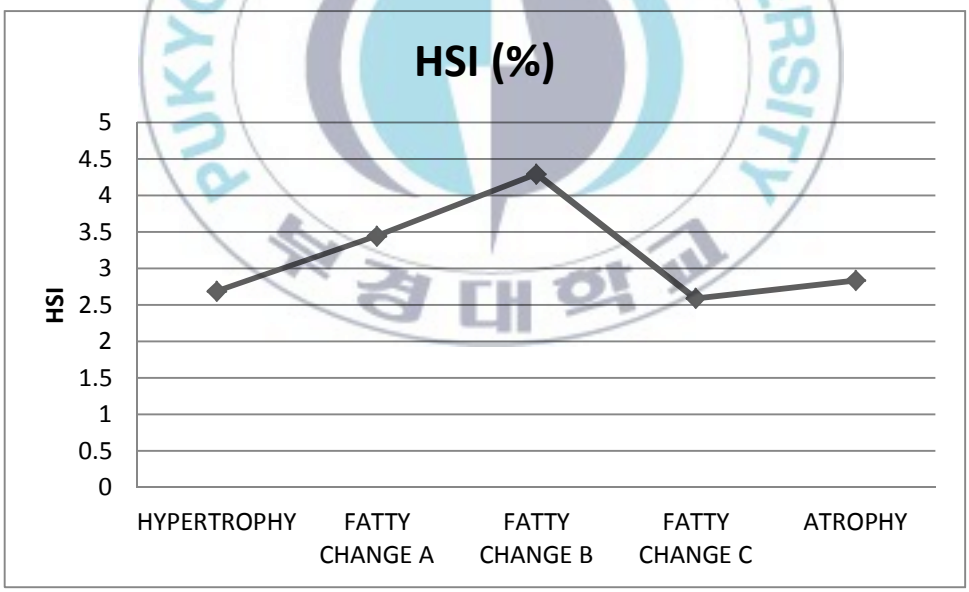

Fig.6. Hepatosomatic index (HSI) of fish dependant on the liver status.

3.5. NBT assay

The one way ANOVA proves there is no significant difference between the value of controls and samples (Fig.7).

Table 3. Average values of NBT index of control and sample.

	N	NBT± SE
Sample	50	0.15± .007
Control	50	0.13± .007

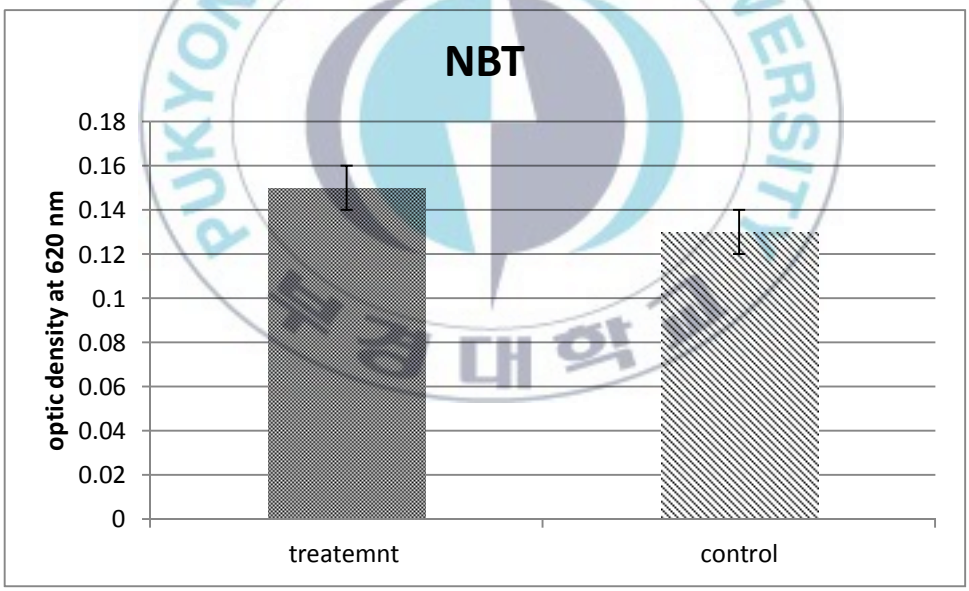

Fig.7. NBT index of fish in sample and control groups.

