A DISSERTATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Studies on vitamin E and myoinositol nutrition for parrot fish (*Oplegnathus fasciatus*)

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Department of Marine Life Science

GRADUATE SCHOOL

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Studies on Vitamin E and Myoinositol Nutrition for Parrot Fish (*Oplegnathus fasciatus*)

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요 약 문

최근 양식산업에 있어서 새로운 양식대상어종으로 각광받고 있는 돌돔(*Oplegnathus fasciatus*)은 넙치와 조피볼락에 비해 성장이 다소 느린 단점을 가지고 있으나 질병에 대한 내병성이 강한 고부가가치의 유망한 양식대상 어종이다. 따라서 이 연구는 돌돔사료 내 비타민의 적정함량 규명을 통한 돌돔양식에 대한 기초연구자료 제공과 양식에 소요되는 비용 절감을 위해 수행되었다.

이 연구는 Part Ⅰ과 Ⅱ로 나누어 수행되어졌으며, Part Ⅰ에서는 돌돔치어 사료 내 비타민 E 적정함량과 비타민 E 의 단계적인 첨가에 따른 어류의 면역력에 미치는 영향을 알아보기 위해 수행되었다. Part Ⅱ에서는 돌돔치어 사료 내 myo-inositol 단계적인 첨가에 따른 어류 성장 및 면역력에 미치는 영향을 알아보기 위해 수행되었다.

돌돔치어 사료 내 비타민 E 요구량과 비타민 E 첨가에 따른 면역력을 알아보기 위해 수행한 사양실험(Part Ⅰ)은 12 동안 수행되었으며, 반 정제 사료원이 기초인 총 6 개의 실험사료는 비타민 E 함량을 각각 0, 25, 50, 75, 100, 500 mg/kg diet 으로 기초사료에 첨가하였다. 12 동안의 사양실험 결과, 비타민 E 가 25ppm 첨가되어진 실험구는 대조구에 비해 유의적으로 높은 성장률과 사료효율을 나타내었으나, 생존률과 전어체 함량은 사료 내 비타민 E 첨가에 유의적인 영향을 받지 않았다. 실험어류의 간 내 비타민 E 함량은 사료 내 비타민 E 첨가 함량의 증가에 따라 유의적인 증가를 보였으며, 실험사료를 섭취한 어류의 면역력을 알아보기위해 수행한 respiratory burst, myeloperoxidase 활성 분석 결과에서도 사료 내 비타민 E 첨가 함량의 증가에 따라 유의적으로 높은 활성을 나타내었다. 한편, *Vibrio anguillarum* 을 이용한

공격 실험의 결과에서는 75ppm 이상의 비타민 E 를 섭취한 실험구들에서 다른 실험구들에 비해 높은 생존률을 나타내었다.

돌돔 치어 사료 내 myo-inositol 첨가에 따른 어류의 성장 및 면역력에 미치는 영향을 알아보기 위해 수행한 사양실험(Part Ⅱ)은 18 주동안 수행되었다. 실험사료 내 myo-inositol 은 0, 100, 200, 400, 800 mg/kg diet 으로 첨가되어졌으며, 사양실험은 3 반복으로 수행되었다. 18 주 동안의 사양실험 결과, myo-inositol 이 첨가되어진 실험구들은 myo-inositol 첨가되어지지않은 대조구에 비해 유의적으로 높은 성장률과 사료효율을 나타내었다. 그러나 사료 내 myo-inositol 의 첨가는 어류의 생존률에는 유의적인 영향을 미치지 않았으며, 혈액학적 분석 (hematocrit, asparatate aminotransferase, alanine aminotransferase, plasma triglyceride, cholesterol)과 면역학적 분석 (myeloperoxidase activity) 결과에서도 모든 실험구에서 유의적인 차이를 발견하지 못했다. 한편, 실험어류 간 내 myo-inositol 농도는 사료 내 myo-inositol 함량 증가와 함께 높아지는 경향을 나타내었으며, 사료 내 200ppm 첨가 실험구에서 가장 높은 값을 나타내었다. 어류 간 내 지방함량에 있어서는 사료 내 myo-inositol 함량이 증가함에 따라 유의적으로 낮은 지방함량을 나타내었다. Broken-line regression 모델을 이용하여 어류의 성장률과 간 내 myo-inositol 농도를 기초로 한 돌돔 치어 사료 내 myo-inositol 요구량은 약 94 와 121 mg/kg 으로 각각 분석되어졌다. 이와 같은 연구 결과를 바탕으로 할 때 돌돔 치어에 있어서 최적의 성장과 원활한 지방대사를 위한 사료 내 myo-inositol 요구량은 대략적으로 100mg/kg 으로 사료되어진다.

결론적으로, 돌돔치어 사료 내 최적의 성장을 위한 비타민 E 요구량은 약 40 mg/kg 으로 판단되어지며, 돌돔치어 사료 내 고농도(500 mg/kg)의 비타민 E 첨가는 어류의 면역력을 향상 시킬 수 있을 것으로 여겨진다. 또한, 돌돔치어에 있어서 최적의

성장과 원활한 지방대사를 위한 사료 내 myo-inositol 요구량은 대략적으로 100mg/kg 으로 여겨진다.

SUMMARY

Parrot fish, *Oplegnathus fasciatus*, has been identified as one of the candidate for marine intensive aquaculture in Korea, China and Japan. However, little information in nutrition is available for the fish. Particularly, dietary requirements of vitamin E and inositol, which have been reported as the most expensive ingredients for normal growth of fish (Gaylord et al., 1998). Therefore, the aim of the first feeding trial was to determine the dietary requirement of vitamin E and its effects on innate immune responses and disease resistance of juvenile parrot fish. The second feeding trial aimed to elucidate essentiality of inositol in diet for juvenile parrot fish, and its effects on liver lipid metabolism.

The present work consists of two chapters. The first chapter covers the requirement of vitamin E and its effect on immune responses in parrot fish. The second chapter covers the requirement of dietary myo-inositol and its physiological nutrition in parrot fish. In the first chapter, a 12-week feeding trial was conducted using a flow-through system to determine the dietary requirement of vitamin E for parrot fish in relation to growth performance, and to examine the effects of vitamin E on immune responses and disease resistance. Six semi-purified diets (E0, E25, E50, E75, E100 and E500) supplemented with 0, 25, 50, 75, 100 and 500 mg DL-α-tocopheryl acetate/kg diet, respectively, were fed to triplicate groups of parrot fish juveniles (mean body weight 20.15±0.09 g). At the end of feeding trial, growth performance and feed utilization and vitamin E deposition in liver were evaluated. Respiratory burst, lysozyme and myeloperoxidase (MPO) activities were analyzed. The fish fed with graded dietary vitamin E levels were challenged with *Vibrio*

anguillarum. Significant higher weight gain, specific growth rate, protein efficiency ratio and lower feed conversion ratio were observed in fish fed diet E25. Survival did not differ among the treatments. Addition of vitamin E to the basal diet did not significantly affect whole body protein, crude fat, ash and moisture. An increase in the supplementation of vitamin E in the diet caused an increment in the deposition of vitamin E in liver tissue. Respiratory burst and MPO activities significantly increased with increment of dietary vitamin E levels. Serum lysozyme activity was not significant different among experimental diets. In challenge test, the dietary treatments E500, E100, E75 registered the highest survival of 13.89, 5.56 and 5.56%, respectively.

In the second chapter was conducted a study to estimate the essentiality and requirement of *myo*-inositol (MI) for juvenile parrot fish, *Oplegnathus fasciatus*. MI was supplemented 0, 100, 200, 400 and 800 mg/kg diet in the basal diet providing 0, 83, 212, 410 and 943 mg MI/kg diet respectively. Each diet was fed to triplicate groups of juvenile parrot fish (initial body weight, 11.5 g). After 18 weeks of a feeding trial, fish fed diets with supplementation of MI had significantly higher weight gain and specific growth rate than those of fish fed the basal diet. Supplementation of dietary MI did not affect survival of parrot fish. Dietary MI affected blood hematocrit, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, while plasma triglyceride and myeloperoxidase (MPO) activity were not affected. Liver MI concentration of the fish fed the experimental diets was significantly increased by dietary supplementation of MI. The liver MI concentrations were highest in fish fed the ≥212 mg MI/kg diet and lowest in fish fed the MI free diet. Liver lipid concentrations were significantly decreased as

increment of dietary MI supplementation. The dietary requirement of MI based on weight gain percentage and liver MI concentrations were analyzed by broken-line regression and indicated that the requirement for dietary MI in juvenile parrot fish is about 94 and 121 mg/kg diet, respectively. Therefore, we recommend the addition of *myo*-inositol approximately 100 mg/kg diet to prevent a potential problem by an abnormal lipid metabolism and to maximize the growth performances in juvenile parrot fish.

In conclusion, the findings in this study suggest that an optimum level of the dietary vitamin E would be approximately 40 mg/kg for the maximum growth performances. It is suggested that over 500 mg vitamin E/kg diet could improve the immune responses of the fish. Also, the dietary supplementation of myo-inositol is needed to maximise the growth performance and to prevent abnormal lipid metabolism in juvenile parrot fish. The suggested dietary requirement of myo-inositol is about 100 mg/kg diet.

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BACKGROUND

Vitamin E, known as tocopherols, belongs to the group of fat soluble vitamins, such as vitamin A, D and K. Eight naturally occurring tocopherol derivatives have been isolated. And the most important tocopherol is ∝-tocopherol which has highest vitamin E activity (Halver and Hardy, 2002). Tocopherols are sensitive to ultraviolet light and in free forms, they are excellent antioxidants. Vitamin E plays the most vital role as a metabolic antioxidant in preventing the oxidation of unsaturated phospholipids in cellular membranes. Chew (1996) reported that the major antioxidant in blood is vitamin E. It reacts with peroxyl radicals produced from polyunsaturated fatty acids oxidation in membrane phospholipids or lipoproteins to form a relative stable lipid hydroperoxide, thereby reduces the harmful lipid free radicals and protect the tissues from free radicals' attack. Chew (1996) proposed the above reaction might be one of possible mechanisms by which vitamin E enhances the immune response *in vivo*. In addition, vitamin E also acts as physiological antioxidant that protects the other oxidative vitamins and indirectly enhances innate immune response. An interrelationship between the vitamins E, C and A was reported by Dam and Granados (1945) and Dam et al. (1952). Vitamin E is also involved in the maintenance of normal permeability of capillaries and heart muscle.

