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HISTOPATHOLOGICAL HEALTH ASSESSMENT OF FARMED OLIVE FLOUNDER *PARALICTHYES OLIVACEUS*

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Abstract

A histopathological health assessment for olive flounder *Paralicthyes olivaceus*, with different size based on total body length and weight, was carried out. Two samplings were performed: (I) on 4th October 2010 and (II) on 25th January 2011. Two groups of fishes were administered for the study from the same culture system and age group- the big sized fishes (fast growing) and the small sized fishes (slow growing). Clinical record, blood chemistry, total protein (Tp), hemoglobin (Hb), hematocrit (Ht), functional indices i.e., hepato somatic index (HSI) and hepato hypertropic index (HHI), and histological studies of different organs (liver, stomach, intestine, spleen, kidney and heart) were performed for each individual of each group. Moderate to severe fatty change of liver were observed in both groups of fishes in case of sampling I. Moderate to severe atrophy of liver were observed in both groups of sampling II. Focal epicarditis were observed in sampling I for big sized fishes, and vacuolative changes of gastric gland of big sized fishes were observed in sampling II under light microscopic examination. Tp, Ht, and HHI were recorded significantly higher (p values ranged from 0.001 to 0.032) for big sized fish group in sampling I. In sampling II only HHI was found significantly higher ($p = 0.004$) in big sized fish group. Thus, it can be concluded that fast growing individuals are more vulnerable to disease based on the histopathological assessment. This information will be helpful to prevent and control diseases for different age groups of olive flounder by taking different management practices.

Keywords: Fatty change, epicarditis, vacuolative change, olive flounder, *Paralichthyes olivaceus*.

Introduction

Fish disease is a common problem in aquaculture. A basic understanding of the nature of fish disease is important for the farm operator. So farm operators should be well equipped to prevent and handle diseases outbreak. Fish like other animals are prone to a variety of diseases. Fish also can suffer from environmental and nutritional diseases. Fish disease is the result of interaction among fish, pathogen and stressful environment (Snieszko, 1974). Disease outbreaks occur when there are adverse physiological changes, which can have either infectious or noninfectious causes. Pathological alterations in the body are important evidence of physiological changes. A clear understanding of histopathology and path physiology is necessary to determine the real causes of diseases. For the above mentioned cases histological changes occur comparing to the normal situation. That is the interest of the histopathological study. Previously, animal histology aimed primarily to morphologically to clarify the fine structures of the body. However, more recently, the main aims of histology changed to focus on the study of the functions of the body at the tissue level and the clarification of the physiological functions from the view point of cellular correlation. The main purpose of histopathology is to diagnose disease from pathological changes in tissue level (Patino and Takashima, 1995). Histopathological analysis appears to be a very sensitive parameter and is crucial in determining cellular changes that may occur in target organ. Therefore, a histological investigation may prove to be cost effective tool to determine the health of fish population, hence reflecting the health of an entire aquatic ecosystem (Velkova-Jordanoska and Kostoski, 2005).

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Liver is the main focus of the present study. Liver is the most functional organ of fish as well as other animals, as nutrients are stored, absorbed and processed for the use of other organ through liver. It also plays a pivotal role for gathering, transforming and accumulating metabolites. Liver has an important role in maintaining body's metabolic homeostasis that includes processing of carbohydrate, protein, lipids and vitamins. The liver also plays a key role in detoxification and in the synthesis of serum protein like albumin, fibrinogen, complement factors and acute phase proteins (Patino and Takashima, 1995, Akiyoshi and Inoue, 2004). Stomach and intestine are also focused for the study as they have strong relation with liver. Other organs (kidney, heart and spleen) and blood chemistry are also histopathologically considered as biomarkers for assessing the susceptibility of disease.

The olive flounder (*Paralichythys olivaceus*) is a key marine finfish species cultured in the flow-through system of land based facilities in coastal areas of Korea, Japan and China (Fisheries Agency and Japan Sea-Farming Association, 1993). The species was found to be affected by different diseases like scuticociliatosis and lymphocystis diseases (Hossain, 2007; Kim *et al.,* 2004). Limited researches have been conducted regarding histology and histopathology of olive flounder, especially in terms of health assessment based on different age groups and habitats. The aim of the present study was to investigate histopathological health status of olive flounder of the same age and culture system with different sized fishes in terms of total length and body weight to know the susceptibility and intensity of infection for proper health management.