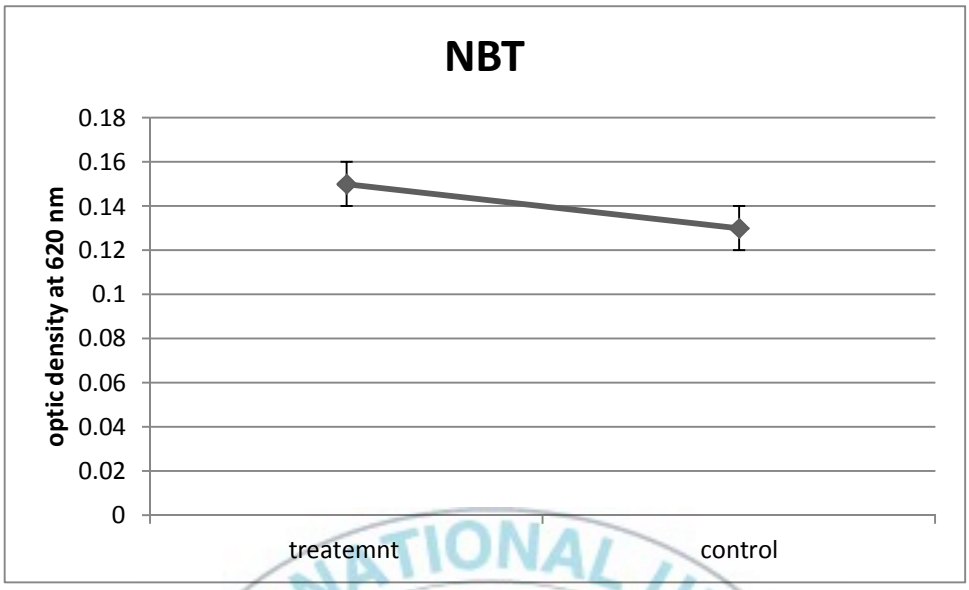

Fig.8. NBT index of fish in sample and control groups.

The NBT express a higher value in the atrophic liver category than the other categories, but statistically there is no significant difference between the different categories (Fig.8).

Table 4. Average values of NBT index of different liver status.

Liver status	N	NBT± SE
Hypertrophy	5	0.13±0.02
Fatty liver A	6	0.14±0.03
Fatty liver B	12	0.14±0.01
Fatty liver C	22	0.16±0.01
Atrophy	5	0.18±0.02
Total	50	0.15±0.01

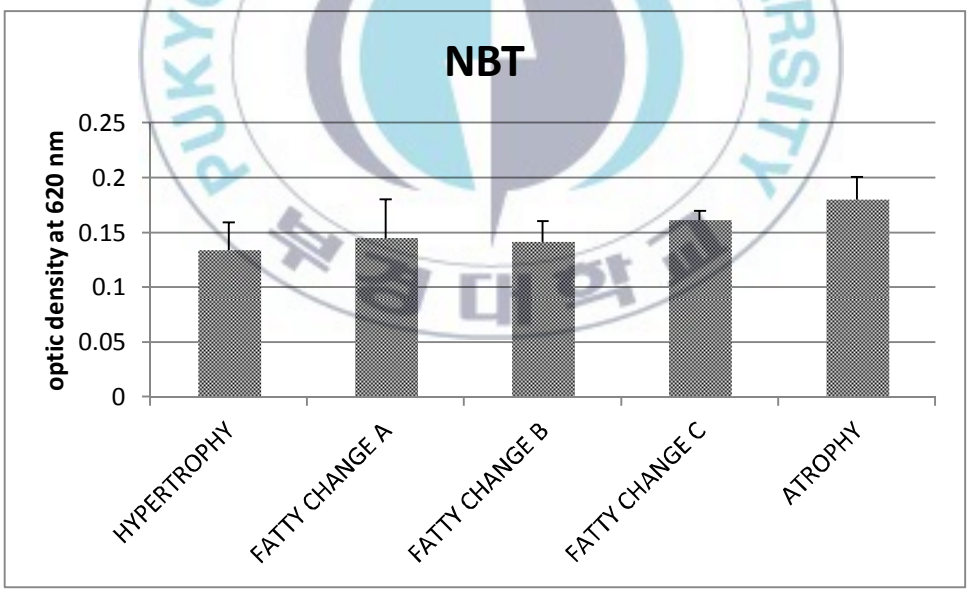

Fig.9. NBT index of fish in different groups.

