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Histological Change in Tissues During Starvation in Olive Flounder, *Paralichthys olivaceus* (Temminck et Schlegel)

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기아시 넙치, *Paralichthys olivaceus* (Temminck et Schlegel)의 조직학적 변화

Advisor: *P*rof. In-Seok *P*ARK

by

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Histological changes in some tissues during starvation in olive flounder, *Paralichthys olivaceus* (Temminck et Schlegel)

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ABSTRACT

I examined the effects of nutritional conditions on histological changes in the hepatocytes, midgut epithelium, and kidney, as well as alterations in hepatocyte ultrastructure in olive flounder, *Paralichthys olivaceus* (Temminck et Schlegel). One group of fish fasted during the 12-week experiment (starved group), a second group was provided with food *ad libitum* (fed group), and a third group was fed a controlled amount of food (control group). At the start of the experiment, ten fish were sampled from each of the control, fed, and starved groups at 4, 8, and 12 weeks after start of the experiment.

Food deprivation resulted in a significant decrease in hepatocyte nucleus size and nuclear height of the midgut epithelium ($P < 0.05$). Kidney melano-macrophages (MMs) with dark brown pigment were randomly distributed in the kidneys of starved fish, increased rapidly after week 4, while deposition levels remained low throughout the experiment in the fed group. These results suggest that catabolic tissue breakdown is a major factor

contributing to the formation of pigments within MMs.

Compared to those of the initial control and fed group during the experiment, hepatocytes underwent marked ultrastructural changes in response to 12 weeks of starvation. The prominent features characterized the hepatocytes of the starved group were a reduction in cell and nucleus sizes; apparent loss of nucleoli; condensation of chromatinloss of stored glycogen; reduction of endoplasmic reticulum profiles; increase in the number of electron-dense bodies containing large amounts of iron; and increased mitochondrial size.

These results suggest that the histological changes of ultrastructural alterations in the hepatocytes and midgut epithelium and the degree of MM deposition in the kidney can be used as alternative indicators to identify of starvation in wild and cultured olive flounder.

Key words: Hepatocyte, Hepatocyte ultrastructure, Melano-macrophage, Midgut epithelium, Nuclear area, Nuclear height, Olive flounder, *Paralichthys olivaceus*, Starvation

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Thesis Advisor: Prof. In-Seok PARK, Ph. D.

INTRODUCTION

Most fish undergo periods of starvation due to wintering, spawning, migration, or regional decreases in food resources. Fish can overcome starvation using biochemical, physiological, and behavioral strategies. Endogenous energy from basic metabolic accumulations in the body is spent as fish consume their own tissues to remain alive during starvation (Mustafa & Mittal 1982; Weatherley & Gill 1987; Lee *et al*. 1999; Woo 2005).

During starvation, essential processes in fish are maintained at the expense of accumulated (i.e., completely endogenous) energy reserves, resultingin the progressive depletion and wastage (degrowth) of body tissues (Weatherley & Gill 1987). **T**he observed incidence of starvation is essentially the same based on morphological and histological criteria (**T**heilacker 1986). Morphological analysis is an attractive approach compared to histological examination because of the relative ease and low cost of measuring fish. **O**n the other hand, histological analysis allows the determination of cause and effect relations between body structure and starvation, whereas gross morphological measurements provide an index of starvation that is vulnerable to error and bias in calibration and interpretation (**T**heilacker 1986).

Within the fish body, an immediate response to starvation is observed in the gut (Ehrlich *et al*. 1976). A decrease in gut epithelial cell height and connective tissue has been observed in carp (***Cyprinus carpio***) and pike (*Esox lucius*) larvae (Kostomarova 1962) and in herring (***Clupea harengus***) and plaice (*Pleuronectes platessa*) larvae (Ehrlich *et al*. 1976). **R**estricted food availability also induces a reduction in the dimensions of liver parenchymal cell nuclei

(Alvarez & **C**owden 1966; Baic *et al*. 1979; Love 1980; Storch & Juario 1983; Storch *et al*. 1983; Wang & **T**akashima 1984; Segner 1985; Strüssmann & **T**akashima 1989, 1990; Lee *et al.* 1998; *P*ark *et al*. 1998).

Teleosts contain abundant kidney melanin. Microscopically, melano-macrophages (MMs), which are similar to human macrophages, metabolize toxic substances and perform various immune functions in the kidney (**R**oberts 1975; Agius 1979a, b). Moreover, MMs increase in number in response to various pathological and physiological conditions (*P*almer *et al.* 1992) such as vitamin E deficiency, consumption of humus-based feed (Blazer & Wolke 1983), starvation (Agius & **C**ouchman 1986; Micale & *P*erdichizzi 1990), or aging (Brown & George 1985).

MMs are also reported to differ in shape, number, size, and content (Agius & **R**oberts 1981). Furthermore, researchers have recently examined the possibility of using morphological changes in MMs, i.e., number and melanin content, as a biomarker in aquatic environments (Macchi *et al*. 1992; Wolke 1992). **I**ncreases in the number of MMs in the kidney due to starvation have been studied in rainbow trout (***O****ncorhynchus mykiss* Walbaum), plaice, swordtail (*Xiphophorus helleri* Heckel), tilapia (***T****ilapia zillii* Gervais), and masu salmon (***O****. masou*)(Agius & **R**oberts 1981; Mizuno *et al.* 2002).

Studies have investigated the ultrastructure of the teleost liver (**C**hapman 1981) and the morphological and functional alterations in response to environmental changes (e.g., temperature; Berlin & **D**ean 1967) seasonal variation (Quaglia 1976a, b) physiological changes, such as those caused by starvation (**T**akahashi *et al*. 1977; Langer 1978; Langer & Storch 1978) and the administration of toxic substances such as **DDT** (Weis 1974), polychlorinated biphenyls, Aroclor 1254 (Hacking *et al*. 1978), and heavy metals (Segner 1981).

Love (1970) noted that teleost species show variable tolerance to starvation. Many species are subjected to natural starvation periods during the year and have developed the ability to survive without food. Some species can survive starvation for up to 4 years (El Hakeen 1979), and non-feeding larvae may survive for 1 month (**C**owey & **R**oberts 1978).