Materials and Methods

Two categories of fishes were chosen from same age group and culture system according to their body weight and total length, considering the intensity of infection may differ for age groups and culture systems. Six big sized fish group (fast growing) with the average length of 27.58 cm and 26.70 cm and the average weight of 221.83 g and 404.00 g at sampling I and II, respectively. Six small sized fish group (slow growing) with the average length of 22.58 cm and 22.03 cm and the average weight of 125.83 g and 215.00 g at sampling I and II, respectively were utilized. Six fishes were selected in each group for more accuracy. Liver and stomach were taken as focus organ for the study. Other organ such as, intestine, spleen, heart, body kidney, gill and functional indices i.e. total protein (Tp), hemoglobin (Hb), hematocrit (Ht), hepato somatic index (HSI) and hepato hypertropic index (HHI), were also taken in consideration for clear clarification. To pursue the experiment and processing of tissue for Histopathological study, the following chronological steps were followed.

Sample collection: Fishes were collected from a local fish farm named Dongji fish farm, Gijang in Busan and subsequently carried to the laboratory in polyethylene bag with oxygen in live condition. Samplings were done two times. First one (sampling I) was on 4th October 2010 and the second one (Sampling II) was on 25th January 2011.

History taking: General history regarding culture system, feeding, stocking density and other information were also collected from the farm operators.

Clinical record and blood chemistry: By examining each individual's clinical information were recorded. Blood chemical parameters such as total protein, haematocrit and hemoglobin were also taken after collecting blood from caudal vein.

Dissecting of the fish: Operculum and body cavity were opened by dissecting fishes with sharp scissors and forceps. Photos of the internal organ of each individual were taken with a digital camera system (OLYMPUS E-P2, Japan).

Fixation and refixation: Subsequently after dissection, samples were fixed in Bouin's solution. After 24 hours of fixation samples were cut into suitable pieces with sharp blade and put in labeled cassettes (Categorized as organ and individuals). Then these samples were refixed 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. In the present study tissues were washed and dehydrated through alcoholic grades (70%, 80%, 90%, 95%, 100%, 100% and 100%) and cleaned in xylene.

Embedding: Tissues were embedded with paraffin wax at 58 – 62 ^{o}C.

Sectioning: Embedded blocks were cut at 5 micron in thickness by rotatary type microtome (Reichert-jung820, Leica LTD). The sectioned ribbon was floated on warm water bath (54^{o}C) to flatten out the section. The sections were carefully collected on to a glass slide, allowed to dry fully before proceeding to H&E staining.

Staining: Hematoxylin and eosin (H&E) staining methods were followed to stain the prepared slides.

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

Photography: Photos of the prepared slides of different organ were taken, using the software DP2-BSW (Olympus, Japan).

Calculation of HHI: HHI, hepatohypertrophic index was calculated using the image analyzer (Image pro-plus3, Media cybernetics). At first the number of nucleus in 1,000 µm^2 were counted, using the software and then values were put in the following formula and HHI were calculated.

$$HHI = 1/Log\ (Number\ of\ nucleus\ count\ in\ 1{,}000\mu m^2)$$

Results and Discussion

Necropsy of important organs: Necropsy of the collected samples was done for small and big sized fish groups for sampling I and sampling II (Tables 1 and 2). Ascites, liver congestion, kidney enlargement, kidney membrane rupture, spleen enlargement, gill anemia and presence of parasites were taken into consideration. Ascites were found in big sized fish group in sampling I and sampling II. Liver congestion was pronounced in big sized fish group of sampling II. Four individuals were found with liver congestion in case of big sized fish group for sampling II but not any for small sized fish group. Protozoan parasite *Trichodina* was found in all individuals for the both samplings. Other observations were more or less same.

