Thesis for the Degree of Master of Fisheries Science

Histological Changes of Liver in Overfed Young Nile Tilapia *Oreochromis niloticus*

by

Fasil Taddese WOLEBU

KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

Pukyong National University

February 2014

Histological Changes of Liver in Overfed Young Nile Tilapia *Oreochromis niloticus*

사료의 과잉공급에 의한 나일틸라피아 *Oreochromis niloticus* 간장의 조직형태학적 변화

Advisor: Prof. Min Do HUH

by

Fasil Taddese WOLEBU

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Fisheries Science

KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

Pukyong National University

February, 2014

Histological Changes of Liver in Overfed Young Nile Tilapia

Oreochromis niloticus

A dissertation

by

Fasil Taddese

Approved by :

(Chairman) Professor Sungchul C. BAI

(Member) Professor Young Jin CHANG

(Member) Professor Min Do HUH

February 21, 2014

Table of Contents

List of Figures

List of Tables

Histological Changes of Liver in Overfed Young Nile Tilapia *Oreochromis niloticus*

Fasil Taddese

KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries,

Pukyong National University

Abstract

The adverse effect of overfeeding has been explained only from water quality deterioration perspective so far. Direct influence of overfeeding on liver of fish was given little attention. This study aimed to investigate histopathologically structural responses of liver of Nile tilapia *Oreochromis niloticus* towards overfeeding. Mixed population of *O. niloticus* with mean weight of 55±3.83g obtained from Pukyong National University fish farm and acclimated for one week. Then, the fish were separated into control and treatment groups. Glass aquariums with external biofilters and artificial heaters were used. Control and treatment groups were fed commercial tilapia diet at 3 % and 6 % per body weight feeding level, respectively. Fish were dissected at three time intervals. Tissues were fixed, re-fixed, processed, embedded with paraffin, sectioned and stained with Hematoxylin & Eosin technique for light microcopy. Occurred morphological changes were explained semi-quantitatively using indices. Significant differences ($p<0.05$) of indices were found between control and treatment groups of different weeks. Livers with normal brown and

pale brown color were observed from control groups and treatment groups, respectively. Normal hepatocytes with circular, conspicuous and centrally located nuclei were found from all control groups. Hepatocyte hypertrophy was observed after three weeks of overfeeding. Large hepatocytes with nuclei dislocated to the periphery of the cell dominated in the third week treatment group. Hepatocytes with lipid vacuoles and inconspicuous nuclei were found after five weeks of overfeeding. Some atrophic livers were also observed among fish from the fifth week treatment group. Hence, the findings of this study suggested that overfeeding indeed can cause liver histological alterations in *O. niloticus*.

Key words: *Oreochromis niloticus*, Overfeeding, Liver, Histology, Morphology, Alteration

Introduction

The Nile Tilapia *Oreochromis niloticus* is a freshwater fish belonging to the family Cichlidae. It is commercially important fish species which is being cultured in many countries. It is native to Africa, but was introduced into many tropical, subtropical and temperate regions of the world during the second half of the 20th century (Pillay and Kutty, 2005)

This species is of great interest to fish culture as it is fast growing and tolerant to wide environmental conditions. In general, tilapia including *O. niloticus*, have many attributes that make them ideal candidate for aquaculture, especially in developing countries (El-Sayed, 2006). These include:

1. Fast growth
2. Tolerance to a wide range of environmental conditions (such as temperature, salinity, low dissolved oxygen, etc.)
3. Resistance to stress and disease
4. Ability to reproduce in captivity and short generation time
5. Feeding on low trophic levels and acceptance of artificial feeds immediately after yolk-sac absorption

Currently, *O. niloticus* is being cultured in almost all parts of the world. Tilapia and other Cichilids were on the second rank next to carps, of world aquaculture production during 2010 (FAO, 2012). Thus, tilapia and other cichlids totally contribute a lot to the total global aquaculture production.

In fish culture operations feed and feeding accounts for the highest cost. Optimum feeding is one of the ways through which cost is minimized as well as better growth is achieved. However, the traditional management strategy for maximizing growth is by maximizing feeding (Gao and Lee, 2012). Besides, feeding regimes are known to be one of the most disputed areas in tilapia nutrition (El-Sayed, 2006). Various contradictory feeding regimes, frequencies and mechanisms are mentioned in different findings. It is also common to find recommended feeding frequency as high as 6-8 times per day and up to 5 % per body weight feeding rate for tilapia (Boyd, 2004). Therefore, either unknowingly or knowingly because of misleading information, culturists often try to increase fish production by excessive feeding or overfeeding.

The alimentary canal of teleostean fishes has been widely studied and described morphologically to determine the function of many specialized anatomical structures in relation to the different feeding adaptation of this large group (Cataldi *et al.*, 1987). Liver is the major organ in the digestive system of fish. The teleost liver is relatively large dense organ ventrally located in the cranial region of the general cavity (Jobling, 2012). Its size, shape, and volume are adapted to the space available between other visceral organs (Vicentini *et al.*, 2005). In wild fish it is usually reddish brown in carnivores and lighter brown in herbivores but at certain times of year it may be yellow or even off white. In farmed fishes, where diets generally contain higher levels of lipid, it is usually lighter in color than in the equivalent wild specimen.

Fish liver serves functions similar to those in mammals. Its functions include assimilation of nutrients, production of bile, detoxification, and maintenance of the body metabolic homeostasis that includes processing of carbohydrates, proteins, lipids and vitamins (Genten *et al.*, 2009; Jobling, 2012). Liver also plays a key role in the synthesis of plasma proteins, like albumin, fibrinogen, and complement factors.

The microstructure of the liver varies among species, but there are general traits in majority of species (Ferguson, 2006). The liver of the *O. niloticus* is a large organ and has only two lobes (Vicentini *et al.*, 2005). The left lobe is bigger and spreads throughout almost the entire corporeal cavity.

Nutritional and physiological status of many fishes has been studied by using liver as an indicator. Various authors have described the effects of different nutritional conditions on liver. Caballero *et al.* (1999) studied the effect of lipid level and fish meal quality on liver of gilthead sea bream and found swelling hepatocytes. The effect of different lipid sources on liver of *Pangasius nasutus* was observed by Asdari *et al.* (2011). Histological methods in the assessment of different feed effects on liver and intestine of fish were reviewed and explained by Raskovic *et al.* (2011).

Several authors also explained fish liver alterations as a result of toxic substances. According to Xu *et al.* (2012) hepatic pathway of lipid metabolism in Crucian Carp can be influenced by the adverse effect of trichlorofon. Histopathological effects in fish exposed to the toxins from *Karlodinium micrum* were also investigated by Deeds *et al.* (2006). On a

study which was done to assess the toxic effect of zinc on the liver structure of *O. niloticus*, hepatocyte degeneration and congestion of blood vessels were found (Abdel-Warith *et al.*, 2011). Histopathological changes of *Tilapia zilli* in response to aluminum toxicity were also explained by Hadi and Alwan (2012). Similar histopathological study was as well done on *Tilapia mossambica* in response to cadmium sulphate by Jalaludeen *et al.* (2012). Histopathology of *O. niloticus* due to exposure of other toxic substances like ethanolic extracts of *Ipomoea aquatica* leaf (Ayoola, 2011) and toxic Cyanobacterium *Microcystis aeruginosa* (Fahprathanchai *et al.*, 2007) were studied.

The majority of the researches on histopathology of fishes have focused on the quality of the feed as a cause for liver alterations, not on the quantity. Besides, only water quality deterioration (Masser *et al.*, 1992; Mohanty, 2001) and high cost of production are thought to be the only consequences of overfeeding.

However, Verreth *et al.* (1994) indicated that feeding level can cause an accumulation of lipid in the liver of *Clarias gariepinus*. According to these

authors, feeding level is also the most decisive parameter for larval growth and metabolic performance for the liver than the feed type. Phillips and Podoliak (1957) also mentioned that excessive fat deposition in trout`s liver as well as kidney is always caused by overfeeding. Storch *et al.* (1983) as well indicated that feeding level can provoke alteration of hepatocytes in milkfish fry.

As those previously mentioned investigations indicated, structural alterations of the liver can be caused by the feeding level or amount. In other words overfeeding can induce liver morphological changes. No enough attention was given to overfeeding as a direct stressor to the fish liver. Thus, clear and detailed information about the effect of overfeeding on morphological alterations of liver is lacking. Specifically, studies which show how the liver of *O. niloticus* responds to increased feeding level are scarce. Therefore, the present study aimed to fill the existing information gap.

Objectives

The main objective of this study is to investigate liver histological alterations of the Nile tilapia *O. niloticus* in response to overfeeding. The specific objectives include:-

1. To assess the liver damages and alterations caused by overfeeding
2. As a consequence to describe liver morphological alterations histologically
3. Also to quantify liver structural alterations using semi-quantitative methods, and
4. To determine hepatosomatic index (HSI)

Materials and Methods

The research was carried out at Laboratory of Fish and Shellfish Pathology (LFSP), Department of Aquatic Life Medicine, Pukyong National University, Korea. The whole study had two major components namely, feeding experiment and histopathological investigation. The feeding experiment lasted for a period of five weeks excluding acclimation time.

Fish Source

Mixed sex population of *O. niloticus,* with mean weight of 55±3.83g, were obtained from the recirculating freshwater fish farm of Pukyong National University.

Acclimation

Twelve randomly selected fish were sacrificed before the beginning of feeding trial. The rest 52 fish were acclimated to the experimental condition for about one week after arrival. Commercial diet was used for acclimation with 2 % per body weight feeding level two times per day.

Experimental Design

After acclimation the fish distributed equally and randomly to four glass aquariums. These four aquariums were categorized into two groups, treatment and control. Each group was represented by duplicate.

Feed Source

A commercial tilapia diet with 38 % Crude Protein and 6 % Crude Fat content was purchased from Woosung Feed Co. Ltd., Korea. Composition of the diet (Table 1) was declared by the company. It was extruded pellet and administered by hand feeding.