As suggested by its flattened oval body, the olive flounder, *Paralichthys olivaceus* (**T**emminck et Schlegel), mainly resides in the benthos at a depth of 1020 m, and is widely distributed in Korea and East Asia (**C**hoi *et al.* 2002). Approximately 35 000 tons of this species were farmed in Korea in 2003. **O**live flounder may experience short- or long-term starvation, e.g., directly when feeding is arrested or indirectly due to low water temperature caused by cold water masses or red tides in summer season at Korean seashore. Starvation is an important factor that effects physiological changes in immunity, survival, and growth (Woo 2005).

The purpose of this study was two-fold. **T**he first objective was to provide methods, based on histological observations, that can easily be applied in the aquaculture industry to estimate olive flounder condition. **T**he second objective was to extend our knowledge of the changes in histological structure that occur in olive flounder during starvation. Specifically, **I** investigated histological changes in hepatocytes and the midgut epithelium, ultrastructural changes in hepatocytes, and MM accumulation in the kidney caused by long-term starvation these data were used to determine nutritional indices for olive flounder.

MATERIALS AND METHODS

Ⅰ. Experimental fish

Olive flounder, *Paralichthys olivaceus* (**T**emminck et Schlegel), used in this experiment were hatched in April 2004 at a flounder farm and were subsequently transported to a mariculture farm at the Fishery Genetics and Breeding Laboratory of the Korea Maritime **U**niversity in Busan, Korea. **T**he starvation experiment began in **O**ctober 2004. **I**nitially, fish were weighed to the nearest 0.01 g using an electronic balance (JW-1, Korea), and their body lengths were measured to the nearest 0.01 cm using digital Vernier calipers (**CD**-20**C***P*, Japan).

Ⅱ. Experimental procedure and rearing

Four experimental groups were established: initial control, control, fed, and starved. All fish were fed daily with commercial feed (**T**able 1) at 1~3% of their total body weight for 2 weeks prior to the start of the experiment. **T**he fed and control groups were hand-fed three times daily in 4-h feeding intervals (the first feeding occurred between 0800 and 1200 h, the second between 1200 and 1600 h, and the third between 1600 and 2000 h, but always with 4 h between successive feedings). **T**he control group received feed at 1~3% of their total body weight and fed group received feed *ad libitum*, while the starved group fasted throughout the experiment.

Fish were reared in a recirculating system. **T**wenty fish were placed in each 1.1-ton fiberglass reinforced plastics circular tank (118 cm diameter × 100 cm depth); each experimental group consisted of two tanks of fish. Light was

Table 1. Composition of the diet used in the experiment*

	Content (%)
Crude protein	50.0
Crude fat	8.0
Crude fiber	4.0
Ash	15.0
Calcium	1.5
Phosphorus	1.7
Mineral	3.0

*Purchased from E-Wha Oil & Fat Ind. Co., Busan, Korea.

provided by four 40-W (5400 K) fluorescent bulbs controlled by an electric timer, which kept the photoperiod at a 12:12-h light/dark cycle. No lights were used during the dark period. Water temperature was controlled automatically and held at 22 ± 0.6°C during the experimental period.

The dissolved oxygen concentration was adjusted to 5.0 ppm (Table 2). The rotating rate was 30 times the water volume, and the water change rate involved replenishing two-thirds of the water volume with natural seawater once a week. Table 2 shows other water conditions during the experimental period. Each tank was covered with a net to prevent fish from jumping out, and survival was recorded throughout the experiment.

Ⅲ. Histological analysis of the hepatocyte nuclear area and the nuclear height of the midgut epithelium

Ten fish were removed from the initial control at the start of the experiment and each of the two groups (fed and starved) every 4 weeks during the 12 weeks of the experiment to examine the hepatocyte nuclear area and the nuclear height of the intestinal epithelium. Fish were euthanized within 2 h of sampling using an overdose of lidocaine-HCl (300 ppm at 22°C; *P*ark *et al*. 1988) and immediately dissected on an ice-cold cutting board.

The liver and midgut epithelium were removed and tissue samples were fixed in 10% neutral formalin solution (100 mL formalin, 6.5 g $Na_2HPO_4 \cdot 12H_2O$, 4.5 g KH_2PO_4, 900 mL DW) for 24 h. The samples were then refixed in Bouin's solution for 24 h. Samples were prepared in 6-μm thick paraffin sections, placed on slides, stained with hematoxylin and eosin Y-phroxine B, and observed under a microscope (Axioskop, Zeiss, Germany). *P*hotographs were taken of tissues. The mean hepatocyte nuclear area for each

Table 2. Water parameter (i.e., temperature, salinity, dissolved oxygen, and hydrogen potential) used during the course of this experiment*

*P*arameters	Value
T-N (mg/L)	1.9~4.1
TKN (mg/L)	1.0~1.2
$\mathbf{NH_4^+}$**-N** (mg/L)	0.9~1.1
$\mathbf{NO_3^-}$**-N** (mg/L)	0.9~2.9
T-*P* (mg/L)	0.02~0.78
$P\mathbf{O_4^{3-}}$*-P* (mg/L)	0.004~0.351
$\mathbf{COD_{Mn}}$ (mg/L)	2.0~11.1
Water temperature (℃)	19.4~22.4
Salinity (‰)	34.0~37.0
Dissolved oxygen (ppm)	5.7~8.0
*P*otential of hydrogen (pH)	6.5~7.8

*The values are monitored for 12 weeks. **COD**: **C**hemical oxygen demand; **TKN**: **T**otal Kjehldahl nitrogen; **T-N**: **T**otal nitrogen; **T-***P*: **T**otal phosphorus.

photograph was calculated using an **NI**H **I**mage (Ver. 1.57) system. **T**he mean nuclear height of the middle intestinal epithelium for each individual was measured using an eye piece micrometer at 400 × under a microscope.

Ⅳ. Melano-macrophage deposition

The same ten sampled fish per group (control, fed, and starved) were used to investigate kidney MM accumulation. **T**he kidneys were removed and tissue samples were fixed in 10% neutral formalin solution for 24 h. **T**he samples were then refixed in Bouin's solution for 24 h. Samples were prepared in 6-μm thick paraffin sections, placed on slides, stained with hematoxylin and eosin Y-phroxine B, and observed under a microscope (Axioskop, Zeiss, Germany). *P*hotographs were taken of tissues. **T**he area of MM accumulation was calculated for each kidney using an **NI**H **I**mage (Ver. 1.57) system to determine the rate of accumulation.