Blood chemistry: Blood chemistry parameters: hemoglobin (Hb), total protein (Tp) and hematocrit (Ht) were taken in consideration for the present study. Mean observed values for Hb, Tp and Ht were 4.46 ± 0.69 g dl^{-1}, 2.59 ± 0.059 g dl^{-1} and 34.83 ± 5.91% for big sized fish group and 5.028 ± 1.19 g dl^{-1}, 1.44 ± 0.19 g dl^{-1} and 26.5 ± 5.68% for small sized fish group respectively in case of sampling I. For sampling II values of Hb, Tp and Ht were 5.96 ± 0.88 g dl^{-1}, 2.45 g dl^{-1}and 32.33% for big sized fish group and 4.61 ± 1.58 g dl^{-1}, 2.43 g dl^{-1} and 30.16% for small sized fish group, respectively. Mean value of Tp and Ht were significantly higher (p values ranged from 0.001 to 0.032) in big sized fish group of sampling I. Higher total protein (Tp) and hematocrit (Ht) in big-sized fish of sampling I may be due to high protein utilization capacity and high immunity than the small-sized fish. In case of sampling II mean values of Tp and Hb were observed a little bit higher in big sized fish group compared to small sized fish group (Tables 3, and 4).

Total protein in blood was found significantly higher in big sized fish group in case of sampling I. This may be due to overfeeding of artificial feed by big sized fishes. Blood Tp was positively correlated with feeding level in rainbow trout, *Onchorhynchus mykiss* (Storebakken *et al.* 1991),while other studies have shown declines in fish plasma Tp during fasting (Navarro and Gutierrez, 1995; Wagner and Congleton, 2004). On the contrary, Coz-Rakovac *et al.* (2008) found no correlation between Tp values and the feeding regime. This observation was consistent with the present study for sampling II. For sampling II, Tp value was almost same for big and small sized fish group.

The haematocrit was used to measure the ratio of erythrocytes to plasma, and effectively measure the packed cell volume of the erythrocytes contained in the blood (Blaxhall, 1972). Without the knowledge of the normal range of hematological parameters, it is difficult, if not impossible, to differentiate between the normal and pathological state (Barnhart, 1969). However as a part of the quantitative health assessment index for rapid evaluation, a normal range of 30 - 45% is considered for fish in general (Adams *et al.*, 1993). In the present study mean Ht values for big and small sized fish groups were 34.83 ± 5.91 and 26.5 ± 5.68, respectively in case of sampling I. In case of II, Ht values were 32.33 ± 3.01 and 30.16 ± 3.65 for big and small sized fish groups, respectively. All these values consistent

with the above mentioned range except small sized fish group in sampling I. In sampling I, Ht was significantly higher ($p < 0.05$) in big sized fish group comparing to the small sized.

Table 1. Necropsy of different organs of fish in sampling I.

Histological feature	No. of individuals	
	The big sized	The small sized
Ascites	2	0
Liver congestion	0	2
Irregular color of liver	3	6
Kidney enlargement	0	0
Spleen enlargement	0	0
Paleness of gill	1	0
Presence of parasites (Trichodina)	6	6

***Functional indices*:** Functional indices such as hepato somatic index (HSI) and hepato hypertropic index (HHI) were studied. Mean values for the HSI and HHI were 0.87 ± 0.15 and 3.07 ± 0.38 for big sized fish group and 0.86 ± 0.09 and 2.40 ± 0.18 for small sized fish group, respectively for sampling I. In case of sampling II mean values for HSI and HHI were 1.35 ± 0.19 and 3.14 ± 0.53 for big sized fish group and 1.46 ± 0.75 and 2.27 ± 0.22 for small sized fish group, respectively. Mean HSI was found more or less same between groups in both samplings. But significant ($p < 0.01$) differences were found between groups in both samplings in case of HHI (Table 5).

The mean HSI value was species-specific and correlates with the amount of fat deposition (Chiba *et al.,* 1976; Oguri, 1985; Anelo *et al.,* 1993; Brusle *et al.,* 1996). In osteichthyes, the HSI is normally calculated to be between 1 - 2 % (Brusle *et al.,* 1996). It has also been found that HSI is highly sensitive to the nutritional status of the fish and correlates with the quantity and quality of feed (Hung *et al.,* 1990). In the present study, the mean values for HSI for both groups (small and big sized) in both sampling were calculated. Those values were consistent with the normal range although a little bit minimum values were calculated lower than 1% in case of sampling I. But for sampling II, all values fall in normal range. No significant differences were observed between groups.