Table 1.Nutritional composition of the feed (Woosung Feed Co. Ltd., Korea)

Crude Protein	Crude Fat	Crude Fiber	Ash	P	Ca
38%	6%	18%	6%	0.8%	1.8%

Aquarium Set Up

A recirculating system which comprises four 170 liter glass aquariums was used for the feeding experiment. External biofilters (PhilGreen Ef-1300, China) were used to recycle the water. Each aquarium in the treatment group was fitted to two external biofilters. In the control group, only one biofilter for every aquarium was used. Water temperature was maintained and monitored by submerged water heaters and thermometers. Twin water proof compact pH meter (B-212, Horiba, Japan) and Dissolved Oxygen meter (Oxyguard, Denmark) were used to measure pH and dissolved oxygen, respectively. Water was replaced two times per day for the whole feeding period. Intensively aerated water at a temperature of $27\pm1\ ^{0}C$ was used for replacement.

Scoop net was used for mechanical removal of fecal matter immediately after release. Regular cleaning of biofilters and checkups were done to prevent clogging and to maintain filtering efficiency. All optimum water quality requirements of *O. niloticus* were maintained, besides, 12D and 12L hours were kept. Water temperature of aquariums was maintained at 27 ± 1

^{0}C. Dissolved oxygen was 6-7 mg/l and pH was within the range of 7.2-7.5. Ammonia level kept below 1mg/l.

Feeding

Feed was provided constantly as a percentage of fish body weight, not based on satiation. The latter involves feeding of the fish all they will ingest in a reasonable period of time (Robinson *et al.*, 1998). Fish in the treatment group were fed four times per day at 09:00, 12:00, 15:00 and 18:00 hours 6 % of their body weight. The feed was weighed and divided into four parts equally. While those fish in the control category were fed two times per day 3 % of their body weight at 09:00 and 18:00 hours in the same manner. The treatment feeding level was set by defining overfeeding as feeding the fish beyond the optimum or standard feeding level. Feeding level for the control group, 3 % body weight, was in accordance with the optimum requirement of young *O. niloticus* (El-Sayed, 2006). The 3 % per body weight feeding allowance for *O. niloticus* is also suggested by Chowdhury (2011) and NRC (1993).

The fish in the treatment group were fed more frequently than the control ones to maximize their appetite. In frequent feedings each subsequent meal is known to increase the stomach volume (Riche *et al.*, 2004) and the rate of evacuation is assumed to be faster when the stomach volume increases. Then, each evacuation brings appetite back and let the fish eat more. Feeding level adjustment was made for both control and treatment groups every two weeks in accordance with their body weight gain. However, the percent per body weight feeding allowance kept constant.

Dissection

Benzocaine was used at a concentration of 50mg/l (Coyle *et al.*, 2004) to euthanize the fish before dissection. Fish were dissected before the beginning of feeding trial, at third week and at fifth week periods. Body weight and length measurements were taken prior to dissection. Fish were not fed 24 hours before the time of necropsy. Dissection was done using scissors and forceps. Individual livers were weighed and images of internal organs were taken using digital camera (Olympus E-P2-Japan) during dissection. Hematocrit and hemoglobin were determined immediately after euthanizing the fish during the zero week and fifth week dissection times.

Fixation and Re-fixation

Subsequently after dissection, liver tissue samples were fixed in Bouin's solution. After 24 hours samples 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.

Tissue processing

All fixed 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.

Embedding

Tissues were embedded with paraffin wax at 58- 62 ^{0} C.

Sectioning

A portion of embedded blocks of each tissue sample were sliced into 5 micro meter sections using a rotary type microtome (Reichert –Jung 820,

Leica, Germany). The sectioned ribbon was floated on warm water bath (54 ^{0}C) to flatten out the section. The sections were carefully collected on to a glass slide and allowed to dry fully before proceeding to H&E staining.

Staining

Hematoxylin and Eosin (H&E) staining method was followed to stain the tissue sections. The following consecutive steps were followed to perform:

1. Xylene (1) - 3 minutes
2. Xylene (2) - 3 minutes
3. Xylene (3) - 3 minutes
4. Ethyl alcohol 100 % - 1 minute
5. Ethyl alcohol 95 % - 1 minute
6. Ethyl alcohol 90 % - 1 minute
7. Ethyl alcohol 80 % - 1 minute
8. Ethyl alcohol 70 % - 1 minute
9. Washing with flowing tap water - 10 minutes
10. Hematoxylin - 3 minutes
11. Washing with tap water – 1 minute

12. HCL (Acid alcohol) - 2 times dipping
13. Washing with tap water – 1 minute
14. Ammonia water – 4 times dipping
15. Washing with flowing tap water – 15 minutes
16. Eosin- 2 minutes
17. Ethyl alcohol 70-80 % - 4 times dipping
18. Ethyl alcohol 90 % - 1 minute
19. Ethyl alcohol 95 % - 1 minute
20. Ethyl alcohol 100 % - 1 minute
21. Ethyl alcohol 100 % -1 minute
22. Xylene +Alcohol – 3 minutes
23. Xylene (1) - 3 minutes
24. Xylene (2) -3 minutes
25. Xylene (3) - 3 minutes

Mounting

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

Photography

Histological examination of liver structures was done using light microscope (U-MDOB, Olympus optical Co. Ltd., Japan). Images of the prepared slides of different organs were taken using the DP2-BSW (Olympus, Japan) software.

Determination of Liver Reaction Index (LRI)

Captured images of prepared slides were viewed and examined on Image-pro plus software version 6.0 (Media Cybernetics, Inc., USA). Liver morphological alterations as a result of overfeeding were investigated. Descriptions (Takashima and Hibiya, 1995; Bernet *et al.*, 1999) were used to illustrate the observed morphological changes on liver. Four expected alterations of liver were identified and described (Table 2).

Although histopathology is descriptive science and assessment of changes always relies on the investigator`s experience, somehow it is better to quantify the changes for the statistical analysis. This will help to strengthen the histomorphological descriptions with statistical evaluation. Hence, a

semi-quantitative histopathological assessment method was adopted and modified from Bernet *et al.* (1999). Among indices proposed by the mentioned authors, only reaction index of an organ was taken and modified to assess the extent of liver reaction of individual fish towards overfeeding.

Table 2.Description of liver alterations (Bernet *et al.*, 1999)

Alteration	**Description**
Plasma alterations	Change in cellular plasma caused by degenerative fatty vacuolization, granular degeneration, glycogen degeneration, deposits and intracellular accumulation of substances caused by degenerative process
Nuclear alterations	Changes in the nuclear shape and structure
Atrophy	Reduction in number or volume of cells and/or decreasing of intracellular substances
Hypertrophy	Enlargement of cell volume or tissue

Index calculation was done using scoring of importance factor and score value. The alterations were classified into three importance factors (w) (Table 3 & 5). Values were assigned to these importance factors by considering the extent of reversibility of the liver alterations. For the reason that a widespread necrosis in Eel`s liver as a result of carbon tetrachloride was found to be regenerated completely within one month (Takashima and Hibiya, 1995) and since the expected alterations in this study were not as severe as necrosis, it was assumed that all selected and expected alterations in the present study are reversible. However, the extent of reversibility was assumed to be different for different alteration types.

Score values (a) were given based on the extent of occurrence of liver alterations. Every alteration was assessed using a score ranging from 0 to 6 (Table 4). Multiplication of importance factor (w) and score value (a) was used to quantify the specific alteration of the liver. All four selected liver alterations were quantified for every individual fish.

The sum of the multiplication of importance factor (w) and score value (a) was used to calculate the liver reaction index (LRI) of liver for every

individual fish. Hence, this index was determined using the formula below as a sum of the multiplied importance factors and score values of the alterations to assess the quality of liver morphological changes in response to overfeeding.

$$\text{Liver reaction index (LRI)} = \sum_{\text{alt}} (a \times w)$$

Where:

a= score value

w= importance factor

alt= alteration

Table 3.Importance factor description modified from Bernet *et al.* (1999)

Value	Description
1	Easily reversible- if exposure to stressor (overfeeding) ends
2	Moderately reversible- reasonably changed cell and assumed to take longer time to regenerate than the easily reversible
3*	Reversible- severely altered cell and will take longer time to regenerate than the other types

* This importance factor was not used in this study.

Table 4.Score Value Description adopted from Bernet *et al.* (1999)

Value	Description
0	Unchanged
2	Mild occurrence
4	Moderate occurrence
6	Severe occurrence

Table 5.Assigned importance factor (w) for selected alterations (Bernet *et al.*, 1999)

Importance factor (w)	Alterations
1	Plasma alterations and deposits
2	Nuclear alterations
2	Atrophy
1	Hypertrophy

Hepatosomatic Index (HSI) Determination

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

$$\text{Hepatosomatic Index (HSI)} = \frac{\text{Liver weight (g)}}{\text{Body weight (g)}} \times 100$$

Hepatohypertrophic Index (HHI) Determination

The HHI was calculated using the following formula (Lee, 2008). This index was adopted from Laboratory of Fish and Shellfish Pathology (LFSP), Pukyong National University, Korea. It has been used so far for histopathological health evaluation of fish and shellfish.

$$\text{Hepatohypertrophic Index (HHI)} = \frac{1}{\log (n)}$$

Where:

n = number of nucleus in 1000 μm^2 of hepatic tissue

Data Analysis

Histopathological description of morphological alterations and statistical results were used to present the research findings. Comparisons were made between controls and treatments as well as between different weeks` results. 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 all groups.

Results

Gross Liver Observation

Almost all of fish livers during the first dissection, before the start of feeding experiment, appeared to be normal (Fig. 1.A). No external abnormality was observed. Similar normal livers with brown color were observed from fish fed at their optimum requirement during the third and fifth week necropsies (Fig. 1.B & Fig. 3).