Ⅴ. Analysis of hepatocyte ultrastructure

For electron microscopy, small portions of the excised hepatopancreas of initial control, fed and starved fish at the conclusion of the experiment were pre-fixed in a cold 2.5% glutaraldehyde solution (pH 7.5) for 2 h. **T**he tissue was pre-fixed for 2 h at 4°**C** in 2.5% glutaraldehyde solution buffered by 0.1 M phosphate buffer solution (*P*BS, pH 7.2). After washing with *P*BS for 10 min, the samples were post-fixed in 1% osmium tetroxide (**O**s**O**$_4$) for 2 h at 4°**C**. Samples were rewashed with *P*BS, then serially dehydrated with ethanol from 50 to 100% and embedded in Epon 812.

Sections (0.5 ㎛ thick) were cut using an ultramicrotome (LKB, **N**ova, Sweden) and then stained with toluidine blue to determine the investigation

region. **T**he sections were double-stained with uranylacetate and lead citrate solution and examined using a transmission electron microscope (JEM 1200 E-*X* **II**, 60-80 kv, JE**O**L, **T**okyo, Japan).

Ⅵ. Statistical analysis

One-way analysis of variance (A**NO**VA) was used to evaluate the effect of feeding and starvation on the change in hepatocyte nuclear area, intestinal epithelium height, and MM deposition in the kidney, followed by Student-**N**ewman-Keuls multiple range tests.

RESULTS

At the start of the experiment, the average body length and weight of olive flounder, *Paralichthys olivaceus* (**T**emminck et Schlegel) were 22 ± 0.9 cm and 100 ± 11.7 g, respectively, in all groups. After 12 weeks, the starved group rapidly lost vitality, and thus, the experiment was terminated. **T**he accumulated survival was 89 ± 1.7% in the initial control group, 90 ± 1.2% in the fed group, and 77 ± 2.4% in the starved group in each duplicated tanks (Fig. 1).

Starvation brought about regressive changes in hepatocytes and the midgut epithelium. **T**able 3 shows changes in the hepatocyte nuclear area and midgut epithelium height during the experimental period. At the beginning of the experiment, the hepatocyte nuclear area and nuclear height of the midgut epithelium were 7.9 ± 0.90 μm^2 and 7.1 ± 0.13 μm, respectively, in all groups

($P > 0.05$, **T**able 3, Fig. 2a, b). Shrinkage of the hepatocyte nucleus and the nucleus of the midgut epithelium was not in the fed group, but proceeded steadily in the starved group ($P < 0.05$, **T**able 3, Figs. 2a, b). At 12 weeks, the hepatocyte nuclear area and nuclear height of the midgut epithelium were 4.6 ± 0.50 μm^2 and 4.3 ± 0.21 μm, respectively, for the starved group, which differed significantly from the values of the fed group and those at the start of the experiment ($P < 0.05$, **T**able 3, Figs. 2a, b).

The liver was considered normal when hepatocytes had clear, distinct nuclei, as observed initially and in the fed group (Fig. 3). **I**n the starved group, liver atrophy was apparent: the liver appeared shrunken and contained darkly stained hepatocytes composed of evenly stained cytoplasm

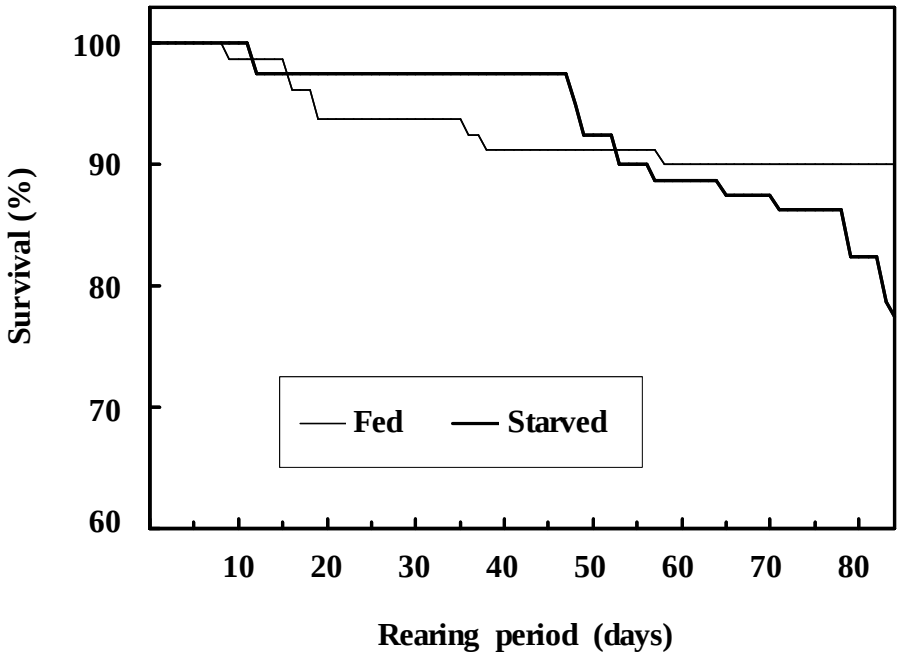

Fig. 1. Mean survival of duplicated experiment of olive flounder, *P. olivaceus* fed and starved during the experiment.

Table 3. The change of hepatocyte nuclear area and nuclear height of midgut epithelium in fed group and starved group in olive flounder, *P. olivaceus*

	Group	Experiment period (weeks)*			
		0	4	8	12
Hepatocyte nuclear area (μm^2)	Fed	7.9±0.90[a]	9.2±0.89[a]	10.4±0.99[a]	11.0±1.00[a]
	Starved	7.9±0.90[a]	6.3±0.54[b]	5.2±0.51[b]	4.6±0.50[b]
Nuclear height of midgut epithelium (μm)	Fed	7.1±0.13[a]	7.4±0.20[a]	7.5±0.22[a]	7.7±0.13[a]
	Starved	7.1±0.13[a]	6.0±0.20[b]	5.5±0.25[b]	4.3±0.21[b]

*The value are means±SD (n=10) of duplicated groups. Means in columns a same superscript letter are not significantly different (P>0.05).