Another functional index known as hepato hypertrophic index (HHI) was studied. This index was first introduced in fish and shellfish pathology laboratory, in the Department of Aquatic Life Medicine, Pukyong National University, South Korea. Actually, this is a parameter used to describe the hepatic function. Limited references were found regarding HHI. Incidence rate and severity of green liver syndrome were increased with the increasing of HHI values (Lee, 2008). In present study HHI values were found significantly higher in big sized fish group compared to the small sized in both samplings. The higher HHI values obliterate the Disse space and sinusoids, which may lead to hypertrophy, fatty change and necrosis of liver. Finally, it may lower the immunity of the fish and make more vulnerable to disease.

***Histopathological features under light microscope*:**

***Liver*:** Histological slides prepared from liver tissue were investigated under light microscope for both samplings. The main alterations observed were atrophic hepatocyte, moderate to severe fatty change in sampling I for small and big sized fish groups. There was no clear difference between small and big sized fish groups (Table 6 and Fig. 1). In case of sampling II, the main feature was mild to moderate atrophy of hepatocycte. No clear difference was observed between small and big sized fish groups (Table 7 and Fig. 2).

***Stomach*:** In case of sampling I, stomach was almost normal except one individual with vaculative change and atrophy of gastric gland and two individuals with immunological activation for big sized fish group. But for small sized fish group, all individuals were normal (Table 6). In case of sampling II, vacuolative changes of gastric gland were conspicuous in big sized fish group with pearl like white round space in all individuals. All individuals of small sized fish group were observed almost normal. Clear and distinct difference was observed in stomach between two groups in case of sampling II (Table 7 and Fig. 3).

***Intestine*:** Histological slides prepared from intestine were investigated under light microscope. No alterations were observed except two individuals with presence of inflammatory cell in big sized fish group in case of sampling I (Table 6). For sampling II all individuals were normal for both groups (Table 7). No distinct difference was observed between groups regarding intestine for both samplings.

Table 2. Necropsy of different organs of fish in sampling II.

Histological feature	No. of individuals	
	The big sized	The small sized
Ascites	3	1
Liver congestion	4	0
Irregular color of liver	1	2
Kidney enlargement	1	2
Spleen enlargement	1	3
Paleness of gill	0	2
Presence of parasites (Trichodina)	6	6

Table 3. Functional indices for big sized and small sized fish groups of sampling I.

Sample No.	Length (cm)	Wt (g)	Hb (g/dl)	Tp (g/dl)	HSI (%)	Ht (%)	HHI
Big sized fish							
1	27	220	4.95	3.74	0.95	33	2.97
2	25	193	4.04	2.15	1.14	35	2.63
3	27	205	5.44	2.5	0.73	46	3.32
4	31	254	4.62	2.36	0.75	35	3.67
5	26.5	213	4.29	2.64	0.85	30	3.10
6	29	246	3.46	2.18	0.85	30	2.72
Mean ± SD	27.58±2.10	221.83±23.72	4.46±0.69	2.59±0.59	0.87±0.15	34.83±5.91	3.07±0.38
Small sized fish							
1	22.5	129	6.76	1.66	1.01	36	2.35
2	23	140	5.44	1.46	0.86	26	2.51
3	23	115	3.62	1.66	0.87	19	2.29
4	23	114	3.71	1.35	0.88	25	2.48
5	22.5	138	5.44	1.14	0.72	24	2.12
6	21.5	119	5.2	1.42	0.84	29	2.63
Mean ± SD	22.58±0.58	125.83±11.51	5.028±1.19	1.44±0.19	0.86±0.09	26.5±5.68	2.39±0.18

Table 4. Functional indices for big sized and small sized fish group of sampling II.