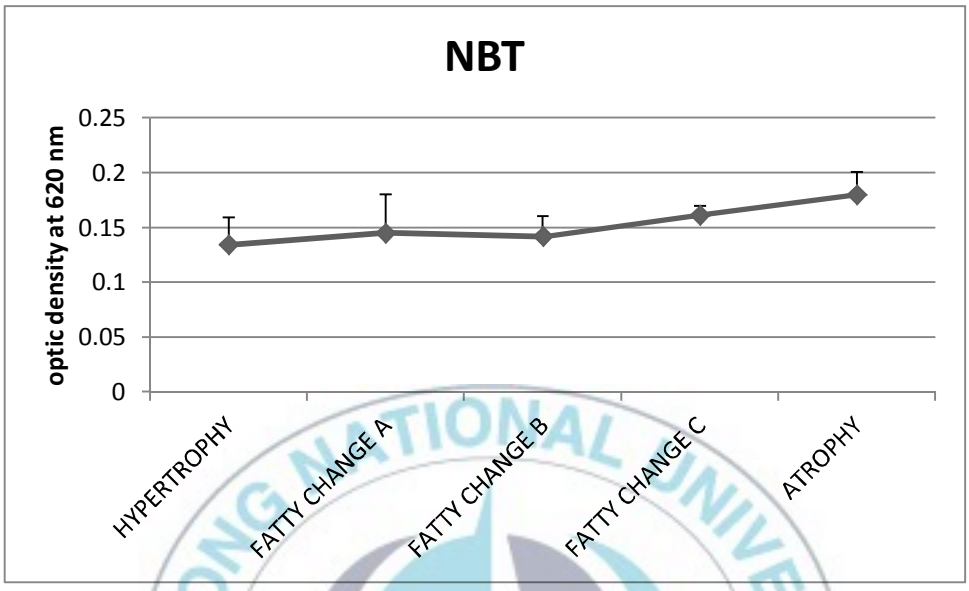

Fig.10. NBT index of fish in different groups.

4. Discussion

In the present study, hypertrophic hepatocytes with glycogen vacuoles and lipid vacuoles (Fig. 2, A) were observed with a percentage of 10%, other fish livers showed different degrees of fatty condition (Fig.2.B, C, D) with prominent lipid vacuoles as clear spaces or foamy cytoplasm with peripherally located nuclei. Depending on the fatty degree livers were classified in three groups (light grade A: 12%, mild grade B: 24%, and severe grade C: 44%). Also atrophic livers (the cytoplasm of hepatocytes shows glycogen and lipid depletion) were present with 10 % of the total sample (Fig.3, D).

Endemic hypertrophy of hepatic cells occurring in culturing fish farms is caused by absorption of sufficient amount of nutrients from intestine(Fontagné *et al.*, 1998). This cellular hypertrophy can cause narrowing of Disse space and sinusoidal space which leads to mechanical hindrance of blood microcirculation and exchange of nutrients and metabolites (Hwang, 2011). Resultantly hepatic cells fall into hypoxic state. Hypoxia can cause lipid accumulation by obliterating normal discharge from hepatic cells as lipoprotein. Accumulation of lipid droplets in

hepatocytes was inferred from the appearance of vacuoles as round, single or coalescing droplets after hematoxylin and eosin staining (Takashima, 1995).

According to definition of hypertrophy on the text book, hypertrophy is enlargement of cells and/or organs. Therefore hypertrophy of liver and its parenchymal cells should be included in this category of term. However the underlying mechanism is unique and different from the original concept of definition on the text that hypertrophy is a cellular response which occurs during increased functional demand (Takashima, 1995, Vinay Kumar, 2012). From this concept, the term hypertrophy seems not appropriate to be applied to endemic hypertrophy of liver of farming fish.

Eventually hepatocytes became in a hypofunctional condition. Decreased workload and low blood supply are known to cause atrophy to cells(Takashima, 1995, Vinay Kumar, 2012).

There are few references about the immune and hepatic function except for comments of immunoglobulin production. In addition reviews about the pathological significance are very scarce.

As I referred earlier, liver is responsible for over 90% body homeostasis. This important fact should be introduced and connected to concept of body immune.