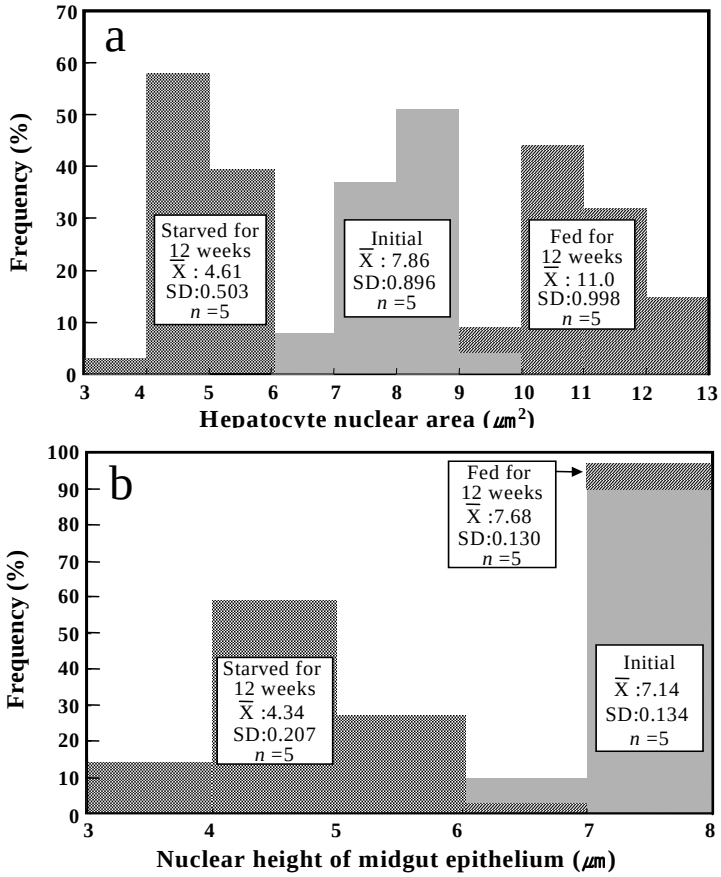

Fig. 2. Frequency distribution of (a) hepatocyte nuclear areas and (b) nuclear height of midgut epithelium from the same individuals depicted in histological appearance of hepatocyte and middle intestinal epithelium in olive flounder, *P. olivaceus*. **N**uclear areas and middle intestine epithelium heights from two replicate photographs of each individual were pooled and grouped in 1-μm^2 area classes and 1-μm length class, respectively.

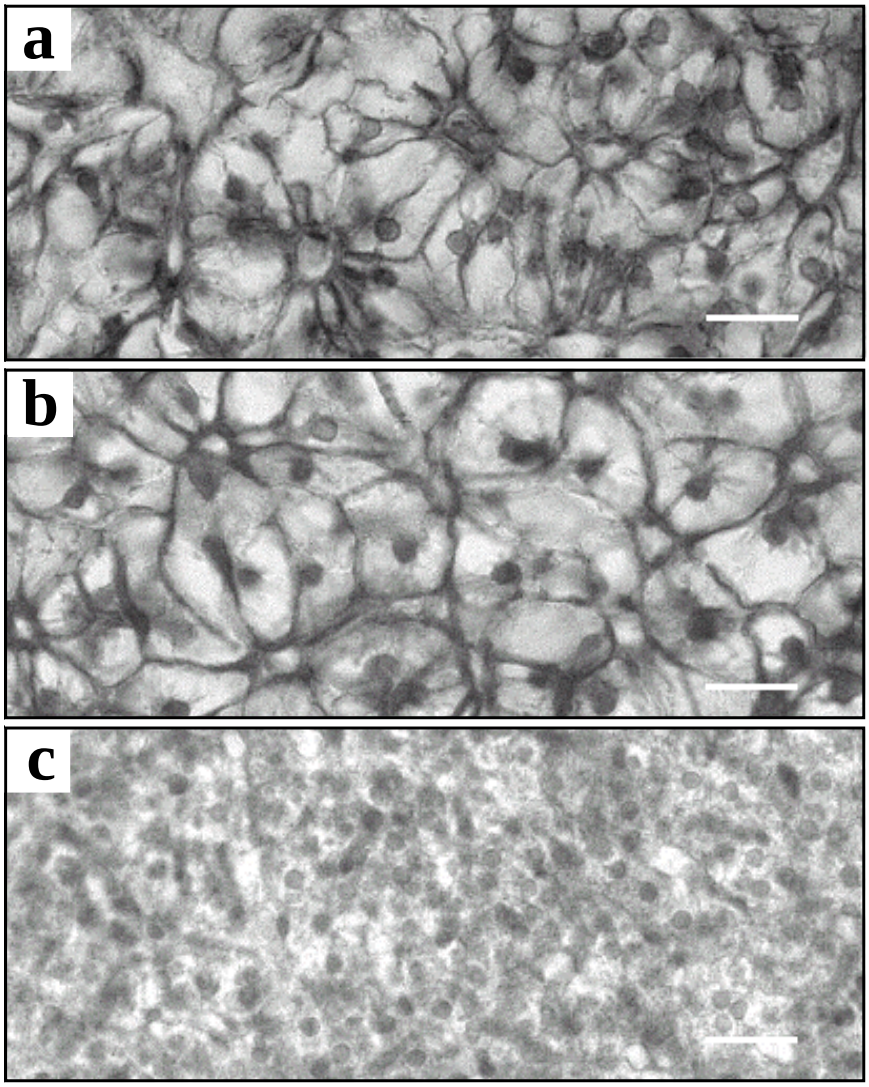

Fig. 3. Histological appearance of hepatocytes of (a) initial control, (b) fish fed for 12 weeks, and (c) fish starved for 12 weeks in olive flounder, *P. olivaceus*. Bars represent 5 ㎛. **N**ote the reduction in hepatocyte nuclear size in starved group.

with indistinct, irregular nuclei (Fig. 3). **I**n the starved group, very few intracellular spaces were observed in the cytoplasm of the hepatocytes in contrast, extensive intracellular space was observed in the fed group (Fig. 3). *P*resumably, these intracellular spaces are areas where glycogen and fat are stored within the cell. **T**he nucleus of the midgut epithelium had a higher density, irregular shape, and was larger in the starved group compared to the fed group and compared to the start of the experiment (Fig. 4).