Sample No.	Length (cm)	Wt (g)	Hb (g dl^{-1})	Tp (g dl^{-1})	HSI (%)	Ht (%)	HHI
Big sized fish							
1	27.6	395	6.43	2.71	1.34	36	3.50
2	25.8	371	6.69	3.59	1.37	30	2.87
3	29.3	493	6.69	2.37	1.4	35	2.72
4	24.7	360	4.66	2.33	1.22	28	3.92
5	27.1	457	5.048	1.49	1.68	32	3.32
6	25.7	348	6.26	2.22	1.1	33	2.48
Mean ± SD	26.7±1.64	404±58.26	5.96±0.88	2.45±0.68	1.35±0.19	32.33±3.01	3.14±0.53
Small sized fish							
1	21.8	184	7.36	2.1	0.68	26	2.21
2	22.7	175	4.18	2.1	1.14	33	2.09
3	22.6	212	5.08	1.38	1.14	28	2.05
4	22.8	258	2.62	2.25	2.53	35	2.48
5	20.7	267	4.66	2.94	1.02	32	2.18

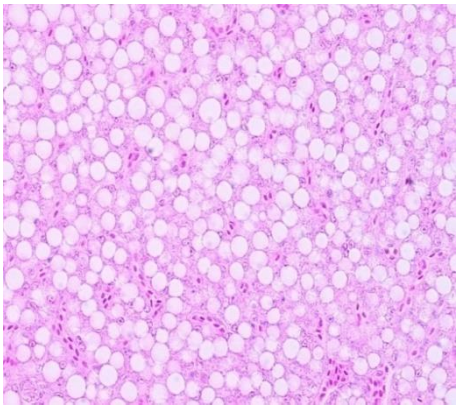
6	21.6	194	3.81	3.81	2.28	27	2.59
Mean ± SD	22.03±0.82	215±38.89	4.61±1.58	2.43±0.83	1.46±0.75	30.16±3.65	2.27±0.022

Table 5. Comparative analysis of blood chemistry and functional indices (Hb, Tp, Ht, HSI and HHI).

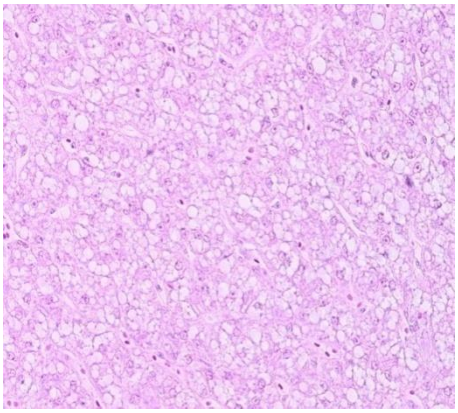
Sampling No.	Parameters	Big sized (Mean±SD)	Small sized (Mean±SD)	P-value
I	Hb (g dl^{-1})	4.46±0.69	5.028±1.19	0.342
	Tp (g dl^{-1})	2.59±0.59	1.44±0.19	*0.001
	Ht (%)	34.83±5.91	26.5±5.68	*0.032
	HIS (%)	0.87±0.15	0.86±0.09	0.839
	HHI (%)	3.068±0.38	2.39±0.18	*0.003
II	Hb (g dl^{-1})	5.96±0.88	4.61±1.58	0.099
	Tp (g dl^{-1})	2.45±0.68	2.43±0.83	0.961
	Ht (%)	32.33±3.01	30.16±3.65	0.28
	HIS (%)	1.35±0.19	1.46±0.75	0.72
	HHI (%)	3.14±0.52	2.27±0.22	*0.004

Significant level (P-Value<0.05)

***Kidney*:** Meningomyelocele (MMC) was increased both in size and number for all individuals of both samplings for small and big sized fish groups. Two individuals from sampling I and one individual from sampling II for big sized fish group were with hydrophic degeneration (Tables 6 and 7).