Various deficiency symptoms of vitamin E including poor growth performance, low food conversion ratio, increase of erythrocyte fragility, muscle dystrophy, xerophthalmia and anemia have been observed in some fish species,

including yellowtail, common carp and rainbow trout. Muscle dystrophy and xerophthalmia were discovered in yellowtail and common carp by Hashimoto et al. (1966), and Sakaguchi and Hamaguchi (1969). Impaired erythropoiesis, fragmentation of erythrocytes, susceptibility to stress of handling, and exudative diathesis appeared in salmon and rainbow trout. Inadequate levels of vitamin E produce several non-specific cell degenerative conditions in rainbow trout when fish are fed diet containing larger amount of polyunsaturated fatty acids (Blazer and Wolke, 1984). Vitamin E, that is required for fish, depends on the amount and type of polyunsaturated fatty acids in diet, the form of the vitamin, the method of diet preparation and the storage condition used (Woodall et al., 1964; Watanabe et al., 1970). A dietary increase of polyunsaturated fatty acids will increase supplementation of vitamin E in diet. Shiau and Shiau (2001) established two optimum dietary vitamin E requirement of juvenile hybrid tilapia to be 40 to 44 mg/kg and 60 to 66 mg/kg in the diets contained 50 and 120 g lipid/kg diet, respectively. On the other hand, hypervitaminosis, a common symptom, is often occurred in fish which ingests an excessive amount of fat soluble vitamins. Hypervitaminosis of vitamin E is more discrete than that of vitamins A and D. Over doses with vitamin E commonly occur when fish are fed fish meal or fish viscera which are enriched with fish oil. High concentration of dietary vitamin E (5,000 mg of DL-∝-tocopherol/kg of diet) reduced concentration of erythrocytes in trout blood (Poston and Livingston, 1969).

Several parameters, including erythrocyte fragility and lipid peroxidation, have been used to determine dietary vitamin E status in fish. Woodall et al. (1964) reported that erythrocyte fragility was an effective parameter to indicate the

physiological status of vitamin E in fish. Meanwhile, lipid peroxidation in liver microsomes has been used to access vitamin E status in rainbow trout and channel catfish (Cowey et al., 1981; Wilson et al., 1984; Gatlin et al., 1986). And vitamin E has been well demonstrated to be an essential dietary nutrient for all fish studied (NRC, 1993). Its requirements for salmon, rainbow trout, yellowtail, and Korean rockfish were 30-120 mg/kg, 25-100 mg/kg, 119 mg/kg, and 45 mg/kg, respectively (Woodall et al., 1964; Halver, 1972; Lall et al., 1988; Hamre and Lie, 1995a; Hung et al., 1980; Watanabe et al., 1981a; Cowey et al., 1983; Shimeno, 1991; Bai and Lee, 1998).

Inositol, a water soluble vitamin-like, is an essential nutritional factor which is required in megadoses in diet for fish. Inositol can be found in seven optically inactive and two active isomers. One of the active forms is myo-inositol which appears in crystalline powder and is soluble in water. Myo-inositol has a lipotropic activity. It prevents the accumulation of cholesterol, a type of fatty liver diseases, and is involved in homeostasis of normal lipid metabolism (West et al., 1966) and prevention of alopecia in mice (Wolley, 1940). Myo-inositol also is an important component in phospholipids' structures in animal tissues (Stetten and Stetten, 1946). Particularly, phosphatidylinositol was found to be involved in signal transduction of various metabolic processes (Mathews and van Holde, 1990; NRC, 1993).

In fish, metabolic synthesis of inositol occurs in different degree in liver, kidney and other tissues (Aoe and Masuda, 1967; Deng et al., 2002). However, de novo synthesis in some fish is not sufficient to support their metabolic needs and thus require an exogenous source of the vitamin. Dietary requirement of inositol depends on fish species. Salmon and common carp have been shown to have high

requirement of inositol for maximum growth performance and liver deposition. Dietary supplementation of inositol was reported to enhance growth performance and heal skin lesion in common carp (Aoe and Masuda, 1967). Meanwhile, channel catfish does not require the supplementation of exogenous inositol in diet due to sufficient endogenous synthesis of inositol in intestinal microflora (Burtle, 1981; Burtle and Lovell, 1989).

Some inositol deficiency symptoms including distended stomachs, prolonged gastric emptying period, edema, dark coloration and decreased cholinesterase and aminotransferase activities were reported in trout, salmon, common carp, red sea bream, Japanese eel, yellowtail, catfish, and Japanese parrot fish (McLaren et al., 1947; Kitamura et al., 1967; Halver, 1970; Yone et al., 1971; Arai et al., 1972; Ikeda et al., 1988; Hosokawa, 1989). Inefficiency in digestion and food utilization, which leads to poor growth performance and distended abdomens, are the most common deficiency signs of inositol in fish. Rainbow trout fed a diet without inositol showed large accumulation of neutral lipids in the liver, increased levels of cholesterol and triglycerides (Holub et al., 1982).

Parrot fish, *Oplegnathus fasciatus*, has been identified as one of the candidate for marine intensive aquaculture in Korea, China and Japan. However, little information in nutrition is available for the fish. Particularly, dietary requirements of vitamin E and inositol, which have been reported as the most expensive ingredients for normal growth of fish (Gaylord et al., 1998). Therefore, the aim of the first feeding trial was to determine the dietary requirement of vitamin E and its effects on innate immune responses and disease resistance of juvenile parrot fish. The second

feeding trial aimed to elucidate essentiality of inositol in diet for juvenile parrot fish, and its effects on liver lipid metabolism.

Part I

Dietary vitamin E requirement in juvenile parrot fish, *Oplegnathus fasciatus*, and its effect on immune response

ABSTRACT

A 12-week feeding trial was conducted using a flow-through system to determine the dietary requirement of vitamin E for parrot fish in relation to growth performance, and to examine the effects of vitamin E on immune responses and disease resistance. Six semi-purified diets (E0, E25, E50, E75, E100 and E500) supplemented with 0, 25, 50, 75, 100 and 500 mg DL-α-tocopheryl acetate/kg diet, respectively, were fed to triplicate groups of parrot fish juveniles (mean body weight 20.15±0.09 g). At the end of feeding trial, growth performance and feed utilization and vitamin E deposition in liver were evaluated. Respiratory burst, lysozyme and myeloperoxidase (MPO) activities were analyzed. The fish fed with graded dietary vitamin E levels were challenged with *Vibrio anguillarum*. Higher weight gain, specific growth rate, protein efficiency ratio and lower feed conversion ratio were observed in fish fed diet E25. Survival did not differ among the treatments. Addition of vitamin E to the basal diet did not significantly affect whole body protein, crude fat, ash and moisture. An increase in the supplementation of vitamin E in the diet caused an increment in the deposition of vitamin E in liver tissue. Respiratory burst and MPO activities significantly increased with increment of dietary vitamin E levels. Serum lysozyme activity was not significant different among experimental diets. In challenge test, the dietary treatments E500, E100, E75 registered the highest survival of 13.89, 5.56 and 5.56%, respectively.

INTRODUCTION

Vitamins represent the most expensive ingredient in fish diets for normal growth (Gaylord et al., 1998). Vitamin E is a lipid-soluble vitamin that comprises eight naturally ocurring tocopherols. Among them, α-tocopherol has the highest biopotency (NRC, 1993). Vitamin E function as a metabolic antioxidant, preventing the oxidation of biological membranes and lipoproteins. It enhances immunity and maintains flesh quality, normal resistance of red blood corpuscles to haemolysis and permeability of capillaries and heart muscle (Halver, 2002). It has been demonstrated to be an essential dietary nutrient for all fish studied (NRC, 1993), which includes 30-120 mg/kg diet for salmon (Woodall et al., 1964; Halver, 1972; Lall et al., 1988; Hamre and Lie, 1995a), 25-100 mg/kg diet for rainbow trout (Woodall et al., 1964; Hung et al., 1980; Watanabe et al., 1981a; Cowey et al., 1983), 25-50 mg/kg diet for channel catfish (Murai and Andrews, 1974; Wilson et al., 1984), 100 mg/kg diet for common carp (Watanabe et al., 1970), 25-66 mg/kg diet for tilapia (Roem et al., 1990; Shiau and Shiau, 2001), 50-100 mg/kg diet for Nile tilapia (Satoh et al., 1987), 45 mg/kg diet for Korean rockfish (Bai and Lee, 1998), and 119 mg/kg diet for yellowtail (Shimeno, 1991). The level and state of oxidation of polyunsaturated fatty acids, the presence of selenium and other antioxidants could affect the dietary vitamin E requirement (Hung et al., 1981; Cowey et al., 1983; Gatlin et al., 1986). An increment of dietary polyunsaturated fatty acids (PUFA) will produce an increase in the requirement of vitamin E in carp (Watanabe et al., 1981b; Schwarz et al., 1988) and Atlantic salmon (Hamre and Lie, 1995b). Shiau and Shiau (2001)

established two optimum dietary vitamin E requirement of juvenile hybrid tilapia to be 40 to 44 mg and 60 to 66 mg in function of 50 and 120 g lipid per kg diets, respectively.

The answer of fish to infectious agents is by nonspecific and specific ways, similar to other vertebrates; being the nonspecific responses more relevant. (Anderson, 1992). Fish phagocytes are considered an important component in the nonspecific defense system and they play an important role in both the initiation and regulation of immunity (Clem et al., 1985). Nutrients like proteins, lipids, vitamins and minerals could affect phagocyte function (Chandra, 1988; Landolt, 1989). Vitamin C and E considered as activators of the phagocyte population play an important role acting as immunostimulants. They improve the nonspecific defense mechanisms and at the same time extend the duration of the specific immune response (Blazer, 1992).

Several deficiencies like erythrocyte fragility, anemia, muscular dystrophy and exudative diathesis have been induced in fish by a vitamin E deficient diet. (NRC, 1993). The above mentioned signs have been described for Chinook salmon (Woodall et al., 1964), Atlantic salmon (Poston et al., 1976), channel catfish (Murai and Andrews, 1974; Lovell et al., 1984; Wilson et al., 1984), common carp (Watanabe et al., 1970, 1981b), rainbow trout (Cowey et al., 1981, 1983; Watanabe et al., 1981a) and yellowtail (Toyoda, 1985).

Parrot fish (*Oplegnathus fasciatus*) is one of the emerging aquaculture species in China, Japan, and Korea. Its high commercial value makes it a promising aquaculture species in the future. Little information is available on the vitamin E nutrition in this species. Therefore, the present feeding trial was performed to

determine the dietary vitamin E requirement and its effects on nonspecific immune responses and disease resistance against *Vibrio anguillarum* in juvenile parrot fish.