Figure 5 shows changes in MM deposition during the experimental period. At the start of the experiment, the degree of MM deposition was 0.14 ± 0.042%, 0.14 ± 0.036%, and 0.14 ± 0.012% in the control, fed, and starved groups, respectively ($P > 0.05$). MMs were dark brown and were present in irregularly scattered masses in the control group (Fig. 6a). **I**n the starved group, MM deposition increased with time: 0.37 ± 0.021% at 4 weeks, 0.90 ± 0.167% at 8 weeks, and 1.01 ± 0.145% at 12 weeks. However, MM deposition ranged from 0.21 ± 0.072 to 0.29 ± 0.080% in the control group and 0.25 ± 0.077 to 0.28 ± 0.060% in the fed group over the experimental period, and did not differ significantly from corresponding values at the start of the experiment

($P > 0.05$, Fig. 5). Although no significant difference was observed among the three groups during the first 4 weeks, the degree of MM deposition was significantly higher in the starved group than in the control and fed groups at 8 and 12 weeks ($P < 0.05$, Fig. 5).

Under the optical microscope, MMs appeared as small to large round or oval structures. Figure 6b shows kidney tissue from the fed group at 12 weeks. Little MM deposition was observed in either the fed or control groups (Fig. 6). **I**n contrast, MM deposition in the starved group at 12 weeks was

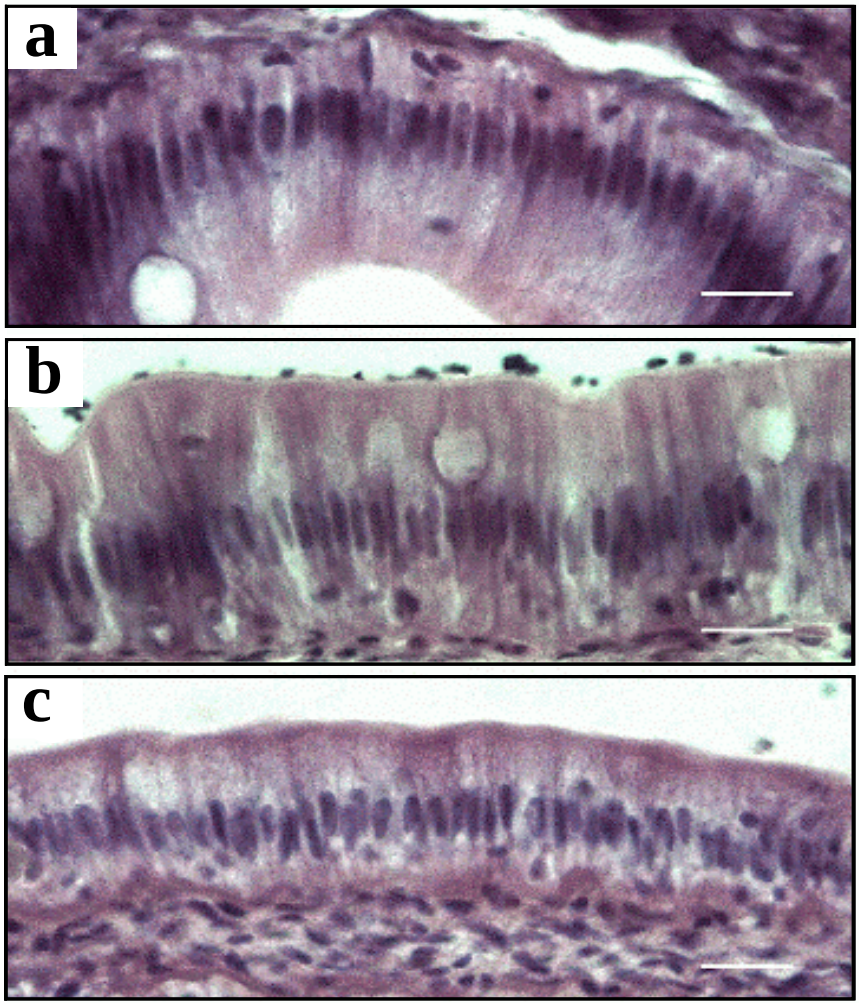

Fig. 4. Histological appearance of midgut epithelium of (a) initial control (b) fish fed for 12 weeks, and (c) fish starved for 12 weeks in olive flounder, *P. olivaceus*. Bars represent 10 ㎛. Note the reduction in nuclear height of midgut epithelium in starved group.

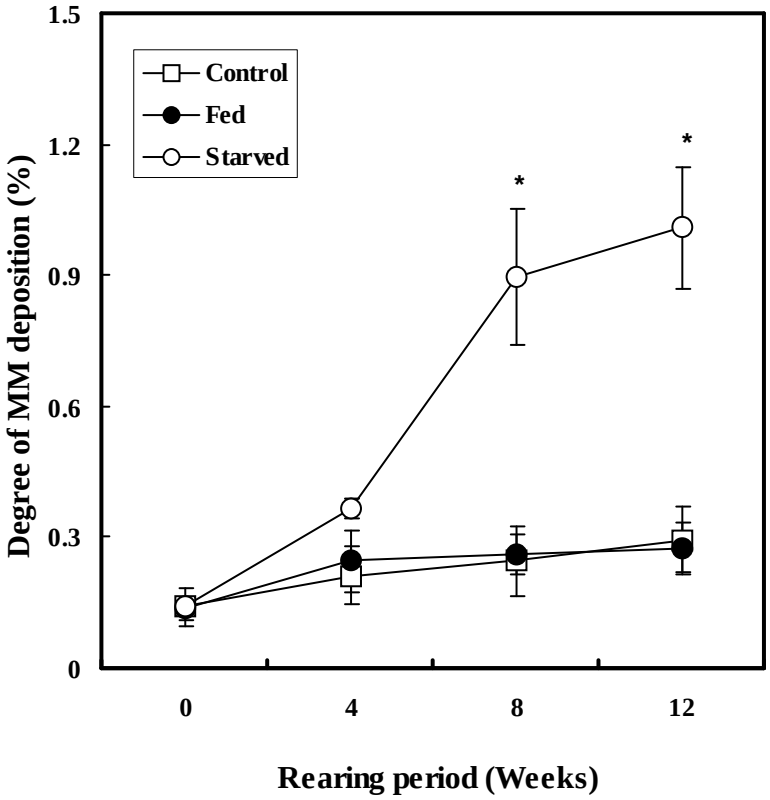

Fig. 5. **C**hanges in melano-macrophage (MM) deposition during the experiment in the control (□), fed (●), and starved (○) groups in olive flounder, *P. olivaceus*. Astrick indicate significant difference from the starved and control group values (P<0.05).