Whereas, after three weeks of overfeeding a relatively large sized livers with pale brown color (Fig. 2.A) were found. Shiny and oily livers were also seen during week three dissection of the overfed fish. Those abnormalities occurred after three weeks of overfeeding also happened to the fish which were overfed for five weeks (Fig. 2.B). The only difference was that in the fifth week overfeeding groups, the extent of occurrence of pale and oily appearances were relatively severe than the third week overfeeding counterparts.

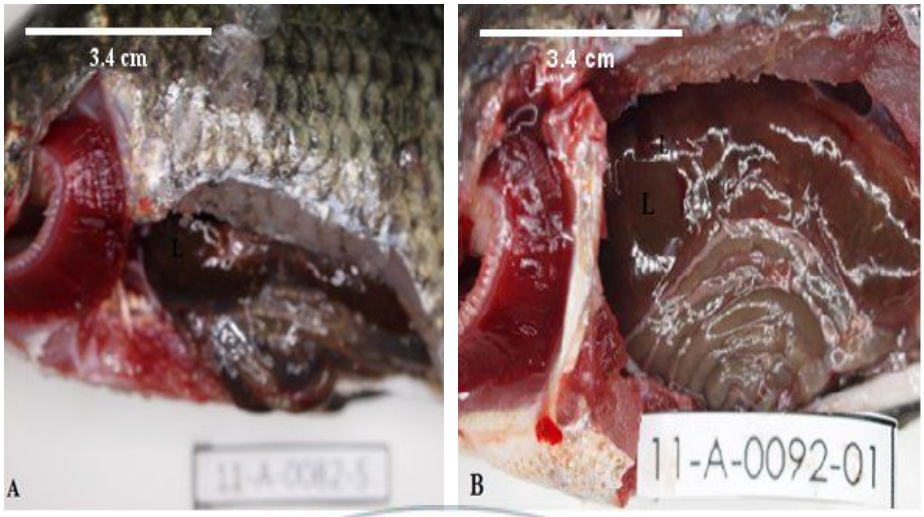


Fig.1. Gross appearance of liver from control groups. **A.** Before the beginning of feeding, **B.** Optimum feeding week three. Olympus, Japan; **L**-liver. Scale bar - 3.4 cm.

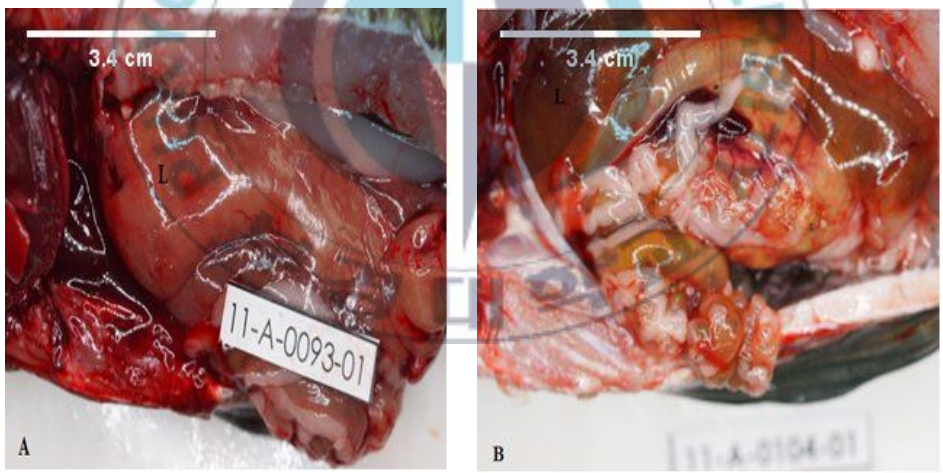


Fig.2. Gross appearance of liver after overfeeding. **A.** After three weeks of overfeeding, **B.** After five weeks of overfeeding. Olympus, Japan; **L** - liver. Scale bar - 3.4 cm.

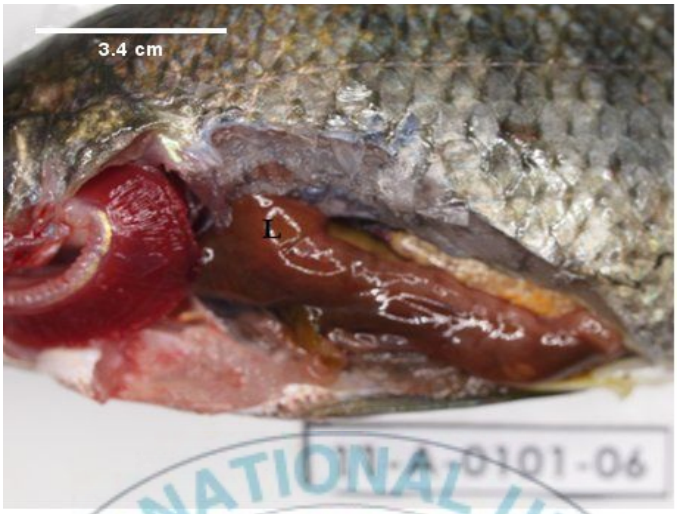


Fig.3. Gross appearance of liver from fifth week control. Olympus, Japan; **L** - liver. Scale bar - 3.4 cm.

Microscopic Liver Observation

Normal irregular shaped hepatocytes with very prominent circular nuclei (Fig. 4.A & B) were found from the fish which were dissected before the start of the feeding experiment. The same kinds of healthy hepatocytes with conspicuous centrally located circular nuclei were found from fish which were under optimum feeding regime during both third (Fig. 4.C & D) and

fifth week periods (Fig. 5.A & B). However, very few hepatocyte hypertrophy incidents were found after three weeks of optimum feeding. Hepatocytes from both overfeeding groups were structurally different from the non-overfed groups. Very large sized hepatocytes with nuclei dislocated to the cells border (Fig. 6.A, B & C) were observed from overfed groups during the third week necropsy.

Moreover, circular lipid vacuoles were also present together with large sized hepatocytes (Fig. 6.D) in some livers of the third week treatment group. Highly decreased hepatocyte volume and severe hepatocyte vacuolization (Fig. 7.A, B, C & D) were found from livers of fifth week overfeeding group.

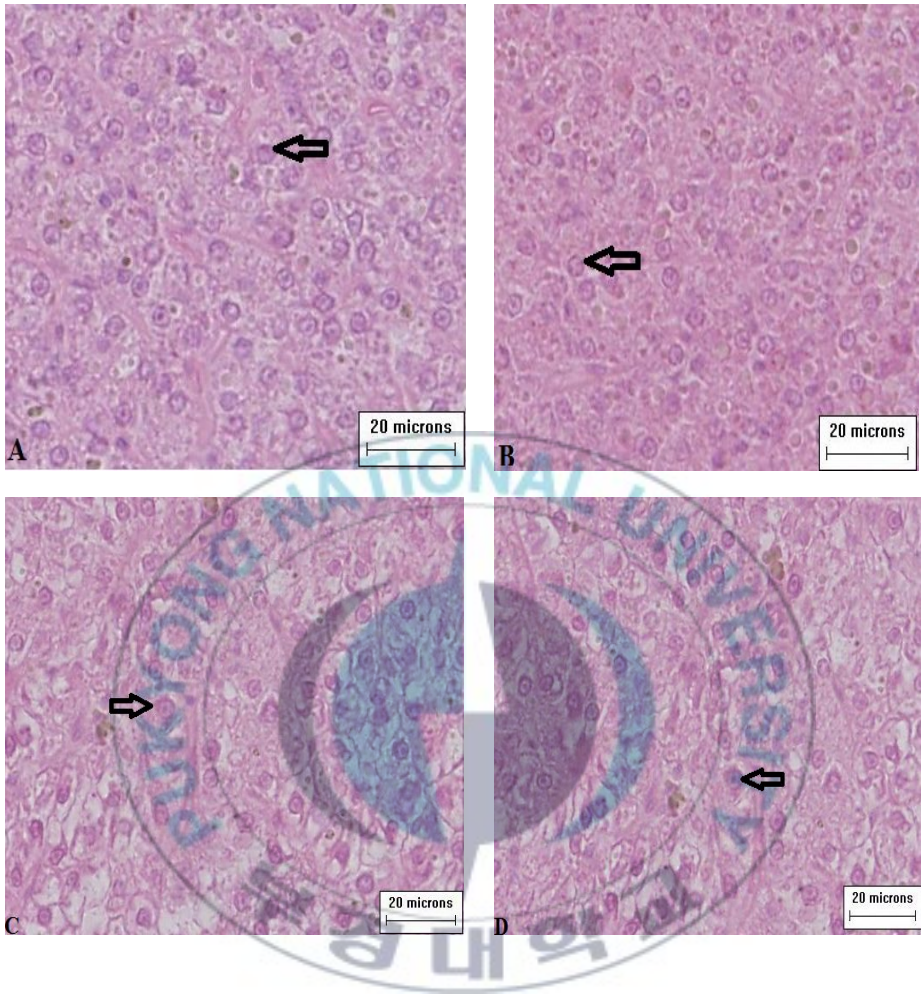


Fig.4. Liver microscopic structure before the beginning of feeding trial (**A** & **B**) and after three weeks of optimum feeding (**C** & **D**). Normal hepatocytes with conspicuous, circular and centrally located nuclei (arrows). (**H&E**; X400; Scale bars - 20 µm).