MATERIALS AND METHODS

Experimental diets

Six experimental diets were prepared (Table 1.1) by the supplementation with 0, 25, 50, 75, 100, 500 mg DL-α-tocopheryl acetate per kg dry diet (designated as diet E0, E25, E50, E75, E100 and E500, respectively). The dietary concentrations of vitamin E analyzed by HPLC were 0, 38, 53, 87, 119 and 538 mg/kg diet for E0, E25, E50, E75, E100 and E500, respectively (Table 1.4). The experimental diets were formulated to contain 48% crude protein and 21.94 MJ available energy/kg diet (Table 1.2). The energy value of the experimental diets was estimated on the basis of mammalian physiological fuel value, i.e., 16.7 kJ/g protein or carbohydrate and 37.7 kJ/g lipid (Lee and Putman, 1973). Defatted fish meal (10% in diets) was added to the experimental diets to enhance palatability to parrot fish. Fish meal was defatted twice (for 48 h) with 70% aqueous ethanol, and thereafter air dried before its inclusion into the experimental diets. Pellets were extruded through the meat chopper machine (SMC-12, Korea) in 3.0 mm diameter size and dried with freeze drier (Operon FDT-8605, Korea) at –40 °C for 24 h, crushed into desirable particles sizes (0.4-3.0 mm), and stored at –20 °C until use.

Experimental fish and feeding trial

Parrot fish juveniles were obtained from a private hatchery (Chang-Hae Fisheries Co.) in Jeju Island and transported to the Marine and Environmental Research Institute, Cheju National University, Korea. During a 2-week conditioning period, fish were fed a commercial feed (Suhyup, Korea) to be adapted to the

experimental conditions. The feeding trial was conducted for 12 weeks in a flow-through system receiving seawater at a rate of 2 L/min. Supplemental aeration was provided to maintain dissolved oxygen near saturation. Three hundred and sixty fish averaging 20.15±0.09 g were randomly distributed into eighteen 60 L tanks as groups of 20 fish. The diets were fed to triplicate groups of fish at 3% of body weight per day, twice a day at 9:00 and 18:00 h, and 7 days a week. Total fish weight in each tank was measured every 3 weeks. Feeding was stopped 24 h prior weighing, and feeding rate was adjusted accordingly.

Sample collection and analysis

At the beginning and the end of the feeding trial, all fish were weighed and counted for calculation of weight gain, feed conversion ratio, protein efficiency ratio and specific growth rate. Four fish from each tank (12 fish per diet) were sampled and stored at –20 °C for whole body proximate analysis. Proximate analysis of whole body was performed using the standard procedures (AOAC, 1995).

Serological assay

For serological assay, six fish per tank were anesthetized using 2-Phenoxyethanol (50 ppm), and blood was taken from caudal veins by 1 ml medical syringes with heparin as anticoagulant. Nitroblue tetrazolium (NBT) assay was used to verify the effect of dietary vitamin E on nonspecific immune response of the fish by the method of Kumari and Sahoo (2005a). Briefly, blood and 0.2% NBT were mixed in equal proportion (1:1), incubated for 30 min at room temperature, then 50 µl was taken out and dispensed in glass tubes. Then, 1 ml of dimethyl formamide

(Sigma) was added and centrifuged at 2000 × g for 5 min. Finally, the optical density of supernatant was measured at 540 nm. Dimethyl formamide was used as the blank.

Serum lysozyme assay was done using a turbidometric method described by Swain et al., (2007). *Micrococcus lysodeikticus* concentration of 0.2 mg/ml (in 0.02 M solution citrate buffer, pH 5.5) was added to serum sample at 10:1 ratio. Absorbance was measured at 450 nm immediately after adding *M. lysodeikticus* suspension. Final absorbance was measured after incubating for 1 h at 25 °C. Lyophilized hen egg white lysozyme (HEWL) was used as standard. Serum lysozyme values are expressed as μg/ml equivalent of HEWL activity. Myeloperoxidase (MPO) assay in serum was measured according to (Quade and Roth, 1997) with slight modification (Kumari and Sahoo, 2005b). Serum (20 μl) was diluted with 80 μl of HBSS without Ca^{+2} or Mg^{+2} in 96-well plates. To which 35 μl of 20 mM 3.3', 5,5'-tetramethylbenzidine hydrochloride (TMB) and 5 mM H_2O_2. The color change reaction was stopped after 2 min by adding 35 μl of 4 M sulphuric acid (H_2SO_4). The optical density was read at 450 nm in a microplate reader.

***Vibrio anguillarum* challenge test**

V. anguillarum challenge test was conducted according to Kettumen and Fjalestad, (2006). *V. anguillarum* (KCTC 2711, Korean Collection for Type Cultures) provided by the Marine Microbiology Laboratory at Department of Aquatic Life Medicine, Cheju National University, was cultured on Marine Broth (MB-2216, Difco), and incubated with shaking for 24 h at 12 °C. The optical density

of the culture was determined to be $OD_{600\ nm}$ =1.5. Based on the previous bath challenge experiments, 2 ml of the bacterial culture was added to 40 L of sea water. Colony forming units (CFU) of the challenge dose was estimated to be $1.1x10^5$ CFU/ml by spreading dilution of the bacterial culture on BS plates. After feeding trial, twelve healthy fish were randomly selected and re-distributed into the same tanks. Water flow was continued after 5 h with aeration. Mortality was recorded daily for 24 days.

Vitamin E analyses

Diet samples were prepared for the analyses of vitamin E. Three gram of each diet sample was homogenized for 3 min (3 times) in 5 mL methanol containing 1% DMSO and 2% acetic acid on ice. The homogenate was centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was transferred to glass tube. The pellet was homogenized again in 5 mL methanol containing 1% DMSO and 2% acetic acid for 3min. The supernatants were combined and then transferred to vaccum dry oven for extraction of α-tocopherol acetate and final volume was adjusted to 10 mL with methanol. The aliquot was filtered with a disposable syringe filter (0.45μm, Whatman, Clipton, NJ, USA) before analysis by HPLC.

Liver samples were prepared for the analyses of vitamin E. Two hundred milligram of frozen liver sample was accurately weighed and homogenized for 3 min (3 times) in 4.5 ml methanol containing 1% H_3PO_4 and 0.45 ml 5% pyrogallol on ice. The homogenate was centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was transferred to a 10 ml volumetric flask and the pellet was homogenized again in

4.5 ml methanol containing 1% H_3PO_4 and 0.45 ml 5% pyrogallol for 3 min on ice. The supernatants were combined and the final volume adjusted to 10 ml with methanol. Mix by inverting the flask 3 times and stored a 1.5 ml aliquot at –20°C. The aliquot was filtered with a disposable syringe filter (0.45μm, Whatman, Clipton, NJ, USA) before analyses by HPLC.

The HPLC system (YoungLin Instrument Co., Ltd., Korea) consisted of a model SDV 50A Vacuum Degasser and Valve Module, a Solvent Delivery Pump model SP 930D, a Scanning Fluorescence Detector Waters 470 Millipore and a CTS 30 Column Oven. The HPLC was operated by conditions of 150 x 3.0 mm Luna 5u C18 column (Phenomenex, CA, USA) at 1.2 ml/m flow rate, 40 °C column temperatures, and 20 μl injection size. The mobile phase contained 93% methanol, 6.5% water and 0.5% H_3PO_4.

Statistical analysis

Data were subjected to one-way ANOVA in SPSS version 11.0. The significant differences between group means were compared using Duncan's multiple test. Data presented are means ± standard deviations (SD). The percentage data of weight gain and specific growth rate were arcsine transformed before the ANOVA analysis. Differences were considered significant at $P < 0.05$.

RESULTS

Inclusion of vitamin E significantly influenced growth performance of juvenile parrot fish. Significantly higher final body weight (Fig. 1.1), weight gain (Fig. 1.2), specific growth rate (Fig. 1.3), and protein efficiency ratio (Fig. 1.4) was found in fish fed 38 mg vitamin E/kg diet (E25 diet), and beyond the level no further increase was observed. Lower feed conversion ratio (Fig. 1.5) and feed intake (Fig. 1.6), was observed in fish fed the E25 diet. No mortality was observed during the 12 weeks of the feeding trial (Fig. 1.7) Addition of vitamin E to basal diet did not significantly affect whole body protein, lipid, ash and moisture (Table 1.3). Condition factor of fish fed the experimental diets for 12 weeks was not significantly different between treatments (Table 1.6).

Nitroblue tetrazolium activity was significantly increased with an increase in dietary vitamin E indicating an improved nonspecific immune response of the fish (Fig. 1.8). Fish fed vitamin E over 87 mg/kg diet (diets E75, E100, and E500) exhibited significantly higher phagocytic activity than the fish fed the control diet deficient in the vitamin E. Vitamin E concentration (Fig. 1.9) in the liver of fish after the 12 weeks of feeding trial was significantly increased with an increase in dietary vitamin E as a dose dependent manner ($Y= 1.0659x+6.0012$, $r^2 = 0.9992$). Serum myeloperoxidase activity (Fig. 1.12) was also increased by the increment of dietary vitamin E level. However, lysozyme activity (Fig 1.11) was not significantly affected by dietary vitamin E level. The erythrocyte fragility was not significantly different among the different dietary treatments except for diet E50 (Table 1.5).

The cumulative mortality over 50% was observed in all dietary groups at day

6 of challenge test with *V. anguillarum* after the feeding trial (Fig. 1.10). Interestingly, the fish groups fed the E500 diet which is a mega dose of vitamin E showed higher survival (14.0, 5.6, 5.6, 0, 0 and 0% for E500, E100, E75, E50, E25, and the control diet, respectively) than the other fish groups from day 7 to the end of the challenge test showing an increased disease resistance against *V. anguillarum*.