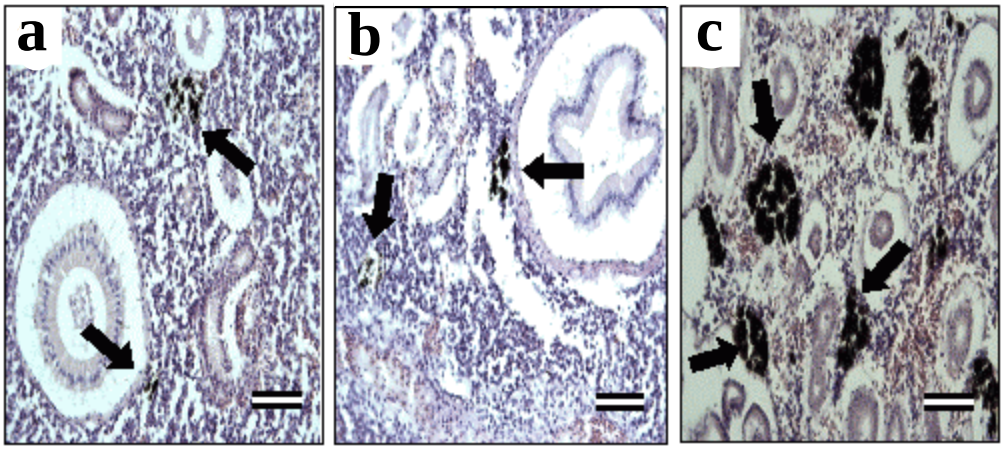

Fig. 6. Histological observations of melano-macrophage (MM) on the kidneys of (a) control, (c) starved and (b) fed group in olive flounder, *P. olivaceus*. **T**he arrows indicate exactly the panels are shown at 400-fold magnification on indicate exactly the increase in degree of MM deposition during starvation. However, kidneys were observed at 100× in the present study. Scale bars are 50 ㎛.

significantly higher than in the fed group ($P < 0.05$, Fig. 6).

The hepatocyte ultrastucture in the initial control, fed and starved groups showed common features: the polyhedral cells contained one large vesicular nucleus the outer element of the nuclear envelope bore many ribosomes situated on the cytoplasmic surface; batches of glycogen particles were prominent in the cytoplasm; the infoldings of the inner mitochondrial membrane were usually lamellar; the endoplasmic reticulum (E**R**) appeared loose and mitochondria with profiles ranging from ovoid to elongate, and occasionally, threadlike, that formed a matrix of comparatively low density were associated with the cisternae of the rough endoplasmic reticulum (**R**E**R**)(Figs. 7a, d).

In the initial control and fed group, the nucleus was round or ovoid and the nucleolus was sometimes visible (Figs. 7a, c). **T**he **R**E**R** appeared to be arranged in parallel stacks of cisternae, usually located around the nucleus and along the plasma membrane. **T**he mitochondria were usually found in close association with the **R**E**R** their shape varied from circular to elongated, with well-developed cristae. Some lipid droplets were present, but the cytoplasm was mainly filled with large glycogen-containing areas. A huge accumulation of large lipid droplets and glycogen invaded the hepatocyte cytoplasm and induced a marked reduction of mitochondria and **R**E**R** (Figs. 7a, c).

Compared to those of the initial control and fed group, in starved group after a starvation period of 12 weeks, the hepatocytes showed changes (Figs. 7b, d), the most prominent of which was the presence of polymorphic inclusion bodies, which could be interpreted as lysosomes. **T**he amount of glycogen and the diameter of the cell nucleus were reduced. A greater proportion of the mitochondria was enlarged and exhibited a pale matrix (Fig. 7b). **T**he

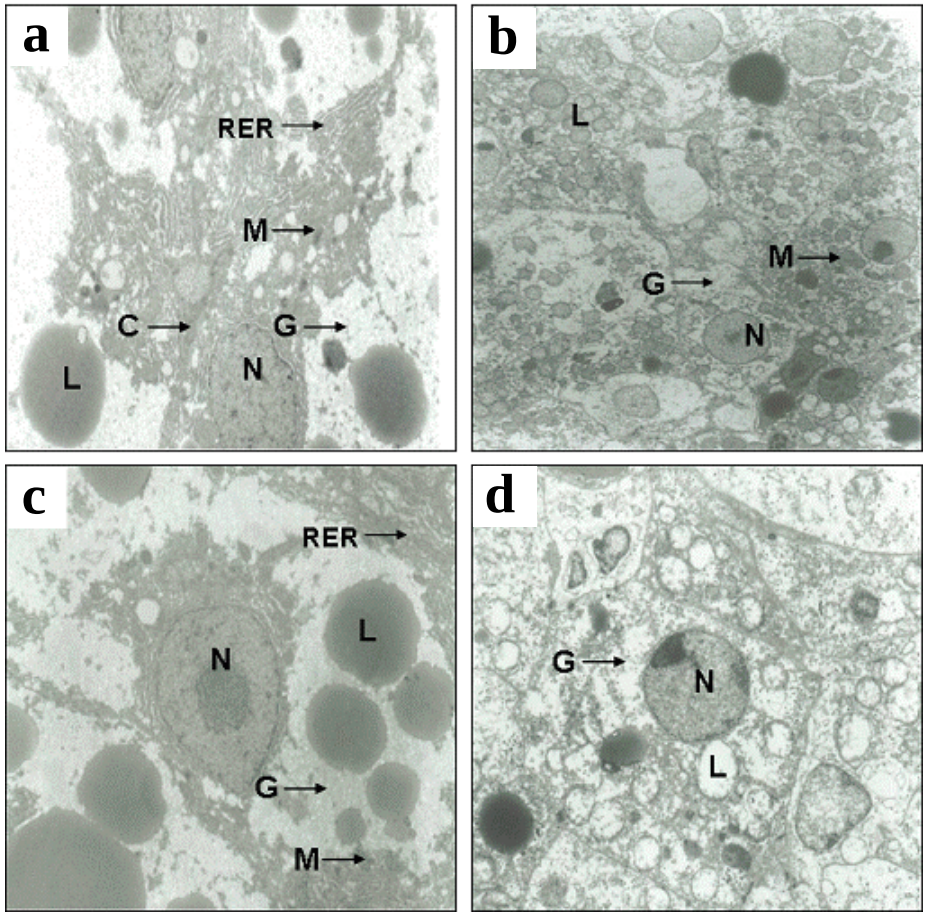

Fig. 7. **I**n electron microscopy, hepatocyte of (a and c) fed and (b and d) starved groups in olive flounder *P. olivaceus*. **C**: bile caniculus, G: glycogen, L: lipid droplet, M: mitochondria. **N**: nucleus, **R**E**R**: rough endoplasmic reticulum. Magnification: starved group (× 2,500); fed group (× 2,000).

mitochondria were large with low electron density, and electron-dense bodies of similar outline and size were observed. **T**he cytoplasm was devoid of glycogen, lipids, and Golgi bodies. **T**he pale and spherical nucleus lacked a nucleolus, but had inflated perinuclear cisternae. **R**emnants of the E**R** were observed, but not in all sections. Bile canaliculi were widened (Fig. 7d).