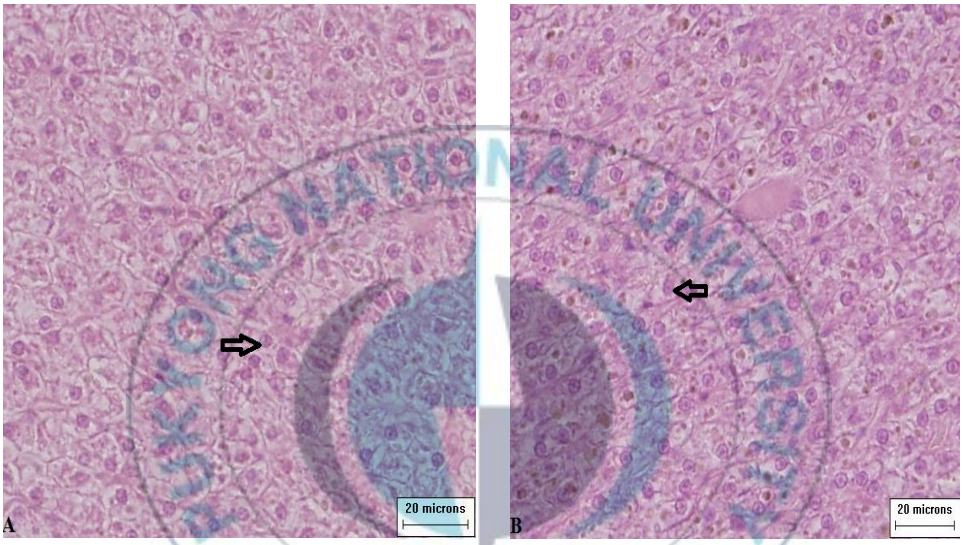


Fig.5. Liver microscopic structure after five weeks of optimum feeding; **A & B** - Normal hepatocytes with circular conspicuous and centrally located nuclei (arrows). (**H&E**; X400; Scale bars -20 μm).

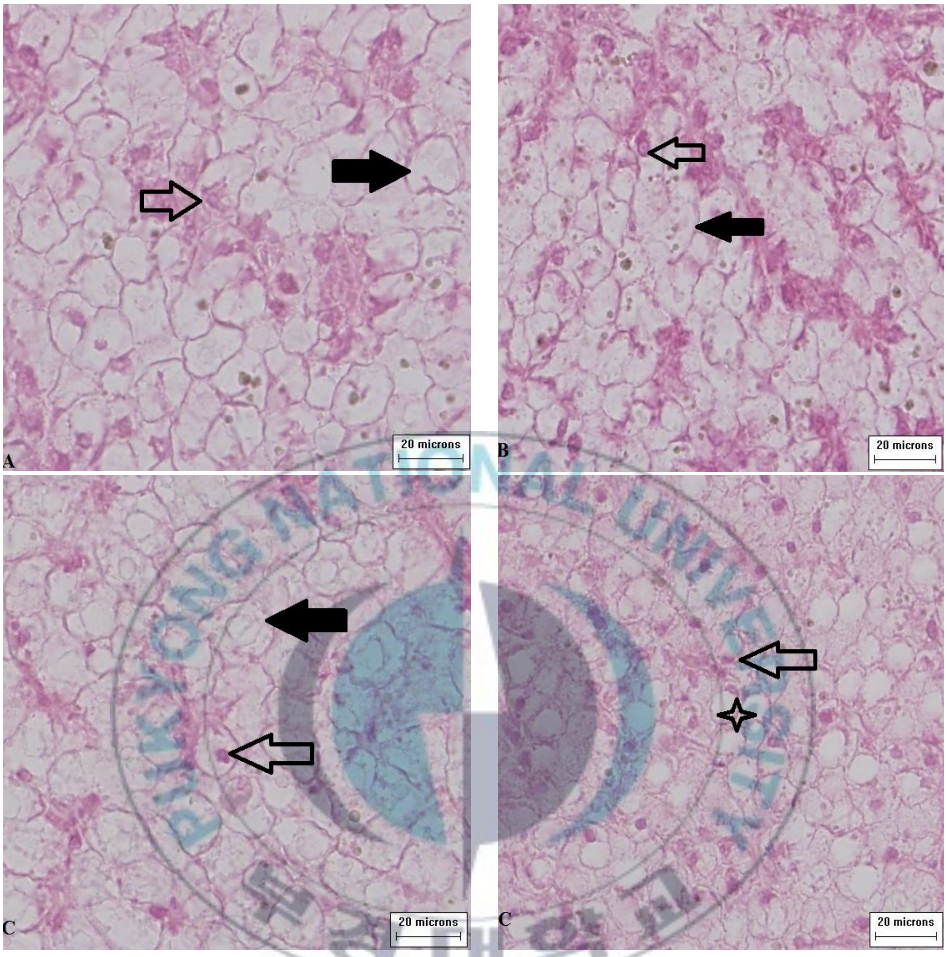


Fig.6. Liver microscopic structure after three weeks of overfeeding; **A**, **B**, **C** & **D** Large hypertrophic hepatocytes (solid arrows) with nuclei dislocated to cell periphery (arrows) and lipid vacuoles (four point star). (**H&E**; X400; Scale bars - 20 μm).

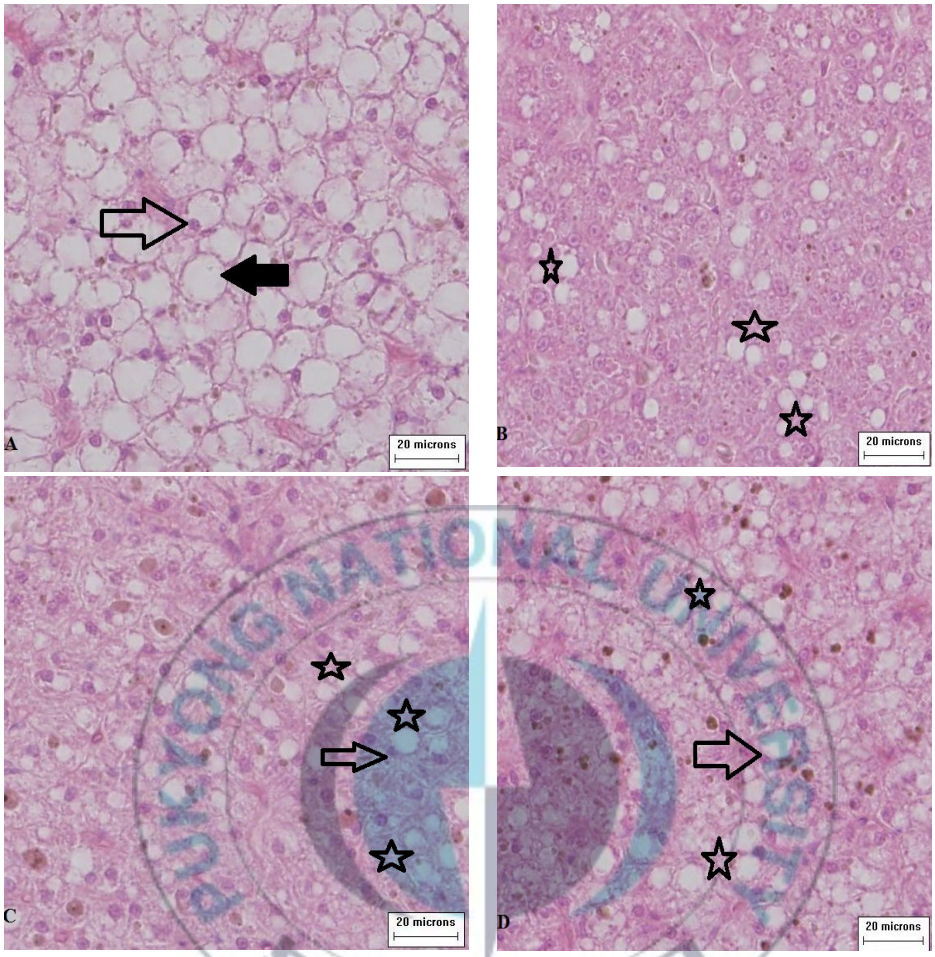


Fig.7. Liver microscopic structure after five weeks of overfeeding. **A** - Large hypertrophic hepatocytes with dislocated nuclei (arrow) & lipid vacuoles (solid arrow); **B**, **C** & **D** - Shrunken hepatocytes with inconspicuous dislocated nuclei (arrow) and circular lipid vacuoles (five point stars) (**H&E**; X400; Scale bars - 20 μm).

Liver Reaction Index (LRI)

The fish which were overfed for five weeks scored the highest mean liver reaction index (Table 6). The second largest mean liver reaction index was from fish under the overfeeding group during week three sampling. Significant variation (*p<0.05)* was observed between groups (Fig. 9). However, those fish dissected before the start of the feeding experiment and those which were fed optimally for five weeks showed no significant (*p<0.05)* difference, moreover, these two groups scored relatively low mean liver reaction index.

Table 6.Mean±SE of liver reaction index (*P<0.05*); mean values with different letters are significantly different

Feeding Group	**N**	**Liver Reaction Index**
Control Week Zero	12	2.67 ± 0.28^{d}
Control Week Three	9	7.56 ± 0.44^{c}
Treatment Week Three	9	16.44 ± 0.44^{b}
Control Week Five	17	2.35 ± 0.19^{d}
Treatment Week Five	16	24.25 ± 1.17^{a}

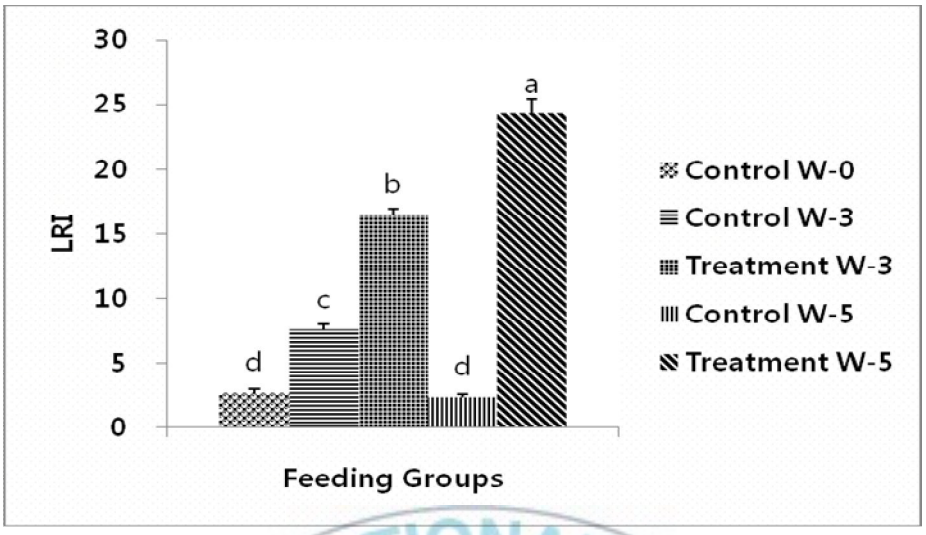


Fig.8. Liver reaction index (LRI) of all groups (*P<0.05*). **W**- Week.