The HSI index, an indicator of hepatomegaly or atrophy, was higher in fatty change B group than the other groups (Fig.5) indicating more accumulation of lipids in the liver. The HSI increases with the increase of the number of lipid vacuoles in the liver (Fig.5) which is in concordance with previous studies (Lambert & Dutil, 1997, Auðunsson, 1999).

Nevertheless the hepatosomatic index did not have significant difference between the different statuses of the liver (Table.2), suggesting that the hepatosomatic index is not a parameter related to the health of fish. Several authors have proven that the hepatosomatic index is not related with the health evaluation of the fish (Tierney & Farrell, 2004).

Macrophages are important cells in disease resistance of fish. They are the principal phagocytic cells in fish (Blazer, 1991) and the first line of defense in nonspecific immunity.

So for the assessment of nonspecific immunity, the respiratory burst was used to detect the generation of reactive oxygen species (ROS) after

stimulation of the macrophages by zymosan(Chung & Secombes, 1988, Secombes, 1990).

There was no difference (P>0.05) in the respiratory between the macrophages stimulated with zymosan and non-stimulated macrophages (Fig.7). At the same time, the NBT indices were not significantly different (P>0.05) among the various status of liver health (Fig.8).

The macrophages and other nonspecific factors can be affected by numerous environmental, physiological and pathological stressors (Kumar *et al.*, 2005, Narnaware *et al.*, 1994, Shoemaker *et al.*, 1997). Furthermore, the phagocytic assay and respiratory burst assay were used to assessment of fish health in many studies (Bols *et al.*, 2001, Biller-Takahashi *et al.*, 2013).

As a result no significant difference in the NBT indices according to the liver health can be explained by the absence of specific stressor, which can cause the stimulation of macrophages. These results can permit to say that use of NBT index as an indicator of macrophage activity should be restricted to presence of specific stressor to macrophages. Strictly speaking, various classical parameters evaluating immunity including NBT indices are not deeply correlated with host immune in the real aspect.

The histological examination of the liver was applied in diverse studies concerning the pollution, the nutritionals effects, and the pathogens effects(Yildirim *et al.*, 2006, Kumar et al., 2005, van Dyk *et al.*, 2009, Marchand et al., 2009, Bernet *et al.*, 1999). But they did not directly correlate the histological findings of liver as a representative organ for host immune state.

From the result of this study, there was no correlation between one of classical immune parameters, NBT index and histological health of liver in culturing rockfish, *Sebastes schlegeli*.

5. Conclusion

The outcome of this study claims the hepatosomatic index (HSI) and the phagocyte oxidative burst (NBT) did not express any significant change to the alteration structural of liver.

From results obtained, it might be concluded that the histopathological assessment of the liver and the other organs could be used as fish health indicator from the point of real meaning of the host immune. Liver structural changes observed in this study are believed to be a good enough to disrupt the normal functioning of the liver. . Taking in consideration the important role the liver plays in physiological homeostasis of fish, it is quite reasonable to think that histologically affected liver can lower the fish resistance to a variety of disease entity.

Therefore the present study emphasizes microscopic structural findings of liver to be a good parameter of health assessment for the fish health and also a good indicator of immunological state.

Further studies are required to be done for better understanding of the concept of health and immunity by comparing the different classical immunological parameters and histological state of liver.

6. Acknowledgements

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7. References

Abreu, J., C. Marzocchi-Machado, A. Urbaczek, L. Fonseca and E. Urbinati, 2009: Leukocytes respiratory burst and lysozyme level in pacu (*Piaractus mesopotamicus* Holmberg, 1887). Brazilian Journal of Biology, **69,** 1133-1139.

Auðunsson, G. A., 1999: The effect of nutritional status of Icelandic cod (*Gadus morhua*) on macroconstituents and trace elements in the liver. Rit Fiskideildar -Journal of the Marine Research Institute **16,** 111-129.

Barton, B. A. and W. P. Dwyer, 1997: Physiological stress effects of continuous- and pulsed-DC electroshock on juvenile bull trout. Journal of Fish Biology, **51,** 998-1008.

Bernet, D., H. Schmidt, W. Meier, P. Burkhardt-Holm and T. Wahli, 1999: Histopathology in fish: proposal for a protocol to assess aquatic pollution. Journal of Fish Diseases, **22,** 25-34.