DISCUSSION

Larsson & Lewander (1973) noted that many fishes undergo natural periods of starvation during the year and have consequently evolved the ability to withstand prolonged food shortages. Such periods may amount to weeks, months, or even years, and may cause extensive loss of energy stores in the body as the fish consumes its own tissues to remain alive (Love 1970; Weatherley & Gill 1987). **D**uring this process, various body constituents may be mobilized at different rates, and similar substrates may be used at different rates in different tissues (Weatherley & Gill 1987).

Within the fish body, an immediate response to starvation appears in the gut and liver, especially where they are involved in energy storage (Ehrlich *et al*. 1976). A decrease in gut epithelial cell height has been observed in carp and pike larvae (Kostomarova 1962), as well as in herring and plaice larvae (Ehrlich *et al*. 1976) during starvation. **T**his also occurred in olive flounder, *Paralichthys olivaceus* (**T**emminck et Schlegel) in this study.

The findings for the hepatocyte nucleus generally agree with those reported by other researchers for starvation in fish (Alvarez & **C**owden 1966; Baic *et al*. 1979; Love 1980; Storch & Juario 1983; Storch *et al*. 1983; Wang & **T**akashima 1984; Segner 1985; Strüssmann & **T**akashima 1989, 1990; Lee *et al.* 1998; *P*ark *et al*. 1998). **I**t appears that the onset of irreversible starvation is preceded by structural alterations in the hepatocytes. Storch *et al*. (1983) concluded that this cell type is particularly useful as an indicator of nutritional status in fish.

Structural changes within the hepatocytes include variation in nuclear size, which results from changes in the non-chromosomal protein content of

the nuclei (Leuchtenberger & Schrader 1951; Alvarez & **C**owden 1966). **T**herefore, kariometry has been used to corroborate qualitative histological findings, and to evaluate the nutritional adequacy of refeeding regimes after a period of starvation (Mizuno *et al*. 2002). **T**he largest nuclei in the fed group coincided temporally with extensive glycogen pools that are indicative of sufficient endogenous or exogenous energy resources.

Under the optical microscope, MMs appear as small to large round or oval structures, which are easily distinguished from the surrounding lymphatic tissue, whereas under the electron microscope, they appear as groups of macrophages (Agius & Agbede 1984). MMs are observed in normal fish,but are more numerous in physiologically abnormal states, i.e., due to disease or stress. Moreover, in these states, the number, size, and shape of MMs vary (Agius 1979b; Agius & Agbede 1984).

The cytoplasm of MMs contains abundant melanin, hemosoderin, lipofuscine (fat free lipochrome), and ceroid, which react positively to *P*AS and Ziehl-**N**eelson reactions. MMs vary in color from yellow to black, mainly due to tissue catabolism (Micale & *P*erdichizzi 1990). **T**he color intensity depends on the type of fish and its age and health status (Agius & **R**oberts 1981; Kranz 1989). **I**ncreased numbers of MMs are related to detoxification, the destruction of and response to endogenous or exogenous toxins, inflammatory response, and iron circulation (Wolke 1992). MMs increase in the kidneys of some teleosts, such as rainbow trout, during starvation (Agius & **R**oberts 1981).

MM deposition ranged from 0.21-0.29% in the control group and 0.25-0.28% in the fed group during the 12-week experiment. Mizuno *et al.* (2002) reported that MM deposition in wild type and hatchery-reared masu

salmon was 0.1-0.2% and 0.1-0.7%, respectively, which is similar to that observed in the control and fed groups of olive flounder. **N**o significant changes were observed histologically in the fed group over the experimental period. However, MM deposition increased significantly in the starved group during the experiment similar to results reported for plaice, rainbow trout, swordtail, tilapia (*Tilapia zillii*, Agius & **R**oberts 1981 *Oreochromis aureus*, Agius & **C**ouchman 1986), sea bream *Diplodus annularis* (Micale & *P*erdichizzi 1990), and masu salmon (Mizuno *et al.* 2002). **R**ates of MM deposition increased from 0.37 to 1.01% with starvation from week 4 to 12. **T**hese results agree with the 0.1-0.9% increase in MM deposition observed in masu salmon by Mizuno *et al.* (2002).

The rate of MM deposition due to starvation was directly related to mortality, which agrees with observations of masu salmon after 60 days of starvation (Mizuno *et al.* 2002). After 60 days of starvation, mortality was 20% (0.9% MM deposition) in the starved group and 15% (0.7% MM deposition) in the fed group (Mizuno *et al.* 2002). Similarly in olive flounder, mortality by the end of the experimental period was 22.5, 10.8, and 10.0% and the respective MM deposition was 1.10, 0.29, and 0.28% in the starved, control, and fed groups, respectively.

The distribution of MM deposition, and their shape, number, and size increase with various pathological and physiological conditions (*P*almer *et al.* 1992) such as vitamin E deficiency, humus-based feed (Blazer & Wolke 1983), and starvation (Agius & **C**ouchman 1986; Micale & *P*erdichizzi 1990). Mortality and MM deposition increased with starvation in olive flounder, suggesting that starvation presents a significant physiological burden to fish.

Increased MM deposition due to starvation has been observed in

various organs of different fishes, e.g., dogfish (*Scyliorhinus canicula*), rainbow trout, plaice, and ***T. zillii***. Agius & **R**oberts (1981) reported that this increase in MM deposition is mainly due to catabolic tissue destruction. **C**hanges in MM deposition are also induced by feed, water conditions, water temperature, age, and season (Blazer *et al.* 1987). **T**hus, further studies are needed to establish the possibility of using different morphological changes, such as the number of MMs expressed, and their size and degree of deposition, as bio- and physiological markers (Macchi *et al.* 1992; Wolke 1992).