DISCUSSION

The present study showed that vitamin E is an essential nutrient for normal growth of juvenile parrot fish. The optimum dietary requirement of vitamin E was found to be approximately 40 mg/kg diet for the fish species. This value is in agreement with vitamin E requirement values for Chinook salmon (Woodall et al., 1964), Atlantic salmon (Lall et al., 1988), channel catfish (Murai and Andrews, 1974; Lovell et al., 1984; Wilson et al., 1984), and rainbow trout (Hung et al., 1980; Cowey et al., 1983) of 30, 35, 25-50, and 25-50 mg/kg, respectively. (Bai and Lee, 1998) reported that a high dose of dietary vitamin E (over 500 mg/kg) could result in negative growth performance and hematology in a marine fish, Korean rockfish. In the study, juvenile Korean rockfish exhibited lower hematocrit and hemoglobin as well as poor growth performance and feed utilization compared to the fish fed an optimum dietary level of vitamin E (45 mg/kg). The toxic effects by a high or mega dose of dietary vitamin E have been reported with respect to growth performances in other fish species such as, brook trout fry (Poston and Livingston, 1969), African catfish (Baker and Davies, 1996), and rainbow trout (Kiron et al., 2004). In the present study, however, none of adverse effects on growth performances was observed. This might be an case study showing the fact that vitamin E requirement varies depending on fish species, size, age and other conditions (Hung et al., 1981).

In the present study, increased levels of dietary vitamin E produced an increase in vitamin E deposition in liver tissue (Fig. 1.9). This is a very common result on this vitamin and similar results were reported in many fish species (Boggio

et al., 1985; Murata and Yamauchi, 1989; Frigg et al., 1990; Gatlin et al., 1992; Bai and Gatlin, 1993) and terrestrial animal (Marusich et al., 1975; Bartov and Bornstein, 1977; Rethwill et al., 1981; Lin et al., 1989).

The carcass compositions were not affected by the inclusion of vitamin E. (Gatta et al., 2000) found similar results. Sau et al. (2004) found no difference in lipid, ash and moisture content in rohu fry.

Vitamin E plays an important role in the fish immune response (Putnam and Comben, 1987). However, little is known about its *in vivo* effects and further work is required to determine the optimum dietary vitamin E dose for optimal functioning of fish immune system. The present work attempted to address this lack of information and provided an approximate dietary dose of vitamin E for an improved immune system in parrot fish. High dietary vitamin E level is definitely required by parrot fish to maintain their adequate or increased immunity than for growth in the case of no adverse effect by its high or mega doses. Based on the results for NBT and MPO activity, it was clear from the current study that the optimum dietary vitamin E level would be over 500 mg/kg for the improvement of nonspecific immune response, (Fig. 1.8, 1.12) and disease resistance against *V. anguillarum* (Fig. 1.10). Similar results suggesting a high level of the vitamin for immunity were also reported in grouper (Lin and Shiau, 2005), Atlantic salmon (Lygren et al., 2001), rainbow trout (Clerton et al., 2001; Pearce et al., 2003; Kiron et al., 2004; Puangkaew et al., 2004), flatfish (Pulsford et al., 1995), gilthead seabream (Mulero et al., 1998; Ortuno et al., 2000; Cuesta et al., 2001; Ortuno et al., 2003), and golden shiner (Chen et al., 2004). Nevertheless lysozyme activity in parrot fish showed a trend to increase with an increment of dietery supplementation levels of vitamin E, except for diet E 25, the

differences were not statistically significant. The similar results were obtained for rainbow trout (Puangkaew et al., 2004; Kiron et al., 2004). In Atlantic salmon (Fevolden et al., 1994), found a negative correlation between elevated lysozyme and resistance to two bacterial pathogens.

The higher values of MPO activity related to be dependent on vitamin E supplementation in parrot fish , are similar to other immunostimulants used in other fish species like vitamin C in Asian catfish (Kumari and Sahoo, 2006). Other immunostimulants like lactoferrin, glucan, levamisole as well as vitamin C feeding to healthy subgroup of other fish species significantly increased MPO content. (Siwicki, 1989; Ortuno et al., 1999; Findlay and Munday, 2000; Sahoo and Mukherjee, 2001ab; Kumari et al., 2003; Kumari and Sahoo, 2005b). Erithrocyte fragility did not show significant differences between fish receiving the lowest and highest concentrations of tocopherol, as reported by Cowey et al. (1981), likely due to the duration of the feeding trial and the size of the fish (Table 1.5).

A higher dose of dietary DL-α-tocopheryl acetate produced higher survival in the *V. anguilarum* challenged fish. Higher survival was obtained in dietary treatment E500 with a survival of 14%, followed by dietary treatment E100 and E75 both with 5.6% survival (Fig. 1.10). The course of mortality following the experimentally induced vibriosis showed that a high dietary vitamin E level over 500 mg/kg could increase the resistance of parrot fish juvenile against *V. anguilarum*. The suggested optimum dietary requirement of vitamin E could approximately be over 500 mg/kg with respect to the immune system in juvenile parrot fish, even though the finding in the present study do not give a further information on the accurate dietary vitamin E requirement for the best immune responses. Other pathogens could give different

results in different challenge tests.

In conclusion, vitamin E should be supplemented in the diets for parrot fish. The findings in the present study suggest that an optimum level of the dietary vitamin E would be approximately 40 mg/kg for the maximum growth performances. However, it is suggested that over 500 mg vitamin E/kg diet could improve the immune responses of the fish. Further studies are needed to be carried out to determine more reliable and accurate levels of dietary vitamin E for both growth performance and immune responses in parrot fish.

Table 1.1. Formulation of experimental diets (% dry matter, DM)

	Diet					
Ingredients	**E0**	**E25**	**E50**	**E75**	**E100**	**E500**
Fish meal (defatted)	10.0	10.0	10.0	10.0	10.0	10.0
Casein (vitamin-free)	35.0	35.0	35.0	35.0	35.0	35.0
Gelatin	10.0	10.0	10.0	10.0	10.0	10.0
CMC	2.0	2.0	2.0	2.0	2.0	2.0
Dextrin	22.0	22.0	22.0	22.0	22.0	22.0
Mineral Mix[1]	3.0	3.0	3.0	3.0	3.0	3.0
Vit. Mix (vit-E free)[2]	3.0	3.0	3.0	3.0	3.0	3.0
Squid liver oil	14.0	14.0	14.0	14.0	14.0	14.0
Vitamin E	0.0	0.0025	0.0050	0.0075	0.01	0.05
Cellulose	1.0	0.9975	0.9950	0.9925	0.99	0.95

[1]Mineral premix (g/kg of mixture) $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate,40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3.6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$,2.0; $CoCl_2.6H_2O$, 1.0.

[2]Vitamin premix (g/kg of mixture) L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 0.0;thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-amino benzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

Table 1.2. Proximate composition of the experimental diets (% DM)

	Diet					
Ingredients	**E0**	**E25**	**E50**	**E75**	**E100**	**E500**
Dry matter, %	90.85	91.15	91.12	91.01	90.23	90.82
Protein, % DM	52.00	52.25	52.34	52.40	52.15	49.33
Lipid, % DM	11.33	11.27	11.23	12.07	11.73	13.20
Ash, % DM	3.91	3.91	3.85	3.88	3.87	3.46
Gross energy, MJ/kg DM	21.95	21.95	21.96	21.96	21.96	21.97

DM: dry matter

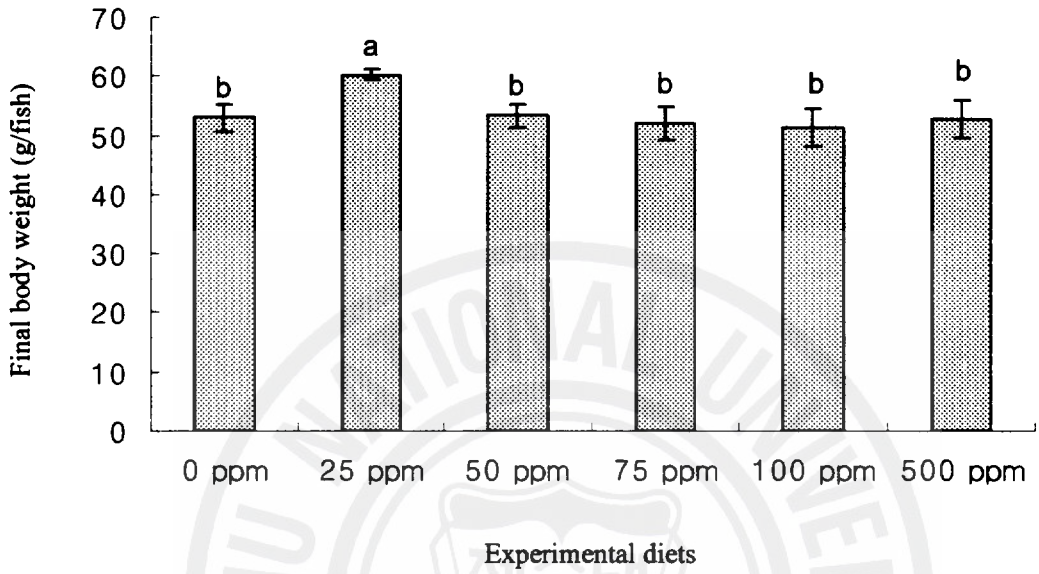

Figure 1.1. Final body weight (g / fish) of juvenile parrot fish fed the experimental diets for 12 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

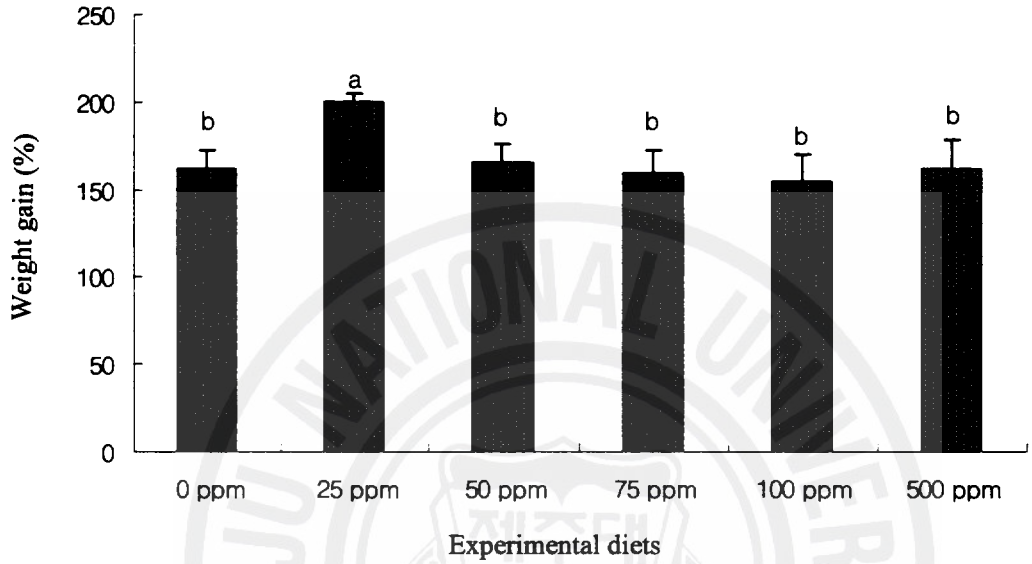

Figure 1.2. Weight gain (%) of juvenile parrot fish fed the experimental diets for 12 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