Biller-Takahashi, J., L. Takahashi, M. Saita, R. Gimbo and E. Urbinati, 2013: Leukocytes respiratory burst activity as indicator of innate immunity of pacu *Piaractus mesopotamicus*. Brazilian Journal of Biology, **73,** 425-429.

Blazer, V. S., 1991: Piscine Macrophage Function and Nutritional Influences: A Review. Journal of Aquatic Animal Health, **3,** 77-86.

Bols, N. C., J. L. Brubacher, R. C. Ganassin and L. E. J. Lee, 2001: Ecotoxicology and innate immunity in fish. Developmental & Comparative Immunology, **25,** 853-873.

Chung, S. and C. J. Secombes, 1988: Analysis of events occurring within teleost macrophages during the respiratory burst. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, **89,** 539-544.

Duncan, D. B., 1955: Multiple Range and Multiple F Tests. Biometrics, **11,** 1 - 42.

Fasil.T.W, 2013: Histological Changes of Liver in Overfed Young Nile Tilapia *Oreochromis niloticus* Master., Pukyong National University.

Ferri, J., N. Topić Popović, R. Čož-Rakovac, B. Beer-Ljubić, I. Strunjak-Perović, F. Škeljo, M. Jadan, M. Petrić, J. Barišić, M. Šimpraga and R. Stanić, 2011: The effect of artificial feed on blood biochemistry

profile and liver histology of wild saddled bream, *Oblada melanura* (Sparidae). Marine Environmental Research, **71,** 218-224.
Fevolden, S. E., T. Taugbøl and J. Skurdal, 1994: Allozymic variation among populations of noble crayfish, *Astacus astacus L.*, in southern Norway: implications for management. Aquaculture Research, **25,** 927-935.
Fontagné, S., I. Geurden, A.-M. Escaffre and P. Bergot, 1998: Histological changes induced by dietary phospholipids in intestine and liver of common carp (*Cyprinus carpio L.*) larvae. Aquaculture, **161,** 213-223.
Han, H. J., D. Y. Kim, W. S. Kim, C. S. Kim, S. J. Jung, M. J. Oh and D. H. Kim, 2011: Atypical Aeromonas salmonicida infection in the black rockfish, *Sebastes schlegeli* Hilgendorf, in Korea. Journal of Fish Diseases, **34,** 47-55.
Hwang, S., 2011: Microcirculation of the Liver. In: D. C. Madoff, M. Makuuchi, M. Nagino and J.-N. Vauthey (eds.), Venous Embolization of the Liver. Springer London.
Jobling, M. R. J. R., 2012: Fish pathology. Aquaculture International, **20**.
Jung, S. J. and M. J. Oh, 2000: Iridovirus-like infection associated with high mortalities of striped beakperch, *Oplegnathus fasciatus* (Temminck et Schlegel), in southern coastal areas of the Korean peninsula. Journal of Fish Diseases, **23,** 223-226.
Kim, K. H., Y. J. Hwang and S. C. Bai, 1999: Resistance to Vibrio alginolyticus in juvenile rockfish (*Sebastes schlegeli*) fed diets containing different doses of aloe. Aquaculture, **180,** 13-21.
Kim, S. M., J. B. Cho, S. K. Kim, Y. K. Nam and K. H. Kim, 2004: Occurrence of scuticociliatosis in olive flounder *Paralichthys olivaceus* by *Phiasterides dicentrarchi* (Ciliophora: Scuticociliatida). Diseases of Aquatic Organisms, **62,** 233-238.
Kumar, S., N. P. Sahu, A. K. Pal, D. Choudhury, S. Yengkokpam and S. C. Mukherjee, 2005: Effect of dietary carbohydrate on haematology, respiratory burst activity and histological changes in *L. rohita* juveniles. Fish & Shellfish Immunology, **19,** 331-344.
Lambert, Y. and J. D. Dutil, 1997: Condition and energy reserves of Atlantic cod (*Gadus morhua*) during the collapse of the northern Gulf of St. Lawrence stock. Canadian Journal of Fisheries and Aquatic Sciences, **54,** 2388-2400.

Lee, M. K., 2008: Pathological studies on green liver syndrome in farmed grey mullet, *Mugli cephalus*. Ph.D., Pukyong National University.

Marchand, M. J., J. C. van Dyk, G. M. Pieterse, I. E. J. Barnhoorn and M. S. Bornman, 2009: Histopathological alterations in the liver of the sharptooth catfish *Clarias gariepinus* from polluted aquatic systems in South Africa. Environmental Toxicology, **24,** 133-147.