As in other species (Kostomarova 1962; Ehrlich *et al.* 1976), these results show that in general, an immediate response to starvation will be seen in the gut of the fish. **T**here was a significant reduction in the nuclear height of the midgut epithelium in olive flounder after 12 weeks of starvation. A similar result was observed by Kostomarova (1962) in starved carp and pike, and by Ehrlich *et al.* (1976) in starved herring and plaice, where food restriction caused a reduction in the nuclear height of the midgut epithelium.

Liver structure and ultrastructure have recently been identified as relevant parameters with which to detect and assess the toxicity of several xenobiotics in fish (Braunbeck *et al.* 1990; Arnold *et al.* 1996), but their use in fish nutrition is much more rare, except in nutritional studies (Segner & Braunbeck 1988; Abi-Ayad & Kestemont 1994).

Microvesicular hepatocellular degeneration was reported in hybrid striped bass fed experimental and practical diets (Brown *et al.* 1993). **I**n Eurasian perch (*Perca fluviatilis*), hepatocyte ultrastructure was noticeably affected by dietary treatments (Kestemont *et al.* 1996). **T**he progressive increase in glycogen storage and lipid droplets, both in size and abundance, and the concomitant reduction in essential organelles involved in the

oxidation processes and protein synthesis, such as **R**E**R** and mitochondria, can be considered preliminary signs of impaired liver function. Lipid storage, however, appeared to be limited to large droplets into the cytoplasm itself, without the formation of liposomes, i.e., lipid droplets that are formed within the cisternae of the E**R**, or intranuclear lipid inclusions of microvesicular and macrovesicular nature (Baglio & Farber 1965).

The results show that olive flounder has a carbohydrate-oriented liver. **T**he carbohydrates are stored as alpha-glycogen, a predominant feature of hepatocytes in the liver of well-fed fish. **I** also observed that olive flounder undergomarked changes that proceed at an accelerated pace as starvation time is prolonged.

After 12 weeks of starvation, the hepatocytes of olive flounder underwent ultrastructural changes that have previously been described for other species. Widened intercellular spaces, reduction in size, hypertrophy of the lysosomes, reduction in the diameter of the cell nucleus, condensation of the chromatin material, depletion of ribosome-studded E**R** and glycogen (**R**enaud & Moon 1980; Moon & Johnston 1981), and mitochondrial enlargement all appearto be characteristic features of hepatocytes in starved teleosts (Storch & Juario 1983).

Differences among species are observed mainly in the amount of lipid, which may increase or decrease (Langer & Storch 1978; Aster & Moon 1981). **I**ncreased lipid deposits could be indicative of lipoid liver disease (ceroidosis), which can develop in fish fed a diet in which the lipid component has become rancid and the vitamin components are insufficient, e.g., 'trash fish' or pelleted rations (Smith 1979), or a diet with high carbohydrate and low protein content (Hille *et al*. 1980). Glycogen may also

react differently following short periods of starvation. **I**n red and sea bream, for example, the glycogen level was elevated at the onset of starvation, followed by a rapid drop in glycogen reserves (Sakamoto *et al*. 1978).

Storch & Juario (1983) noted the development of large mitochondria in starved milkfish (*Chanos chanos*); this was observed in fingerlings and was more pronounced in fry. **T**he mitochondria occupied a large area in the cytoplasm, exceeding the size of the nucleus and pushing it to the periphery of the cell. However, in olive flounder, starvation did not promote the development of large mitochondria.

In olive flounder, the hepatocytes underwent marked ultrastructural alterations in response to 12 weeks of starvation. **T**he prominent features characterizing the hepatocytes in the starved group were a reduction in cell and nucleus size; apparent loss of nucleoli; condensation of chromatin loss of stored glycogen; reduction in E**R** profiles; increase in the number of electron-dense bodies containing large amounts of iron; and increase in mitochondrial size.

These results suggest that the histological changes of ultrastructural alterations in the hepatocytes and midgut epithelium and the degree of MM deposition in the kidney can be used as alternative indicators for the identification of starvation in wild and cultured olive flounder. **O**ngoing research should investigate the validity of these findings in other fish species.

KOREAN ABSTRACT (국문요약)

이학석사 학위논문

기아시 넙치, *Paralichthys olivaceus* (Temminck et Schlegel)의 조직학적 변화

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기아가 넙치, *Paralichthys olivaceus* (Temminck et Schlegel)의 간세포, 중장 상피세포 및 신장에 미치는 조직학적 영향과 간세포 미세구조에 미치는 영향을 조사하였다. 12주간 기아군은 먹이를 주지 않은 반면, 포식군은 인공사료를 공급하였다. 실험 시작시 대조군, 포식군 및 기아군에서 각 10마리를 표본하였다. 대조군, 포식군 및 기아군에서 실험 시작시 부터 4주 간격으로 3회 표본하였다(n=10).

먹이공급 중단은 핵세포 크기 및 중장 상피세포 핵 크기에서의 유의한 감소를 보였다(P<0.05). Kidney melano-macrophages (MMs)는 포식군인 경우 실험기간 중 그 축적이 낮은 반면, 기아군에서의 MMs는 진한 갈색 색소

를 보이며 신장에서 무작위로 분포하였다. 본 연구 결과 MMs에서의 색소 형성의 주된 요인은 이화작용 중인 조직의 붕괴라고 사료된다.

실험 시작시의 대조군 및 실험기간 동안 포식군의 간세포 미세구조에 비해, 넙치에서의 12주간 기아는 간세포 미세구조의 현저한 변화를 야기하였다. 기아군 간세포의 현저한 특징은 뚜렷한 인의 소실, 염색질의 응축, 저장 글리코겐의 소실, 소포체의 감소, 철분을 다량 함유하는 electron-dense body에서의 증가, 그리고 미토콘드리아 크기의 증가이었다.

본 연구 결과에서 간세포를 비롯한 중장 상피세포 및 신장의 조직학적 변화, 간세포 미세구조적인 현저한 차이 그리고 신장에서의 MM 축적 정도 차이는 본 종의 야생집단과 양식집단에서의 기아 평가 기준이 될 수 있으리라 사료된다.

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