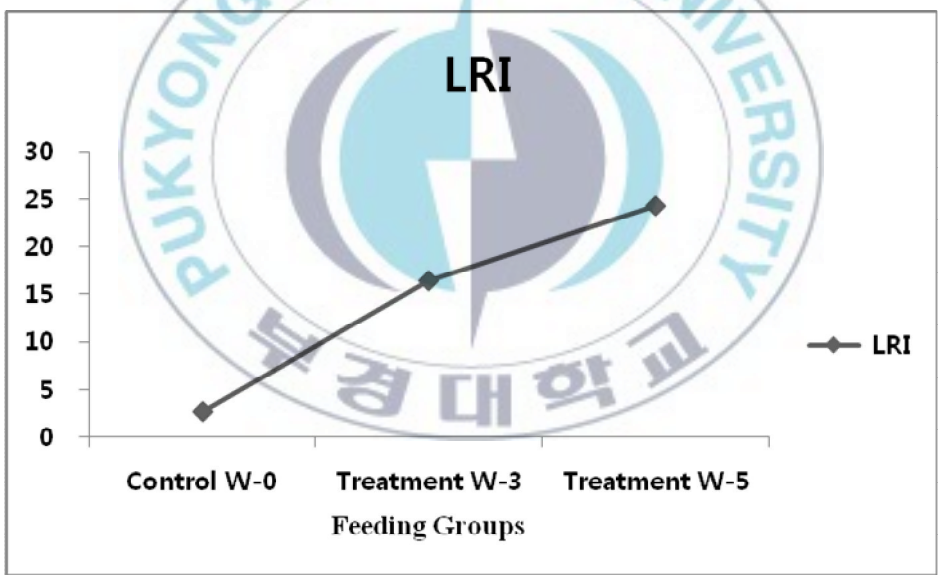


Fig.9. Liver reaction index (LRI) of fish before beginning of feeding trial (week-0) and both overfeeding groups. **W**- Week.

Hepatosomatic Index (HSI)

The hepatosomatic index for the third week overfeeding group was found extraordinarily higher (Table 7) than the other groups. Significant variation (*p<0.05)* between groups were observed. The control groups, before the beginning of feeding experiment and those which were under the optimum feeding level for five week appeared to have no significant (*p<0.05)* variation (Fig. 10). The mean hepatosomatic index for fish which were overfed for five weeks was by far lower (Table 7) than the week three overfeeding group.

Table 7.Mean±SE of hepatosomatic index (*P<0.05*); mean values with different letters are significantly different

Feeding Group	**N**	**Hepatosomatic Index**
Control Week Zero	12	1.04 ± 0.06^{d}
Control Week Three	9	1.95 ± 0.12^{b}
Treatment Week Three	9	3.07 ± 0.24^{a}
Control Week Five	17	1.11 ± 0.08^{d}
Treatment Week Five	16	1.52 ± 0.10^{c}

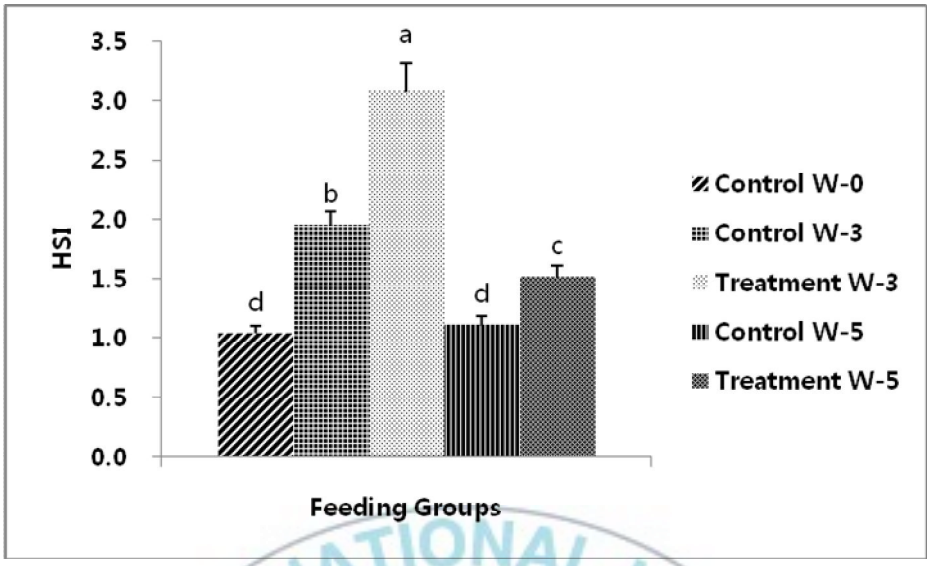


Fig.10. Hepatosomatic index (HSI) of all groups (P<*0.05*). **W**- Week.

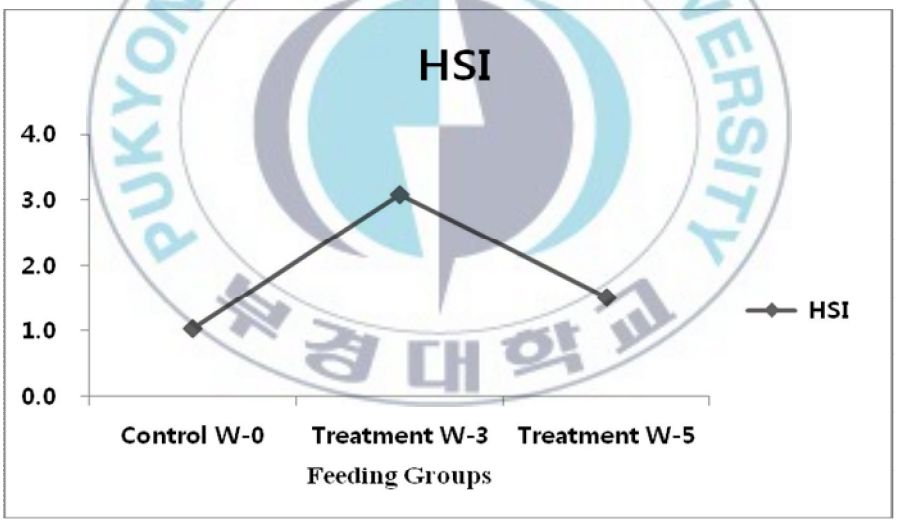


Fig.11. Hepatosomatic index (HSI) of fish before beginning of feeding trial (week-0) and both overfeeding groups. **W**- Week.

Hepatohypertrophic Index (HHI)

Mean hepatohypertrophic index for fish before the start of the feeding experiment and for both groups under optimum feeding regime (Fig. 12) showed no significant ($p<0.05$) difference. On the contrary, those fish which were overfed for three and five weeks found to have higher (Table 8) mean hepatohypertrophic indices. In general, significant ($p<0.05$) variation (Fig. 13) was observed between optimum feeding level category and the overfeeding one. However, the mean hepatohypertrophic index for the fifth week overfeeding group was significantly ($p<0.05$) lower than the third week overfeeding category.

Table 8.Mean±SE of Hepatohypertrophic index ($P<0.05$); means with different letters are significantly different

Feeding Group	N	Hepatohypertrophic Index
Control Week Zero	12	1.02 ± 0.06^{c}
Control Week Three	9	1.12 ± 0.04^{c}
Treatment Week Three	9	1.99 ± 0.27^{a}
Control Week Five	17	0.96 ± 0.03^{c}
Treatment Week Five	16	1.58 ± 0.14^{b}

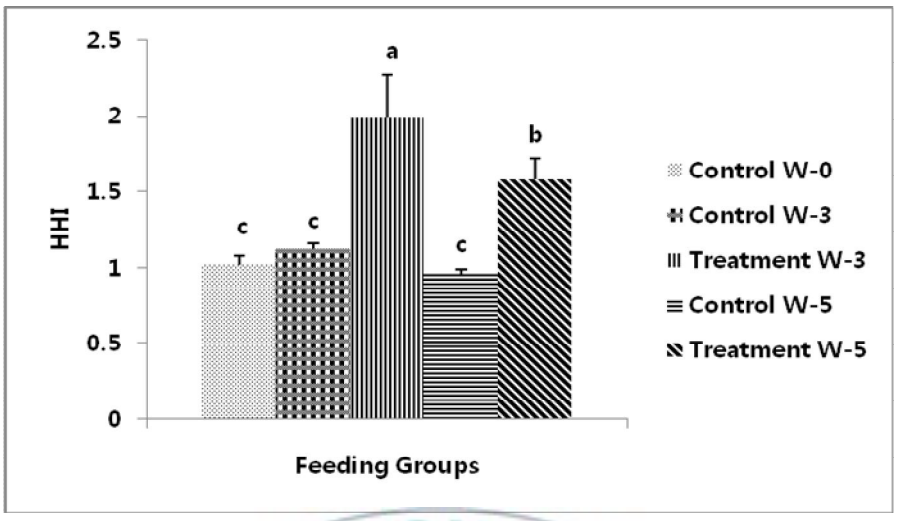


Fig.12. Hepatohypertrophic index (HHI) of all groups (P<*0.05*).

W- Week.