Marcogliese, D. J., M. Fournier, A. Lacroix and D. G. Cyr, 2001: Non-specific immune response associated with infections of lymphocystis disease virus in American plaice, *Hippoglossoides platessoides* (Fabricius). Journal of Fish Diseases, **24,** 121-124.

McHugh, K. J., N. J. Smit, J. H. J. Van Vuren, J. C. Van Dyk, L. Bervoets, A. Covaci and V. Wepener, 2011: A histology-based fish health assessment of the tigerfish, *Hydrocynus vittatus* from a DDT-affected area. Physics and Chemistry of the Earth, Parts A/B/C, **36,** 895-904.

McPhearson, R. M., A. DePaola, S. R. Zywno, M. L. Motes Jr and A. M. Guarino, 1991: Antibiotic resistance in Gram-negative bacteria from cultured catfish and aquaculture ponds. Aquaculture, **99,** 203-211.

Narnaware, Y., B. Baker and M. Tomlinson, 1994: The effect of various stresses, corticosteroids and adrenergic agents on phagocytosis in the rainbow trout *Oncorhynchus mykiss*. Fish Physiol Biochem, **13,** 31-40.

Nya, E. J. and B. Austin, 2009: Use of garlic, Allium sativum, to control Aeromonas hydrophila infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases, **32,** 963-970.

Oh, M.-J., W.-S. Kim, S.-I. Kitamura, H.-k. Lee, B. W. Son, T.-S. Jung and S.-J. Jung, 2006: Change of pathogenicity in Olive flounder *Paralichthys olivaceus* by co-infection of *Vibrio harveyi*, *Edwardsiella tarda* and marine birnavirus. Aquaculture, **257,** 156-160.

Pick, E., J. Charon and D. Mizel, 1981: A rapid densitometric microassay for nitroblue tetrazolium reduction and application of the microassay to macrophages. Journal of the Reticuloendothelial Society, **30,** 581-593.

Pickering, A. D., 1989: Environmental stress and the survival of brown trout, *Salmo trutta*. Freshwater Biology, **21,** 47-55.

Secombes, C. J., 1990: Isolation of salmonid macrophages and analysis of their killing activity. Techniques in Fish Immunology, SOS Publications**,** 139–154.

Shoemaker, C. A., P. H. Klesius, J. J. Evans and C. R. Arias, 2009: Use of Modified Live Vaccines in Aquaculture. Journal of the World Aquaculture Society, **40,** 573-585.

Shoemaker, C. A., P. H. Klesius and J. A. Plumb, 1997: Killing of *Edwardsiella ictaluri* by macrophages from channel catfish immune and susceptible to enteric septicemia of catfish. Veterinary Immunology and Immunopathology, **58,** 181-190.

Takashima, F. H., T, 1995: An atlas of fish histology: normal and pathological features. Kodansha Ltd.

Teh, S. J., S. M. Adams and D. E. Hinton, 1997: Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. Aquatic Toxicology, **37,** 51-70.

Tierney, K. B. and A. P. Farrell, 2004: The relationships between fish health, metabolic rate, swimming performance and recovery in return-run sockeye salmon, *Oncorhynchus nerka* (Walbaum). Journal of Fish Diseases, **27,** 663-671.

van Dyk, J. C., M. J. Marchand, N. J. Smit and G. M. Pieterse, 2009: A histology-based fish health assessment of four commercially and ecologically important species from the Okavango Delta panhandle, Botswana. African Journal of Aquatic Science, **34,** 273-282.

Vinay Kumar, A. K. A., Jon C. Aster, 2012: Robbins Basic Pathology. Elsevier Health Sciences.

Wedemeyer, G. A., 1997: effects of rearing conditions on the health and physiological quality of fish in intensive culture. Fish Stress and Health in Aquaculture., 35–71.

Yildirim, M. Z., A. Ç. K. Benlı, M. Selvı, A. Özkul, F. Erkoç and O. Koçak, 2006: Acute toxicity, behavioral changes, and histopathological effects of deltamethrin on tissues (gills, liver, brain, spleen, kidney, muscle, skin) of Nile tilapia (*Oreochromis niloticus L.*) fingerlings. Environmental Toxicology, **21,** 614-620.