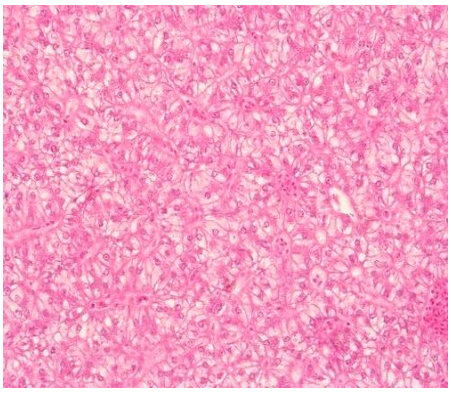


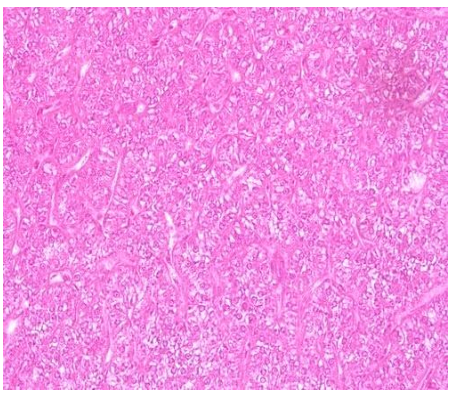
Big sized fish; HHI=2.97



Small sized fish; HHI=2.12

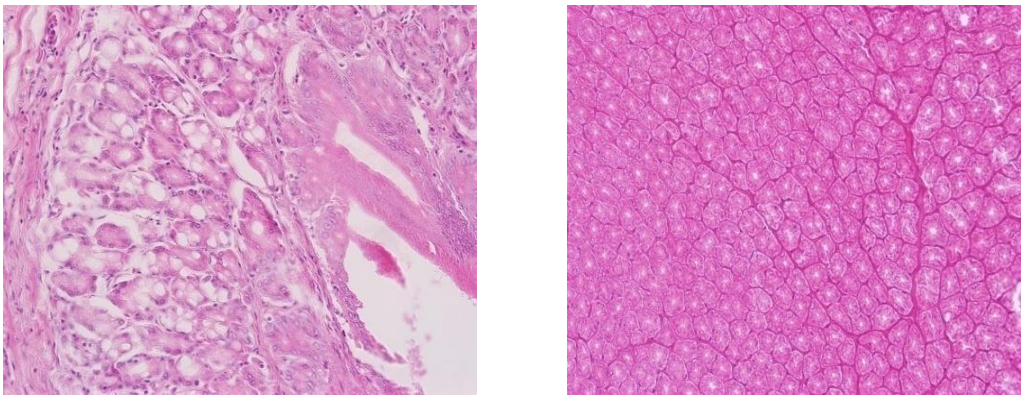
Fig. 1. Comparison of liver tissue between big and small sized fish group (X400) in sampling I.





Big sized fish; HHI=3.32 Small sized fish; HHI=2.59

Fig. 2. Comparison of liver tissue between big and small sized fish group (X400) in sampling II.



Big sized fish Small sized fish

Fig. 3. Comparison of stomach tissue between big and small sized fish group (X400) in sampling II.

Table 6. Microscopy of different organs of fish in sampling I.

Organ examined	Histological feature	No. of individuals	
		The big sized	The small sized
Liver	Atrophic	5	5
	Fatty change	6	5
	Increase of MMC	2	0
Stomach	Vacuolative change	1	0
	Lymphocytic infiltration	2	0
Intestine	Edemateous laminapropriya	3	0
	Lymphocytic infiltration	2	0
	Dialation of blood vessel	0	5
Kidney	Increase of MMCs	6	6
	Hydrophic degeneration	2	0
Heart	Epicarditis	2	0
Spleen	Enlargement of ellipsoid	5	3
	Increase of MMCs(Size/number)	5	6
	Lymphocytic infiltration	1	0

Heart: Epicarditis was the main alterations in heart observed in the present study. For sampling I three individuals from big sized fish group were found with epicarditis but not any from small sized fish group. For sampling II, five individuals from big sized and four individuals from small sized fish group were found with epicarditis. So rate of epicarditis is higher in big sized fish group comparing to the small sized (Tables 6 and 7).

Spleen: Activation of ellipsoids increased MMCs both in size and number, lymphocytic infiltration were the main observation for both sampling for both groups of fishes. No clear distinction was observed between groups for both samplings (Tables 6 and 7).

Fatty change and atrophy of liver were observed in most of individuals of big and small sized fish groups for sampling I. For sampling II both groups were found with atrophic liver. This may be due to intake of excess artificial feed. Cytoplasmic vacuolation of liver of the Saddled bream, *Oblada melanura* in cage associated specimens with artificial feed was pronounced (Ferri, 2011). Similar results also found in populations of mullets

(Coz Rakovac *et al.,* 2008). Fatty degeneration has documented as a significant problem in cultured fish especially in sea bream (Benedito Palos *et al.,* 2008).