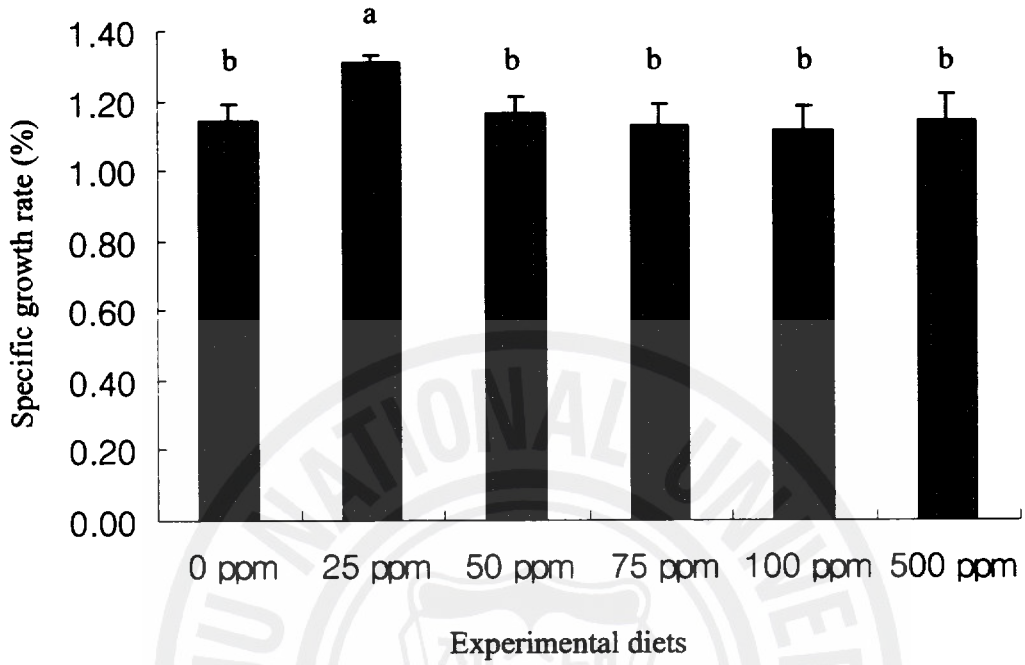

Figure 1.3. Specific growth rate (%) of juvenile parrot fish fed the experimental diets for 12 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

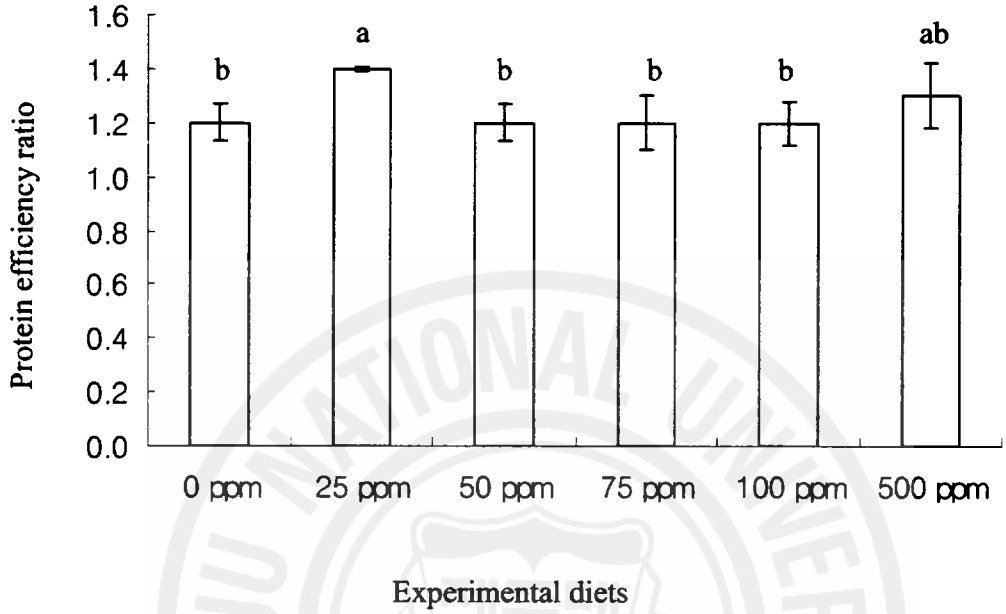

Figure 1.4. Protein efficiency ratio of juvenile parrot fish fed the experimental diets for 12 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

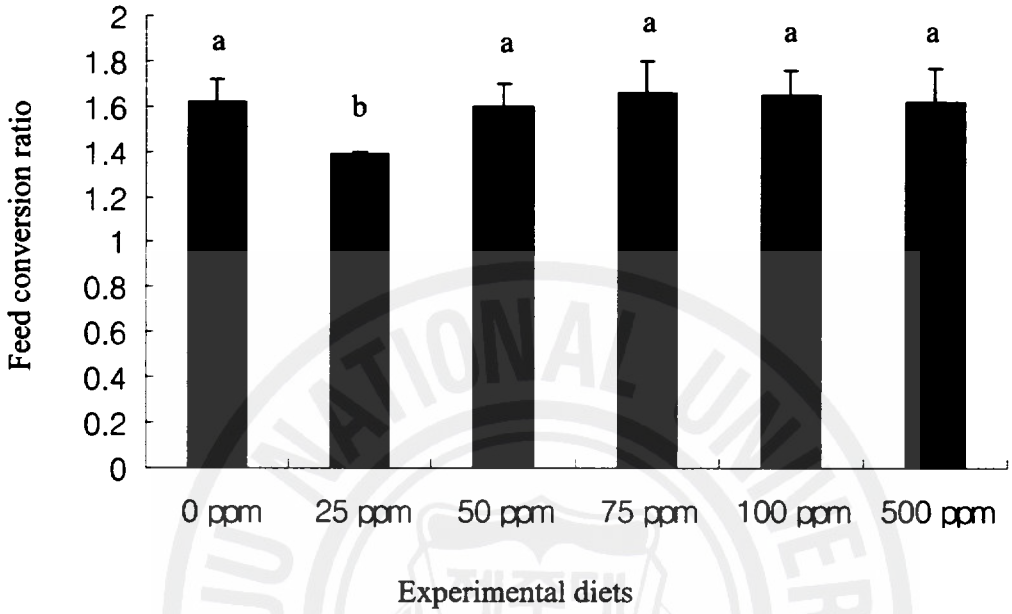

Figure 1.5. Feed conversion ratio of juvenile parrot fish fed the experimental diets for 12 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

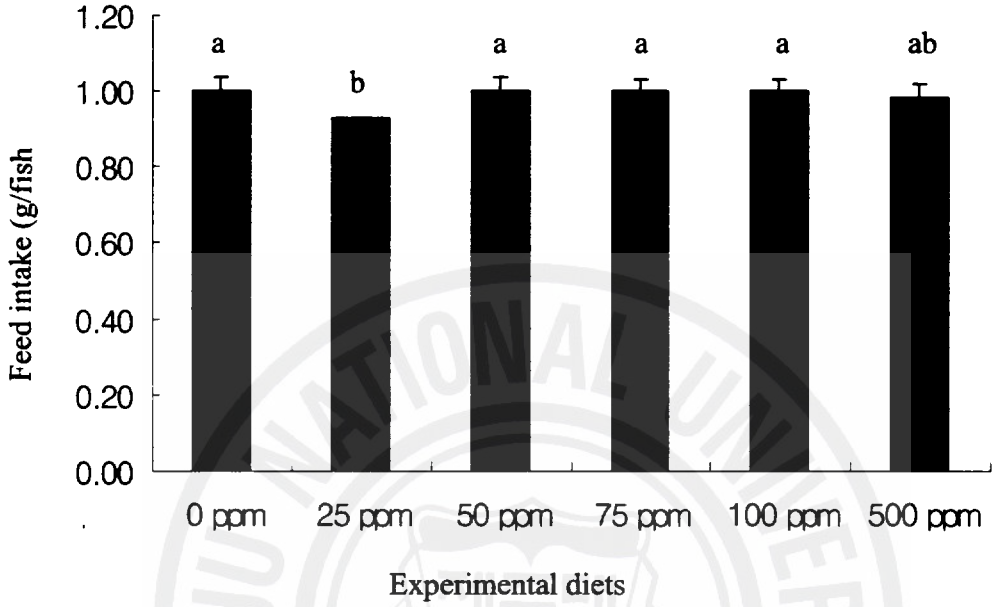

Figure 1.6. Feed intake of juvenile parrot fish fed the experimental diets for 12 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

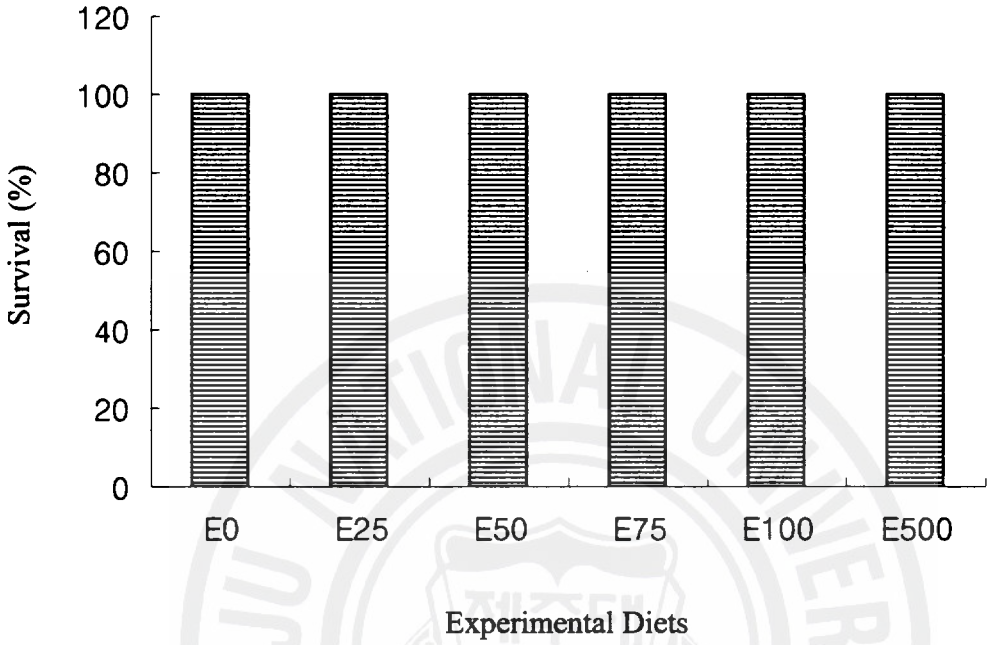

Figure 1.7. Survival (%) of juvenile parrot fish fed the experimental diets for 12 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

Table 1.3. Whole body composition of juvenile parrot fish fed the experimental diets for 12 weeks

Diets	E0	E25	E50	E75	E100	E500
Moisture (%)	72.3±2.0	71.6±0.8	72.2±1.4	72.9±1.4	71.6±0.9	71.0±1.6
Protein (%)	56.9±3.0	58.2±3.2	59.8±4.6	58.9 ±4.6	59.5±4.4	53.5±2.4
Lipid (%)	19.3±2.3	19.7±2.3	20.9±1.1	20.9±5.1	20.7±4.1	23.5±6.1
Ash (%)	4.9 ±0.7	4.3±0.5	4.4±0.4	4.3±0.4	4.9±0.5	4.7±0.9

Values are presented as mean ± SD.