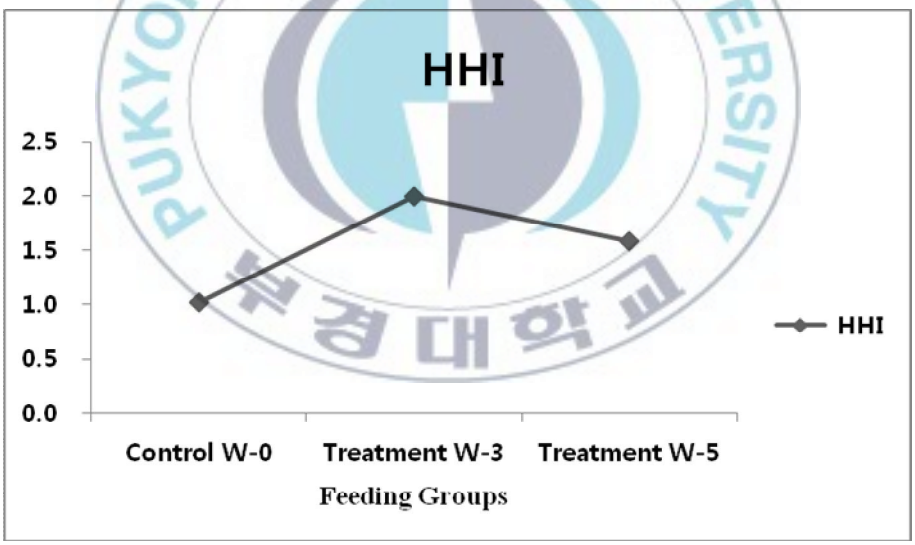


Fig.13. Hepatohypertrophic index (HHI) of fish before beginning of feeding (week-0) and both overfeeding groups. **W**- Week

Hematocrit and Hemoglobin

Mean hematocrit values of fish dissected before the beginning of the feeding experiment (Table 9) appeared to be significantly *($p<0.05$)* lower than other groups. However, no significant *($p<0.05$)* variation was found between fish under fifth week optimum feeding level and those which were being overfeeding for the same period of time. Mean hemoglobin values (Fig. 14) were not significantly different for fish sampled prior to feeding trial as well as for both fifth week feeding groups.

Table 9.Mean±SE of hematocrit and hemoglobin (*$P<0.05$*);

*significantly different from others

Feeding Group	N	Hematocrit (%)	Hemoglobin (g/dl)
Control Week Zero	12	22.5±1.96*	7.12±0.80
Control Week Five	17	37.53±1.32	7. 2±0.56
Treatment Week Five	16	38.5±1.01	7.35±0.63

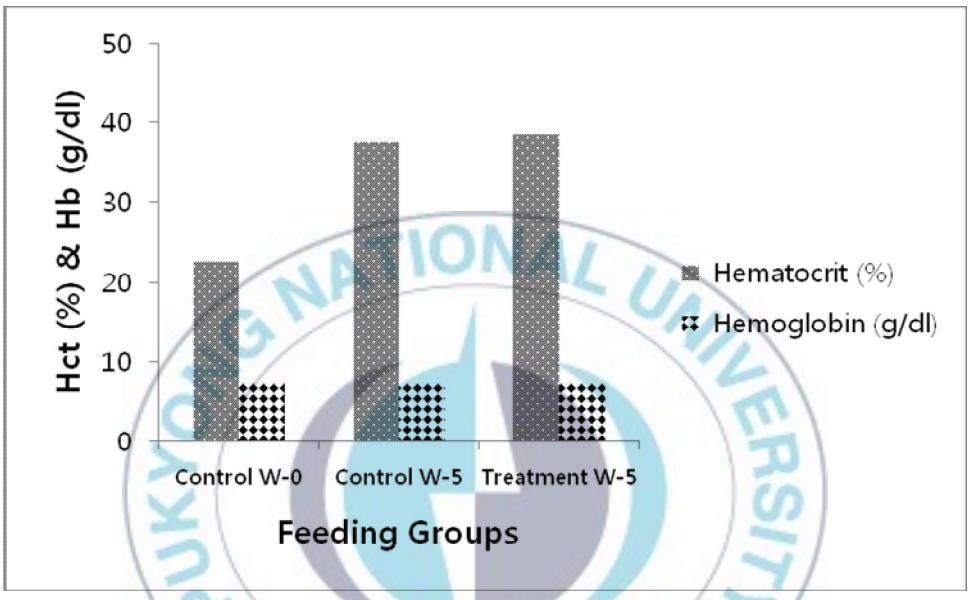


Fig.14. Hematocrit (Hct) and hemoglobin (Hb) values of week-0 control and fifth week feeding groups. W- Week

Discussion

The liver is the largest of the extramural (outside the alimentary canal) organs. Fish liver serves functions similar to those in mammals (Genten *et al.*, 2009). Its functions include assimilation of nutrients, production of bile, detoxification, and maintenance of the body metabolic homeostasis that includes processing of carbohydrates, proteins, lipids and vitamins.

The liver also plays a key role in the synthesis of plasma proteins (Takashima and Hibiya, 1995; Genten *et al.*, 2009; Jobling, 2012). Histological analysis of the digestive system is considered to be a good indicator of the nutritional status of the fish. The intestine and liver are most important organs in digestion and absorption of nutrients from food, and therefore monitoring these organs is considered necessary.

Pathologically, the functional disturbances produced by injury to cells are often mirrored by structural changes, just as, in turn, structural damage may be followed by loss or alteration of some normal function (Woolf, 2000). As a central metabolic organ with main function as digestive gland, it is clear that morphological and structural changes in fish liver affect its normal

functioning. Various stressors are capable of causing injuries to liver cells and affecting the general structure as well as normal functioning of liver. Heavy metals and toxic chemicals such as herbicides are among the stressors known to cause fish liver damage.

Nutritional imbalance is also among factors which cause liver alteration (Storch and Juario, 1983; Caballero *et al.*, 1999). It was indicated that hepatocytes of milkfish fry can alter their structure according to the feeding regime to an extent hitherto unknown among teleost fishes (Storch *et al.*, 1983). On a study which was done to establish a Zebrafish model for diet induced obesity, hepatosteatosis was found as a result of eight weeks of overfeeding with Artemia (Oka *et al.*, 2010).

In the present study, normal sized hepatocytes with no lipid vacuoles and with circular centrally located prominent nuclei (Fig. 4) were observed in almost all livers from the control groups. However, few hepatocyte hypertrophy cases were found within the control group during the third week optimum feeding trial. This hypertrophy was also detected in two of the indices used, LRI and HSI, but not expressed in the HHI. This mild liver

alteration is might be caused by the differences between the poor farming conditions of PKNU fish farm, from which the fish were obtained, and the experimental condition. There was no artificial heater fitted to the recirculating system of the farm and the water temperature of the concrete ponds was, below 16^{o}c, similar to the environmental temperature. For the reason *O. niloticus* has wide tolerance limit for water temperature variations (El-Sayed, 2006), the fish might have been adapted to the cold water which was below its optimum requirement. Considering the water temperature in experimental condition (27$\pm$1^{o}c) and taking into account non stressor-specific nature of cellular stress responses (Jobling, 2012), the slight hypertrophic livers observed during third week control might be results of the fish`s adaptation process to the experimental environment regardless of the one week acclimation.

Results of the fifth week control and week-0, before the beginning of the feeding trial, appeared to be normal. Mean LRI, HSI and HHI values (Fig. 8, 10 & 12) for these groups are not significantly different ($p<0.05$). Healthy hepatocytes with large spherical centrally located nuclei were found from

these groups. Healthy hepatocytes observed in all control groups are consistent with those reported normal by Sadekarpawar and Parikh (2013).

Large hypertrophic hepatocytes (Fig. 6) as well as high mean LRI value were observed after three weeks of overfeeding. Mean HSI and HHI values (Fig. 8 & 12) were higher too in third week`s overfeeding group than the other groups. Higher mean HSI and HHI during third week treatment indicates the extent of hypertrophy occurred after three weeks of overfeeding. High HSI indirectly shows the extent to which hepatocytes were swollen whereas; high HHI indicates very small number of nuclei per $1000\mu m^2$ of hepatic tissue. Hepatocyte vacuolization as a result of lipid vacuoles and atrophy (Fig. 7) were found after five weeks of overfeeding. In fact, feeding level dependent fatty degeneration of liver, which agrees with the findings of the present study, in red Seabream was reported by Mobin *et al.* (2001).

For the reason that general liver histomorphological alterations occurred during week five overfeeding group were relatively severe than the third week counterpart, mean LRI (Fig. 8) appeared to be high for this group.

Hepatocyte size reduction as a result of atrophy contributed to the significantly ($p<0.05$) lower HSI (Fig. 10) of the fifth week treatment group when compared with the third week treatment. In fact, this shows how histological descriptions can be affirmed by the use of indices.

Direct comparison of the present study with the previous ones appeared to be difficult for the reason studies pertinent to the influence of overfeeding on liver histomorphology are rare. However, diet induced liver alterations such as hepatocytic hypertrophy and fatty degeneration which agree with findings of the present study were reported for different fish species (Caballero *et al.*, 1999; Morais *et al.*, 2001; Ruyter *et al.*, 2006; Ferri *et al.*, 2011; Hu *et al.*, 2013).

Similarity between liver morphological alterations of this study and other histopathological studies in response to toxic chemicals was observed. Hypertrophic hepatocytes with disintegrated nuclei in *O. niloticus* was found after acute exposure to zinc chloride (Abdel-Warith *et al.*, 2011) and sub-chronic alachlor (Peebua *et al.*, 2008). Fatty degeneration of liver of *O. niloticus* was also resulted after being exposed to extracts of *Ipomoea*

aquatica leaf (Ayoola, 2011), and toxic Cyanobacterium (Fahprathanchai *et al.*, 2007). Vacuolar degeneration of hepatocytes of *O. niloticus* and tilapia Zilli was observed from El-Salam Canal of Egypt (Mohamed, 2003) as a result of water contamination from agricultural and industrial wastes. Considering hepatocyte structural changes as cellular responses to stressors, the similarity could be occurred because of the non stressor-specific nature of cellular stress responses (Jobling, 2012). In other words, hepatocyte hypertrophy and fatty degeneration induced by various toxic chemicals happened to be similar to the alterations found in the present study. It might be because of the fact that many stressors can provoke similar cellular responses.