Vacuolative changes in the gastric gland in big sized fish group in case of sampling II is the most important observation from the view point of health evaluation of olive flounder (Fig. 3). Vacuolative changes in gastric gland may lead to critical pathological state and could have contributed to increase mortality (Mobin *et al.,*1999). Histological alterations in gastrointestinal tract caused by high stocking density and the resulted social stress were found in the case of Elvers (Willemse *et al.,* 1984) and adult European eels, *Anguilla Anguilla* (Peters, 1982). In the present study stocking density and social stress were the same because both small and big sized fishes were taken from same age group and culture system. Vacuolative changes were observed only in big sized fish group. So this was not the cause of the histological alterations. Blebbing and necrosis of gastric cells, vacuolative changes of gastric glands and necrosis of enterocytes and intestinal wall were clearly found to increase in severity as the feeding level increased in case of Japanese juvenile olive flounder (Mobin *et al.,* 2000). This was the probable cause of vacuolative changes in gastric glands in big sized fish group for the present study. Because big sized fishes take more feed compared to the small sized in competition. Vacuolative change and atrophy of gastric gland is the indicator of high feeding regime. High feeding regime causes hyper secretory activities of gastric gland and leading to vacuolative changes and atrophy of gastric gland. Overfeed leading to dysfunction of liver by mechanically obliterating the microcirculation of hepatic parenchyma.

Table 7. Microscopy of different organs of fish in sampling II.

Organ examined	Histological feature	No. of individuals	
		The big sized	The small sized
Liver	Atrophic	4	4
	Fatty change	1	0
	Increase of MMC	0	1
Stomach	Vacuolative change of gastric gland	6	0
	Lymphocytic infiltration	0	0
Intestine	Edemateous laminapropriya.	0	0
	Lymphocytic infiltration	0	0
	Dialation of blood vessel	0	0
Kidney	Increase of MMCs	4	4
	Hydrophic degeneration	1	0
Heart	Epicarditis	5	4
Spleen	Enlargement of ellipsoid	1	1
	Increase of MMCs (Size/number)	2	3
	Lymphocytic infiltration	1	3

MMCs were increased both in size and numbers in all individuals of both groups and samplings in case of kidney and spleen tissue (Tables 6 and 7). Kidney is the major lymphoid organ in teleosts in addition to the thymus, spleen and mucosa-associated lymphoid tissue. MMCs are distinctive population of pigment containing cells present in the hematopoietic tissue of spleen and kidney (Roberts, 1975; Wolke, 1992). So MMCs were normal in kidney and spleen tissue.

To conclude, a clear histopathological difference between big and small sized fish group were observed in case of stomach with vacuolative changes in gastric gland in big sized fish group for sampling II. HHI values were also found significantly higher ($p < 0.01$) in big sized fish groups in both samplings. Some significant higher (p values ranged from 0.001 to 0.032) values were also observed for Tp and Ht in sampling I for big sized fish group. Vacuolative changes in gastric gland for big sized fishes are an important observation for the present study. Vacuolative changes in gastric gland may lead to critical pathological state and could have contributed to increase mortality. Vacuolative changes and atrophy of gastric gland related to the higher feeding regime. High feeding regime causes hyper secretory activities of gastric glands and leading to vacuolative change and atrophy of gastric glands. Overfeeding, leading to dysfunction of liver by mechanically obliterating the microcirculation of hepatic parenchyma. The visceral organs are important component of the defense system (Fange, 1984). Higher HHI values in big sized fishes implicated over nourished state. Severe increase of HHI values obliterate the Disse space and

sinusoids which lead to hypertrophy, fatty change, atrophy and necrosis of liver. Finally it may lower the immunity of fish and cause more vulnerable to disease. The present study is only based on fish size from same age group and culture system. Impacts of different feeding regimes are also important for health assessment of fishes. Further studies with different feeding regimes and their relationship to fish's susceptibility to disease are required.

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