Table 1.4. Vitamin E content in the experimental diets, and liver of the fish fed the experimental diets for 12 weeks

	Diets					
	E0	E25	E50	E75	E100	E500
Diet	0.00	38.40	52.90	87.40	118.90	537.80
Liver	17.36	55.18	87.78	97.78	104.11	385.18

Values are presented as mean ± SD.

Table 1.5. Erythrocyte fragility of juvenile parrot fish fed the experimental diets for

Diets	E0	E25	E50	E75	E100	E500
O.F.	0.63 ± 0.0002^{ab}	0.61 ± 0.002^{a}	0.69 ± 0.005^{b}	0.65 ± 0.003^{ab}	0.63 ± 0.001^{ab}	0.65 ± 0.001^{ab}

Values are presented as mean ± SD. Value in the same row having different superscript letters are significantly different ($P < 0.05$).

[1]Percent salinity for 50% hemolysis. n=2 means, each based on 4 fish per diet treatment.

O.F. : Osmotic fragility

Table 1.6. Condition factor of juvenile parrot fish fed the experimental diets for 12 weeks

Diets	E0	E25	E50	E75	E100	E500
CF (%)[1]	2.20±0.54	2.09±0.23	2.13±0.59	2.12±0.20	2.05±0.17	2.32±0.27

Values presented are mean ± standard deviation. Values in the same row with different letters are significantly different (P <0.05).

[1]Condition factor = 100 x fish weight (g)/fish length (cm)^3.

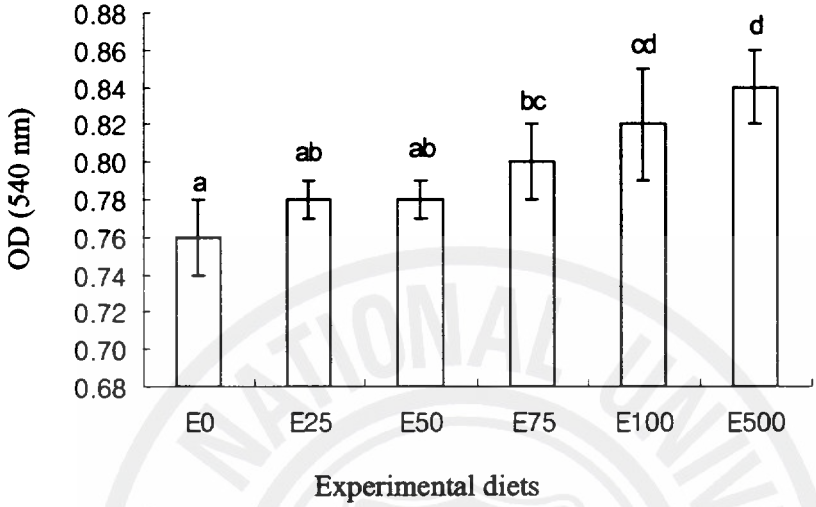

Figure 1.8. NBT activity of juvenile parrot fish fed the experimental diets for 12 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

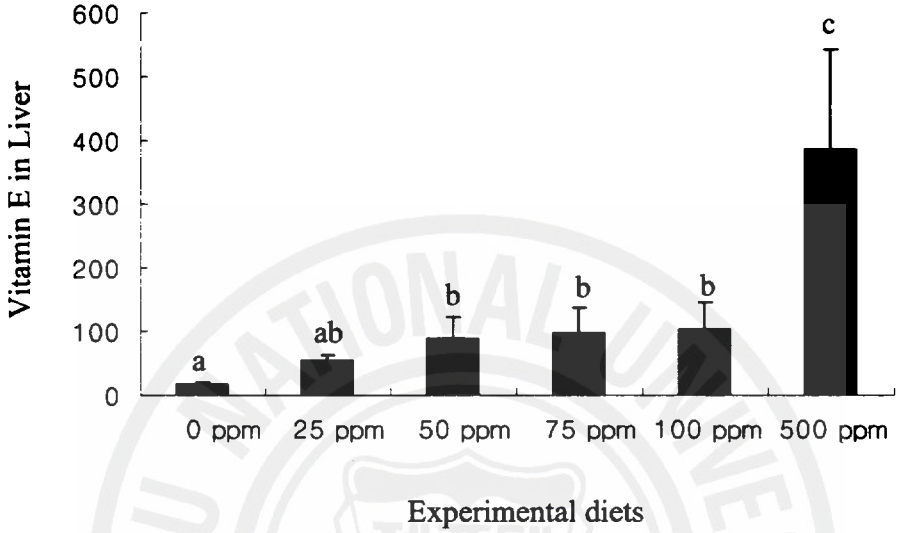

Figure 1.9. Vitamin E level in liver of juvenile parrot fish fed the experimental diets for 12 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

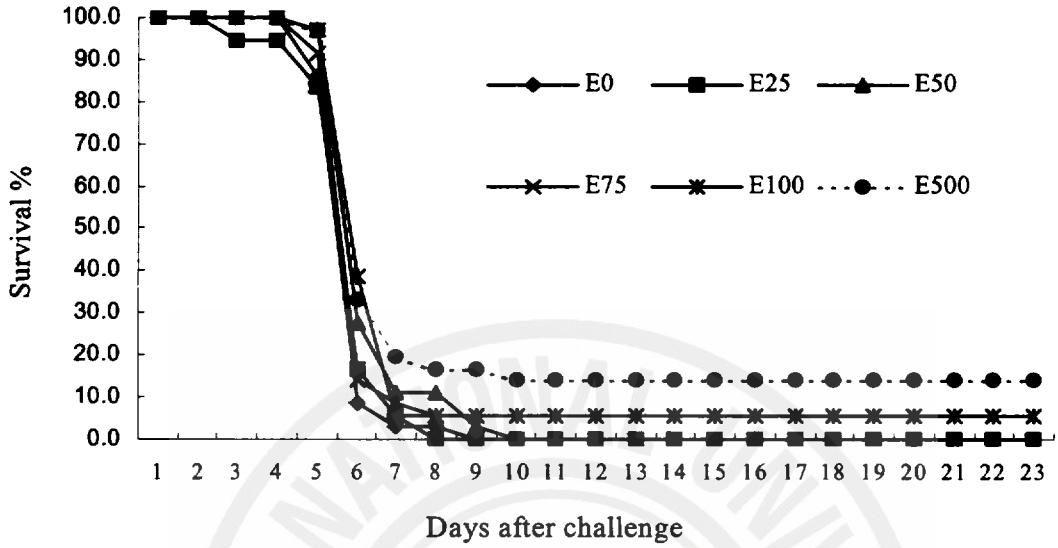

Figure 1.10. Percent survival of juvenile parrot fish under challenge test for 24 days

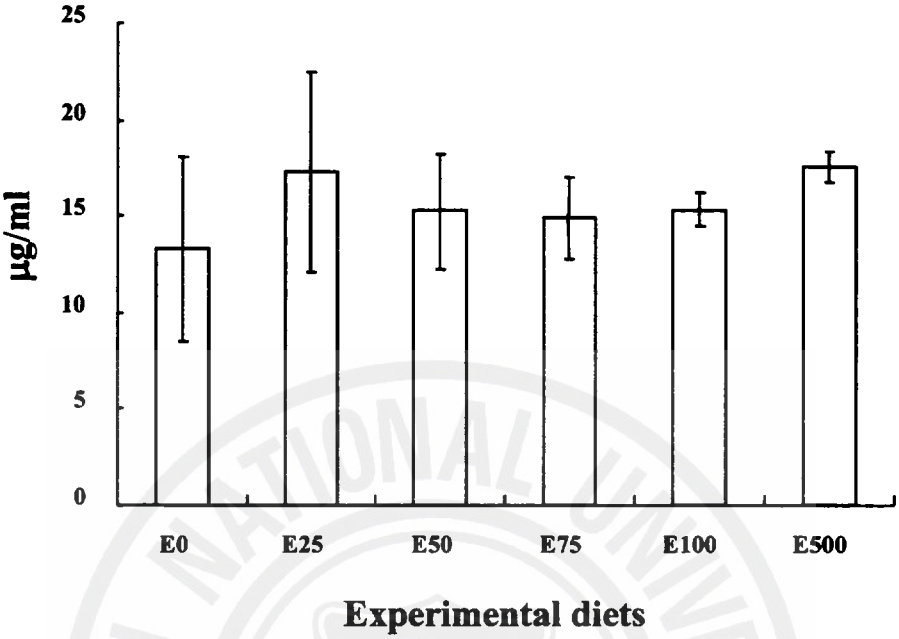

Figure 1.11. Serum lysozyme activity of juvenile parrot fish fed the experimental diets for 12 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