Stressors to the liver are known to disrupt the microcirculation of the hepatic parenchyma. In the present study, overfeeding in the first few weeks might have increased the transport of nutrients, mainly free fatty acids, to liver cells from stomach and intestine via hepatic portal vein. Increased nutrient inflow means increased work load for these cells and this situation puts them on a hyperfunctional condition. It is known that free fatty acids from ingested food are normally transported into hepatocytes, where they

are esterified to triglycerides, converted into cholesterol or phospholipids, or oxidized to ketone bodies.

Hence, esterifying as well as oxidizing the excess inflow of fatty acids and other nutrients might have made hepatocytes very busy. Hypertrophy is a cellular response which occurs during increased functional demand (Takashima and Hibiya, 1995; Kumar *et al.*, 2007). Therefore, the hypertrophy observed in the present study after three weeks of overfeeding might have occurred as a cellular stress response to increased inflow of nutrients to liver cells (Fontagné *et al.*, 1998) and increased workload.

Swelling of hepatocytes as a result of hypertrophy might have caused narrowing of sinusoids and disse spaces. Disse space is perisinusoidal space located between hepatocytes and sinusoids. Hepatocytes get blood and oxygen via sinusoids from hepatic portal vein and artery. The blood reaches to hepatocytes from sinusoids through disse spaces. Narrowing of sinusoids and disse spaces as a result of swelling hepatocytes likely caused obstruction of blood and oxygen flow to hepatocytes (Hwang, 2011) . Increased nutrient inflow and obstructed blood as well as oxygen supply

could lower the metabolizing capacity of hepatocytes. This probably led to deposition of lipid in hepatocytes and fatty degeneration. 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 and Hibiya, 1995).

Eventually hepatocytes become in a hypofunctional condition. Decreased workload and low blood supply are known to cause atrophy to cells (Takashima and Hibiya, 1995; Kumar *et al.*, 2007). For this reason, the lipid vacuolation and atrophy observed in this study after five weeks of overfeeding might have happened because of poor metabolizing capacity of hepatocytes as a result of obstruction of sinusoidal blood flow.

Hemoglobin and hematrocrit values determined before the beginning of the feeding trial were within the normal range (Soltan and El-Bab, 2008). However, no significant ($p<0.05$) difference of mean hemoglobin values was detected (Fig. 15) between week zero control, fifth week control and the fifth week of overfeeding group. Similar trend in hemoglobin was also

observed in *Tilapia mossambica* after mercury exposure (Cyriac *et al.*, 1989).

In fact, the effect of high protein as well as high carbohydrate diets on serum chemistry indicators such as glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), total protein and glucose was reported to be insignificant in juvenile olive flounder (Cho, 2012). Even though further investigation is needed, histopathology might allow detection of problems related to health condition of a fish prior to the expression of those problems in the blood chemistry.

Mean hematocrit values (Fig. 15) of the fifth week feeding groups were found to be significantly ($p<0.05$) higher than the week zero control. It was indicated previously that hematological parameters of adult *O. niloticus* are generally higher than those of the juvenile (Gabriel *et al.*, 2011). Hence, the observed elevated values of hematocrit during the fifth week could be because of the effect of age on hematological parameters of *O. niloticus*.

Conclusion

The outcome of this study claims that overfeeding is a direct threat to liver of *O. niloticus*. Microscopic liver structural changes mainly hepatocyte hypertrophy and lipid vacuolization have been observed. Some livers which are entering to atrophic condition were also found. All these alterations are regarded as histomorphological alterations deviated from the normal being.

Observed morphological changes are affirmed by indices, namely liver reaction index (LRI), hepatosomatic index (HSI) and hepatohypertrophic index (HHI). In addition, the statistical analysis clearly showed the pattern of liver histological alterations.

Therefore, from results obtained, it might be concluded that overfeeding of the Nile tilapia, *O. niloticus* with commercial feed causes morphological alterations to the liver. Liver histomorphological changes observed in the present study are believed to be good enough to disrupt the normal functioning of the liver. Taking in to account the role of liver in the general physiology of fishes, it is not incorrect to conclude that affected liver can lower the fish's resistance to disease outbreaks.

In fact, overfeeding is known to affect the fish`s health indirectly by deteriorating the water quality. However, its direct influence on the wellbeing of fishes and in turn on the aquaculture industry is also obvious.

The present study emphasized on liver microscopic structural changes in response to overfeeding. Similar studies at gene expression level are required to be done for better understanding of the adverse consequences pertinent to excessive feeding in fish. Comparison and correlation of histological as well as immunological assay results are also recommended to be done to clearly show how the healthiness of the fish gets affected by overfeeding.

Acknowledgement

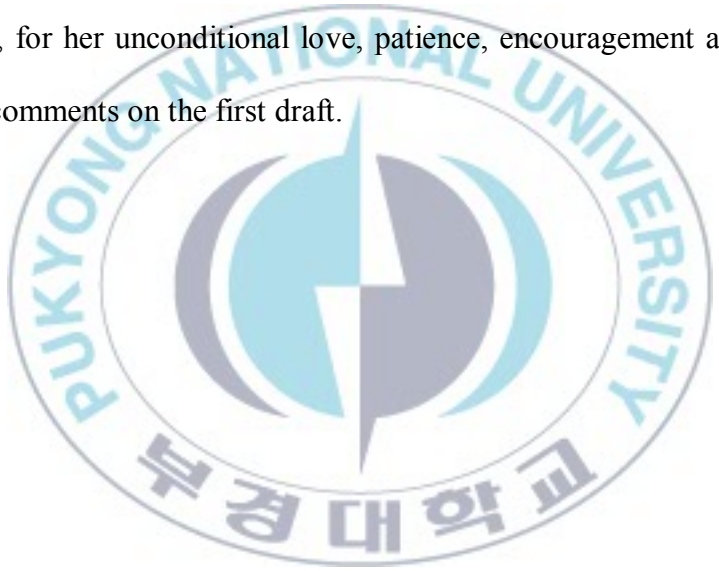
First and foremost, Praise be unto God and His Mother Saint Virgin Mary for everything in my life. Next, I am wholeheartedly thankful to my supervisor Professor Min Do HUH for his support, guidance and appreciation along all the way of research and thesis writing. It is also my pleasure to express my gratefulness to Korea International Cooperation Agency (KOICA) which granted me a scholarship to undertake M.Sc. study.

The debt that I owe to KOICA program coordinator, Dr. Kyoungmi KANG and her assistant Mr. Hyun Ki KIM, cannot be easily described. They were always helpful in every single problem I encountered during my stay in Korea. I must also offer my gratefulness to Professor Hyun Woo KIM who introduced me to Endnote software.

My deep gratitude goes as well to my laboratory captain Kim Bo Sung who taught me histological techniques. I like to extend my gratitude to Najib for his support and friendship. I also want to thank Dr. Mu Kun LEE for his valuable comments.

I am truly thankful to my fellow laboratory members, Seon Ju, Su Yeon, Heung Jun, Eun Young and Semyung. I would also like to say thank you to Paulos for his valuable comments on the second draft as well for introducing me to SPSS. Special thanks go to Mahider for her hospitality and encouragement throughout the whole study period.

Finally, I would like to express my profoundest gratitude to my wife, Edom Wondimu, for her unconditional love, patience, encouragement and for her priceless comments on the first draft.



References

ABDEL-WARITH, A. A., YOUNIS, E. M., AL-ASGAH, N. A. & WAHBI, O. M. 2011. Effect of zinc toxicity on liver histology of Nile tilapia, *Oreochromis niloticus*. *Scientific Research and Essays,* 6**,** 3760 - 3769.

ASDARI, R., ALIYU-PAIKO, M. & HASHIM, R. 2011. Effects of different dietary lipid sources in the diet for *Pangasius nasutus* (Bleeker, 1863) juveniles on growth performance, feed efficiency, body indices and muscle and liver fatty acid compositions. *Aquaculture Nutrition,* 17**,** e883 - e891.

AYOOLA, S. O. 2011. Histopathology of Nile Tilapia (*Oreochromis niloticus*) juveniles exposed to aqueous and ethanolic extracts of Ipomoea aquatica leaf. *International Journal of Fisheries and Aquaculture,* 3**,** 244 - 257.

BERNET, D., SCHMIDT, H., MEIER, W., BURKHARDIT-HOLM, P. & WAHLI, T. 1999. Histopathology in Fish: Proposal for a Protocol to Asses Aquatic Pollution. *Journal of Fish Disease,* 22**,** 25 - 34.

BOYD, C. E. 2004. Farm-Level Issues in Aquaculture Certification: Tilapia. *In:* MEYER, D. E. (ed.). Auburn, Alabama: WWF-US.

CABALLERO, M. J., G. LO´PEZ-CALERO, J. SOCORRO , F.J. ROO, IZQUIERDO, M. S. & FE´RNANDEZ, A. J. 1999. Combined effect of lipid level and fish meal quality on liver histology of gilthead seabream (*Sparus aurata*). *Aquaculture,* 179**,** 277 - 290.

CATALDI, E., CATAUDELLA, S., G. MONACO, ROSS, A. & TANCIO, L. 1987. A study of the histology and morphology of the digestive tract of the sea-bream, *Sparus aurata*. *J. Fish Biol,* 30**,** 135 - 145.

CHO, S.-H. 2012. Effects of Dietary Nutrient on the Biological Index and Serum Chemistry of Juvenile Olive Flounder *Paralichthys olivaceus* Achieving Compensatory Growth. *Fisheries and aquatic sciences,* 15**,** 69 - 72.

CHOWDHURY, D. K. 2011. *Optimal feeding rate for Nile tilapia (Oreochromis niloticus)*. M.Sc., Norwegian University of Life Sciences.

COYLE, S. D., DURBOROW, R. M. & TIDWELL, J. H. 2004. Anesthetics in Aquaculture. Southern Regional Aquaculture Center (SARC).

CYRIAC, P. J., ANTONY, A. & NAMBISAN, P. N. K. 1989. Hemoglobin and hematocrit values in the fish *Oreochromis mossambicus* (peters)

after short term exposure to copper and mercury. *Bulletin of Environmental Contamination and Toxicology,* 43**,** 315 - 320.