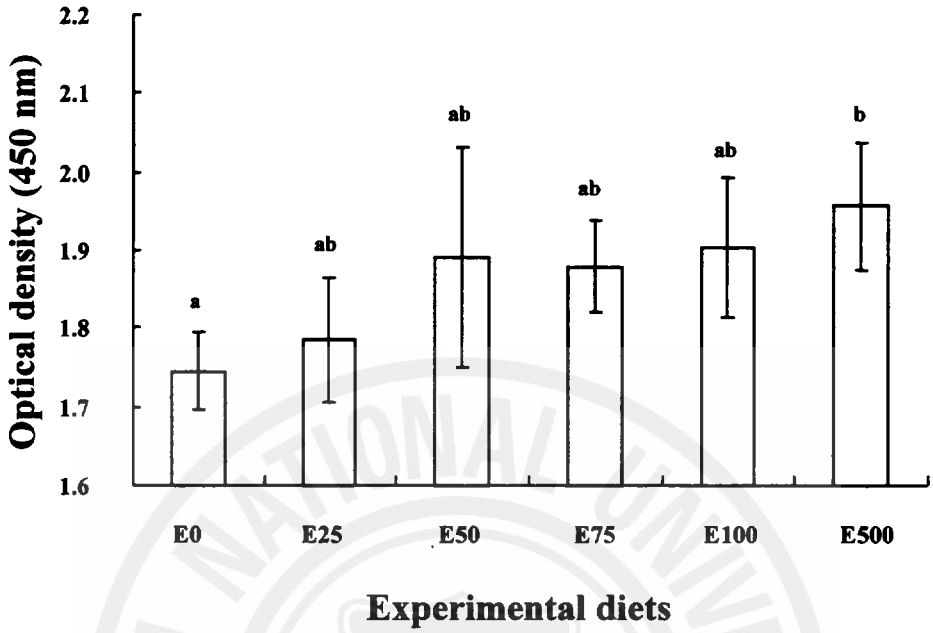

Figure 1.12. Serum myeloperoxidase activity of juvenile parrot fish fed the experimental diets for 12 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

Part II

Dietary myo-inositol requirement for parrot fish *Oplegnathus fasciatus*

ABSTRACT

This feeding trial was conducted to estimate the essentiality and requirement of *myo*-inositol (MI) for juvenile parrot fish, *Oplegnathus fasciatus*. MI was supplemented 0, 100, 200, 400 and 800 mg/kg diet in the basal diet providing 0, 83, 212, 410 and 943 mg MI/kg diet respectively. Each diet was fed to triplicate groups of juvenile parrot fish (initial body weight, 11.5 g). After 18 weeks of feeding trial, fish fed diets with supplementation of MI had significantly higher weight gain and specific growth rate than those of fish fed the basal diet. Supplementation of dietary MI did not affect survival of parrot fish. Dietary MI affected blood hematocrit, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, while plasma triglyceride and myeloperoxidase (MPO) activity were not affected. Liver MI concentration of the fish fed the experimental diets was significantly increased by dietary supplementation of MI. The liver MI concentrations were highest in fish fed the ≥212 mg MI/kg diet and lowest in fish fed the MI free diet. Liver lipid concentrations were significantly decreased as increment of dietary MI supplementation. The dietary requirement of MI based on weight gain percentage and liver MI concentrations were analyzed by broken-line regression and indicated that the requirement for dietary MI in juvenile parrot fish is about 94 and 121 mg/kg diet, respectively. Therefore, we recommend the addition of *myo*-inositol approximately 100 mg/kg diet to prevent a potential problem by an abnormal lipid metabolism and to maximize the growth performances in juvenile parrot fish.

INTRODUCTION

Myo-inositol (MI), the most biologically active isomer of inositol, is a major structural component of phospholipid structures in animal tissues (Cody, 1984). Its primary function appears to serve as a structural element, with the six hydroxy groups available for esterification or for acid salt formation to form an integral portion of cell membranes. The stereo configuration of these cell membrane elements probably plays a major role in cell membrane permeability to various ions and molecules (West et al., 1966). Phosphatidylinositol was shown to be involved in signal transduction in several metabolic processes (NRC, 1993). MI can be synthesized in various animal tissues through a ring closure of glucose-6-phosphate, catalyzed by the enzyme L-myo-inositol-1-phosphate synthase followed by a L-myo-inositol-1-phosphatase catalyzed reaction (Eisenberg, 1967). However, in rodents, the capacity for inositol biosynthesis *in vivo* appears to be limited since dietary MI is needed to prevent the development of MI deficiency (Hayashi et al., 1974; Kukiss and Mookerjea, 1978; Chu and Geyer, 1982, 1983). Dietary MI deficiency induced alopecia, growth retardation and lipodystrophy characterized by an abnormal lipid accumulation in liver and/or small intestine accompanied by a hypolipoproteinemia, increased levels of cholesterol and triacylglycerols but decreased amount of total phospholipids, phosphotidyl-choline, phosphotidyl-ethanolamine and phosphotidyl-inositol (Chu and Hegsted, 1980; Chu and Geyer, 1981, 1983; Holub, 1986)

In fish, nutritional evidence suggests that metabolic synthesis of inositol occurs to some degree in liver, kidney and other tissues (Aoe and Masuda, 1967; Burtle and

Lovell, 1988; Deng et al., 2002). For some fish species, however, de novo synthesis is inadequate to support their metabolic needs and, thus, require an exogenous source of this vitamin. Deficiency symptoms of MI were reported in rainbow trout, chinook salmon, red sea bream, Japanese eel, parrot fish, and yellow tail indicating poor appetite, anemia, poor growth, fin erosion, dark skin coloration, slow gastric emptying, decreased cholinesterase and certain aminotransferase activities (McLaren et al., 1947; Kitamura et al., 1967; Yone et al., 1971; Arai et al., 1972; Ikeda et al., 1988; Hosokawa, 1989).

The dietary essentiality of MI in fish is somewhat unclear. The reported quantitative requirements of dietary MI for some fish species such as Atlantic salmon, common carp and red sea bream range from 300 to 900 mg/kg diet (Waagbø et al., 1998; Aoe and Masuda, 1967; Yone et al., 1971). Other fish species such as channel catfish, Asian sea bass and sunshine bass do not require a dietary supplementation of MI for normal growth and development (Burtle and Lovell, 1988; Boonyaratpalin and Wanakowat, 1993; Deng et al., 2002). These fish species can synthesize sufficient MI to meet their physiological needs (Mai et al., 2001).

Parrot fish, *Oplegnathus fasciatus*, is carnivorous species and has been regarded as emerging aquaculture species because of its advantages of high economic value, excellent meat quality and strong resistance to diseases. However, only few nutritional studies on parrot fish have been reported (Kang et al., 1998; Wang et al., 2003). No data are available on the dietary essentiality of MI for juvenile parrot fish. Therefore, the purpose of this feeding trial was to determine the requirement and/or essentiality of dietary MI and to discuss the effect of MI on lipid metabolism in parrot fish.

MATERIALS AND METHODS

Experimental diets

Five semi-purified diets were formulated to be isonitrogenous and isocaloric with 48% crude protein and 18.8 MJ/kg diet by graded levels of MI (0, 100, 200, 400 and 800 mg/kg designated as M0, M100, M200, M400 and M800, respectively) at the expense of cellulose. (Table 2.1). Ethanol-extracted fish meal was employed in the diets as an attractant to enhance palatability in semi-purified diets (Lee and Dabrowski 2004). Fish meal was extracted two times by 70% aqueous ethanol for 48 h, and dried using an electric fan at room temperature.

Inositol content in the experimental diets was measured according to the enzymatic assay described by (Ashizawa et al., 2000). The analyzed concentration of inositol in the diets M0, M100, M200, M400 and M800 was 0, 83, 212, 410, and 943 mg/kg diet, respectively.

The experimental diets were prepared by thoroughly mixing ingredients with oil and 35% distilled cold water in a mixer (NVM-14-2P, Korea) and pelleting the wet dough by a chopper machine (SMC-12, Kuposlice, Busan, Korea) at 3 mm of diameter. The diets were then freeze dried at –40 °C, crushed into desirable particle sizes (0.4 – 2.0 mm) and stored at –20 °C until use.

Feeding trial and sample collection

Juvenile parrot fish were transported from a private hatchery (at Jeju Island,

Korea) to Marine and Environmental Research Institute, Cheju National University, Jeju, South Korea. All the transported fish were fed a commercial diet for 6 weeks to allow acclimation in the experimental facilities and conditions, and to recover from the stress of transportation. After the acclimation, the fish (initial body weight 11.5±0.01g) were randomly assigned to eighteen 70 L polyvinyl conical tanks (triplicate groups per dietary treatment) at a density of 20 fish/tank. The feeding trial was conducted for 18 weeks in the flow through system supplied with sand filtered seawater. Aeration was also provided to maintain dissolved oxygen levels near to the saturation. The photoperiod was scheduled by 12:12 h light/dark by fluorescent light. Water temperature ranged from 20 to 28 °C according to the seasonal change. Salinity of the water was 34 ppt, dissolved oxygen was ranged from 7.80 to 8.05 mg/L, and pH was 8.02±0.01. The triplicate groups of fish were fed the experimental diets to apparent satiation (twice a day, 9:00 and 17:00 h) for 7 days a week. After feeding, uneaten feeds were removed by siphoning to calculate feed consumption and utilization. Inside of the tanks was routinely cleaned by a sponge to prevent the growth of microflora. The growth of fish was measured every three weeks and feeding rate was adjusted accordingly. Feeding was stopped 24 h prior to weighing.

At the end of 18 weeks of feeding trial, three fish per tank (nine fish per treatment) were randomly selected after 3 and 24 h feeding experimental diets, respectively and anaesthetized with MS-222 solution (100 mg/L) for blood analyses. The blood samples were taken from caudal veins with heparinized syringes. Three fish per tank (nine fish per treatment) were randomly selected for whole body approximate composition. Livers from three fish per tank (nine fish per treatment) were removed and stored at –80 °C for analysis of lipid content.

Analysis

Proximate compositions and growth performance

At the end of feeding trial, all fish in each tank were weighed and counted to compute the weight gain (WG), feed conversion ratio (FCR), specific growth rate (SGR), final mean body weight (FBW), feed intake (FI), protein efficiency ratio (PER) and survival.

Analysis of crude protein, moisture and ash in the experimental diets were performed by the standard procedures (AOAC, 1995). Lipid content in experimental diets and liver was determined using Soxhlet System (SH6, Korea).

Hematological parameters

Hematocrit was determined for three individual fish per tank by microhematocrit technique (Brown, 1980). Hemoglobin was measured in the same three fish by using a Photometer CH100 Plus (Calenzano, Firenze, Italy). Plasma tryglycerides, total cholesterol, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined for three fish per tank by using a Photometer CH100 Plus (Calenzano, Firenze, Italy). Myeloperoxidase (MPO) activity in plasma was measured according to Kumari and Sahoo (2005) with slight modification. Plasma (20 µl) was diluted with 80 µl of HBSS without Ca^{2+} or Mg^{2+} in 96-well plates. To which 35 µl of 20 mM 3,3`,5,5`-tetramethylbenzidine hydrochloride (TMB) (Sigma, USA) and 5 mM H_2O_2 (both substrates of MPO and prepared on the same day) were added. The colour change reaction was stopped after 2 min by adding 35 µl of 4 M sulphuric acid (H_2SO_4). The optical density (O.D.) was

read at 450 nm in a microplate reader.