DEEDS, J. R., REIMSCHUESSEL, R. & PLACE, A. R. 2006. Histopathological Effects in Fish Exposed to the Toxins from *Karlodinium micrum*. *Journal of Aquatic Animal Health,* 18**,** 136 - 148.

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

EL-SAYED, A.-F. M. 2006. *Tilapia Culture,* UK, CABI Publishing.

FAHPRATHANCHAI, P., KANOKPORN SAENPHET SALIKA ARITAJAT, SUPAP SAENPHET, MENG-UMPHAN, K. & SUDWAN, P. 2007. Histopathological and hematological evaluation of Nile tilapia (*Oreochromis niloticus*) exposed to a toxic Cyanobacterium (*Microcystis aeruginosa*). *Southeast Asian J Trop Med Public Health,* 38**,** 245 - 248.

FAO 2012. The State of the world fisheries and aquaculture. Rome, Italy: Food and Agriculture Organization of the United Nations.

FERGUSON, H. W. 2006. *Systemic pathology of fish: a text and atlas of normal tissues in teleosts and their responses in diseases*, Scotian Press.

FERRI, J., TOPIĆ POPOVIĆ, N., ČOŽ-RAKOVAC, R., BEER-LJUBIĆ, B., STRUNJAK-PEROVIĆ, I., ŠKELJO, F., JADAN, M., PETRIĆ, M., BARIŠIĆ, J., ŠIMPRAGA, M. & STANIĆ, R. 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.

FONTAGN , S., GEURDEN, I., ESCAFFRE, A.-M. & BERGOT, P. 1998. Histological changes induced by dietary phospholipids in intestine and liver of common carp (*Cyprinus carpio* L.) larvae. *Aquaculture,* 161**,** 213 - 223.

GABRIEL, U. U., AKINROTIMI, O. A. & ESEIMOKUMO, F. 2011. Haematological Responses of Wild Nile Tilapia *Oreochromis niloticus* after Acclimation to Captivity. *Jordan Journal of Biological Sciences,* 4**,** 225 - 230.

GAO, Y. & LEE, J.-Y. 2012. Compensatory Responses of Nile Tilapia *Oreochromis niloticus* under Different Feed-Deprivation Regimes. *Fisheries and aquatic sciences,* 15**,** 305 - 311.

GENTEN, F., TERWINGHE, E. & DANGUY, A. 2009. *Atlas of Fish Histology,* India, Science Publishers.

HADI, A. A. & ALWAN, S. F. 2012. Histopathological changes in gills, liver and kidney of fresh water fish, Tilapia zillii, exposed to aluminum. *International journal of pharmacy & life sciences,* 3**,** 2071 - 2081.

HTUN-HAN, M., T 1978. The reproductive biology of the dab *Limanda limanda* (L) in the North Sea; gonadosomatic Index; Hepatosomatic Index and condition factor. *J. Fish Biol,* 13**,** 369 - 378.

HU, L., YUN, B., XUE, M., WANG, J., WU, X., ZHENG, Y. & HAN, F. 2013. Effects of fish meal quality and fish meal substitution by animal protein blend on growth performance, flesh quality and liver histology of Japanese seabass (*Lateolabrax japonicus*). *Aquaculture,* 372 – 375**,** 52 - 61.

HWANG, S. 2011. Microcirculation of the Liver. *In:* MADOFF, D. C., MAKUUCHI, M., NAGINO, M. & VAUTHEY, J.-N. (eds.) *Venous Embolization of the Liver.* Springer London.

JALALUDEEN, M. D., ARUNACHALAM, M., RAJA, M., NANDAGOPAL, S., SHOWKET AHMAD BHAT, SUNDAR, S. & PALANIMUTHU, D. 2012. Histopathology of the gill, liver and kidney tissues of the freshwater fish *tilapia mossambica* exposed to cadmium sulphate. *International Journal of Advanced Biological research,* 2**,** 572 - 578.

JOBLING, M. 2012. R. J. Roberts (ed): Fish pathology. *Aquaculture International,* 20**,** 811 - 812.

KUMAR, V., ABBAS, A. K., FAUSTO, N. & MITCHELL, R. 2007. *Robbins Basic Pathology*, Elsevier Health Sciences.

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

MASSER, M. P., RAKOCY, J. & LOSORDO, T. M. 1992. Recirculating Aquaculture Tank Production Systems Management of Recirculating Systems. United States: Southern Regional Aquaculture Center (SARC).

MOBIN, S. M. A., KANAI, K. & YOSHIKOSHI, K. 2001. Effects of Feeding Levels on the Pathological Alterations in the Digestive System and Mortality of Larvae and Juveniles of Pagrus major. *Journal of Aquatic Animal Health,* 13**,** 202 - 213.

MOHAMED, F. A. E.-M. S. 2003. Histopathological studies on some organs of *Oreochromis niloticus*, tilapia zillii and *synodontis schall* from el-salam canal, egypt. *Egypt. J. Aquat. Biol & Fish.,* 7**,** 99 - 138.

MOHANTY, R. K. 2001. Feeding management and waste production in semi-intensive farming of *penaeus monodon* (fab.) at different stocking densities. *Aquaculture International* 9**,** 345 - 355.

MORAIS, S., BELL, J. G., ROBERTSON, D. A., ROY, W. J. & MORRIS, P. C. 2001. Protein/lipid ratios in extruded diets for Atlantic cod (*Gadus morhua* L.): effects on growth, feed utilisation, muscle composition and liver histology. *Aquaculture,* 203**,** 101 - 119.

NRC 1993. Nutrient Requirements of Fish. Washington, D.C.: Subcommittee on Fish Nutrition, National Research Council, National Acad. of Sciences.

OKA, T., NISHIMURA, Y., ZANG, L., HIRANO, M., SHIMADA, Y., WANG, Z., UMEMOTO, N., KUROYANAGI, J., NISHIMURA, N.

& TANAKA, T. 2010. Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. *BMC Physiol,* 10**,** 1 - 13.

PEEBUA, P., KRUATRACHUE, M., POKETHITIYOOK, P. & SINGHAKAEW, S. 2008. Histopathological alterations of Nile tilapia, *Oreochromis niloticus* in acute and subchronic alachlor exposure. *Journal of Environmental Biology,* 29**,** 325 - 331.

PHILLIPS, A. M. & PODOLIAK, H. A. 1957. The Nutrition of Trout: III. Fats and Minerals. *The Progressive Fish-Culturist,* 19**,** 68 - 75.

PILLAY, T. V. R. & KUTTY, M. N. 2005. *Aquaculture Principles and Practices,* India, Blackwell.

RASKOVIC, B., STANKOVIC, M., MARKOVIC, Z. & POLEKSIC, V. 2011. Histological methods in the assessment of different feed effects on liver and intestine of fish. *Journal of Agricultural Sciences, Belgrade,* 56**,** 87 - 100.

RICHE, M., HALEY, D. I., OETKER, M., GARBRECHT, S. & GARLING, D. L. 2004. Effect of feeding frequency on gastric evacuation and the return of appetite in tilapia *Oreochromis niloticus* (L.). *Aquaculture,* 234**,** 657- 673.

ROBINSON, E. H., LI, M. H. & BRUNSON, M. W. 1998. Feeding Catfish in Commercial Ponds. *Southern Regional Aquaculture Center Publication.* Stoneville, Mississippi, USA.: Southern Regional Aquaculture Center (SARC).

RUYTER, B., MOYA-FALC N, C., ROSENLUND, G. & VEGUSDAL, A. 2006. Fat content and morphology of liver and intestine of Atlantic salmon (*Salmo salar*): Effects of temperature and dietary soybean oil. *Aquaculture,* 252**,** 441 - 452.

SADEKARPAWAR, S. & PARIKH, P. 2013. Gonadosomatic and Hepatosomatic Indices of Freshwater Fish *Oreochromis mossambicus* in Response to a Plant Nutrient. *World Journal of Zoology* 8**,** 110 - 118.

SOLTAN, M. A. & EL-BAB, A. F. F. 2008. Effect of dietary replacement of fish meal by mixture of different plant protein sources on growth performance and some blood parameters of Nile tilapia, *Oreochromis niloticus*. *Egypt. J. Aquat. Biol. & Fish,* 12**,** 1 - 16.

STORCH, V. & JUARIO, J. V. 1983. The effect of starvation and subsequent feeding on the hepatocytes of *Chanos chanos* (Forsskal) fingerlings and fry. *Journal of Fish Biology,* 23**,** 95 - 103.

STORCH, V., ST HLIN, W. & JUARIO, J. V. 1983. Effect of different diets on the ultrastructure of hepatocytes of *Chanos chanos* fry (Chanidae: Teleostei): an electron microscopic and morphometric analysis. *Marine Biology,* 74**,** 101 - 104.

TAKASHIMA, F. & HIBIYA, T. 1995. *An atlas of fish histology: normal and pathological features*, Kodansha Ltd.

VERRETH, J., COPPOOLSE, J. & SEGNERBT, H. 1994. The effect of low HUFA- and high HUFA-enriched Artemia, fed at different feeding levels, on growth, survival, tissue fatty acids and liver histology of *Clarias gariepinus* larvae. *Aquaculture* 126**,** 137 - 150.

VICENTINI, C. A., FRANCESCHINI-VICENTINI, I. B., BOMBONATO, M. T. S., BERTOLUCCI, B. L., S. G. & & SANTOS, A. S. 2005. Morphological Study of the Liver in the Teleost *Oreochromis niloticus*. *Int. J. Morphol.,* 23**,** 211 - 216.

WOOLF, N. 2000. *Cell, Tissue, and Disease: The Basis of Pathology*, Saunders W B Company.

XU, W., LIU, W., SHAO, X., JIANG, G. & LI, X. 2012. Effect of trichlorfon on hepatic lipid accumulation in crucian carp *Carassius auratus gibelio*. *J Aquat Anim Health,* 24**,** 185 - 94.