***Myo*-inositol concentration**

Inositol concentration in the experimental diets and liver samples were analyzed according to the enzymatic assay described by Ashizawa et al., (2000). Briefly, inositol was extracted from the sample by perchloric acid (16 %, w/v) and centrifuged at 5,000 × g for 10 min at 4 °C. All supernatants were mixed with 2.0 M K_2CO_3 and centrifuged again. A 100 ml aliquot of the supernatant was added to 10 ml hexokinase reagent, which contained 200 mM Tris-HCl buffer, 400 mM adenosine triphosphate disodium, (the pH of the solution was adjusted to 8.6 by adding 10.0 M NaOH) and 115 U/ml hexokinase. The mixture was incubated at 37 °C for 90 min, heated for 3 min in a boiling water bath to stop the reaction, and added with 20 ml of 4.5 M HCl. After 10 min at 25 °C, 22 µl of 3.0 M K_2CO_3 was added. Measurements were done using a 96-well microplate reader. One hundred ml of sample extract obtained as described above were mixed with 100 µl of MI reagent, which contained 210 mM triethanolamine hydrochloride-32 mM K_2HPO_4-KOH buffer (pH 8.6), 1.2 % (v/v) Triton X-100, 10 mM β-NAD, 1.0 U/ml diaphorase, 0.1 % (w/v) bovine serum albumin and 60 mg /ml INT. After the absorbance of the solution was measured at 492 nm with a microplate reader (Multiskan EX, Thermo Electron, USA), the reaction was initiated by addition of 10 µl 2.1 U/ml MI dehydrogenase dissolved in 20 mM potassium phosphate buffer (pH 7.0) to each well. The mixture was allowed to stand for 30 min at room temperature, and then the absorbance (A) at 492 nm was measured again. From ΔA, an increase in absorbance

during the reaction, MI content was calculated.

Statistical analysis

All experimental diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, multiple were made with Duncan's multiple range test. Statistical significance was determined by setting the aggregate type I error at 5% ($P<0.05$) for each set of comparisons. Data are presented as mean±SD. Percentage data were arcsine transformed before statistical analysis. Dietary *Myo*-inositol requirement of juvenile parrot fish was estimated by broken-line regression method based on weight gain and liver *myo*-inositol concentration. The correlation between MI and lipid of the liver were estimated using Microsoft Excel regression.

RESULTS

Final mean body weight (FBW), weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER), feed conversion ratio (FCR), and survival of juvenile parrot fish fed the experimental diets are presented in (Fig. 2.1~2.6). WG and SGR of the fish fed diets containing MI were significantly higher than fish fed the MI free control diet. In MI supplemented treatment (MI 100 to MI 800), however, growth performance and feed utilization were not significantly different by increased dietary MI level. Survival was not affected by dietary supplementation of MI.

Hematological parameters of juvenile parrot fish 3 and 24 h after feeding are presented in Table 2.2. Hematocrit, hemoglobin, triglyceride level and myeloperoxidase (MPO) activity were not affected by dietary supplementation of MI 3 h after the feeding. However, cholesterol in fish fed MI free diet was significantly lower compared to that of fish fed diets containing MI (Fig. 2.7). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values in fish fed diets containing MI were significantly lower than that of fish fed the MI free diet (Fig. 2.8). After 24 h of feeding, we did not find any significant difference in hematological parameters, except hematocrit value (Table 2.2). Fish fed $\geq$212 mg MI/kg diet had significantly higher hematocrit value than fish fed the control diet.

Fish fed the diets containing MI Whole body protein concentration of fish fed the diets containing MI were significantly lower, compared to that of fish fed the control diet (Table 2.3)

MI concentration in the liver of the fish fed the experimental diets for 18 weeks

was significantly increased by dietary MI supplementation (Table 2.4). Fish fed the diets containing MI showed a decreasing trend in the concentration of liver lipid compared to the fish fed MI free control diets (Table 2.4, Fig. 2.9). Hepatic lipid concentration of fish fed ≥410 mg MI/kg diet was significantly lower than that of fish fed the MI free control diet.

Broken-line analysis was used to estimate the dietary MI requirement for parrot fish. Based on WG (Fig. 2.10), and liver MI concentration (Fig. 2.11), dietary MI requirements for juvenile parrot fish were 94.3 mg/kg and 121.3 mg/kg, respectively. There was a strong negative correlation between hepatic MI and lipid concentration in the liver of the fish fed the experimental diets (Fig. 2.12).

DISCUSSION

The present study clearly demonstrated the essentiality of dietary *myo*-inositol for maximum growth in juvenile parrot fish over 18 weeks. Weight gain and specific growth rate of parrot fish fed MI containing diets were significantly higher compared to fish fed MI free diet. The growth response of parrot fish to dietary MI in the present study agrees with reports for rainbow trout, chinook salmon, common carp, red sea bream, yellowtail, Japanese eel and olive flounder those have been shown to require exogenous source of dietary inositol for normal growth and to prevent clinical deficiency signs (McLaren et al., 1947; Halver, 1953; Aoe and Masuda, 1967; Kitamura et al., 1967; Yone et al., 1971; Arai et al., 1972; Lee et al., 2006). However, this is in contrast with previous results reported for channel catfish (Burtle and Lovell, 1988), Asian seabass (Boonyaratpalin and Wanakowat, 1993) and sunshine bass (Deng et al., 2002). These authors demonstrated that metabolic synthesis of inositol detected in several organs such as liver and brain and/or synthesis of inositol by intestinal microflora were sufficient to meet the requirements. These findings indicate that the essentiality of dietary MI could depend on fish species, sizes, and rearing conditions.

The dietary MI requirement of juvenile parrot fish is about 94.3 mg/kg diet by broken-line regression based on weight gain in the present study (Fig. 2.10). This level is somewhat lower than results of previous studies with several fish species. Values for rainbow trout and common carp ranged from 250 to 500 mg/kg diet based on weight gain and requirements of 300 to 423 mg/kg was estimated for Pacific

salmon and yellowtail based on maximum liver storage of the vitamin (NRC, 1993). Similarly, (Shiau and Su, 2005) reported that the dietary requirement of MI in growing tilapia was about 400 mg/kg diet.

Hematocrits values of fish fed ≥212 mg MI/kg diet had significantly higher than that of fish fed the MI free diet 24 h after feeding (Table 2.2). The higher values in AST and ALT indicate, that more amino acids are transformed or metabolized into waste in tissue, and therefore become indicators for liver function (Ozaki, 1978; Yamamoto, 1981) or for liver damage (Oda, 1990). In this study, dietary supplement of MI demonstrated its effects in lowering ALT and AST, suggesting its function in improving liver protection. Cholesterol in plasma, 3 h after feeding, was significant lower in control diet in relation to fish diets containing MI, however 24 h after feeding, cholesterol in plasma showed a decreasing trend in relation to an increasing inclusion of myo-inositol in diet (Fig. 2.7). Similar to those found in rainbow trout by Holub et al. (1982), who found that trout fed diet devoid of inositol had large accumulation of neutral lipids in the liver, increased levels of cholesterol and triglycerides.

Liver MI concentration of juvenile parrot fish fed the experimental diets for 18 weeks was significantly increased by dietary supplementation of MI Table 2.4. (Shiau and Su, 2005) reported that liver MI concentration was significantly increased with increment of MI in diets for juvenile tilapia. However, liver MI concentrations were similar in Atlantic salmon (Aoe and Masuda, 1967), channel catfish (Waagbø et al., 1998) and sunshine bass (Deng et al., 2002), when fed diets regardless of the level of dietary MI. In the present study, liver MI concentration was responsive to dietary MI level when the dietary MI requirement was met Fig. 2.11. The plateauing

of the liver MI concentration provides additional evidence to support the requirement based on growth. A level of 94 to 121 mg MI/kg diet, therefore, seems to be sufficient to maximize tissue concentration as well as growth of juvenile parrot fish.

Liver lipid concentrations in the parrot fish were found to have a tendency of decreasing as dietary MI concentration increased in the present study (Table 2.4, Fig. 2.9). The result was similar to that of the studies (Peres et al., 2004; Shiau and Su, 2005) which showed high levels of hepatic lipid in MI free groups. (Katayama, 1997) reported that dietary MI and phytate can protect an accumulation of hepatic lipid in rats.

Accordingly, we speculate that hepatic lipid accumulation might be related to an abnormal lipid metabolism as one of MI deficiency signs when dietary MI is insufficient for juvenile parrot fish.

Also a negative correlation between myo-inositol and lipid concentration in the liver of juvenile parrotfish was found (Figure 2.12).

In conclusion, the findings in this study indicate that the dietary supplementation of *myo*-inositol is needed to maximize the growth performance and to prevent abnormal lipid metabolism in juvenile parrot fish. We recommend that the dietary *myo*-ionsitol requirement in juvenile parrot fish is about 100 mg/kg diet.

Table 2.1. Formulation and proximate composition of the basal diet

Ingredients	%
Casein (vitamin free)	38.0
Gelatin	9.0
Fish meal (defatted)	10.0
Dextrin	15.0
Starch	10.0
Squid liver oil	11.0
CMC	0.5
Vitamin Mix.[1]	3.0
Mineral Mix.[2]	3.0
Cellulose	0.5
Chemical analysis	
Crude protein (%, DM)	47.6
Crude lipid (%, DM)	16.1
Ash (%, DM)	4.8
Gross energy (MJ/kg DM[4])	18.8

[1] Vitamin premix (g kg^{-1} of mixture): retinyl acetate, 0.667; cholecalciferol, 0.033; menadione, 0.133; thiamine hydrochloride, 2.667; riboflavin, 2.933; d-pantothenic acid hemicalcium, 9.667; pyridoxine hydrochloride, 2.667; cyanocobalamin, 0.007; niacinamide, 20.000; folic acid, 0.320; d-biotin, 0.133; ascorbic acid, 30.000; α-tocopherol, 6.667.

[2] Mineral mixture was based on the composition of Lee et al., 2003[4] Gross energy of experimental diets was calculated according to gross energy values 5.64 kcal/g protein, 4.11 kcal/g carbohydrate, and 9.44 kcal/g fat respectively (NRC, 1993).

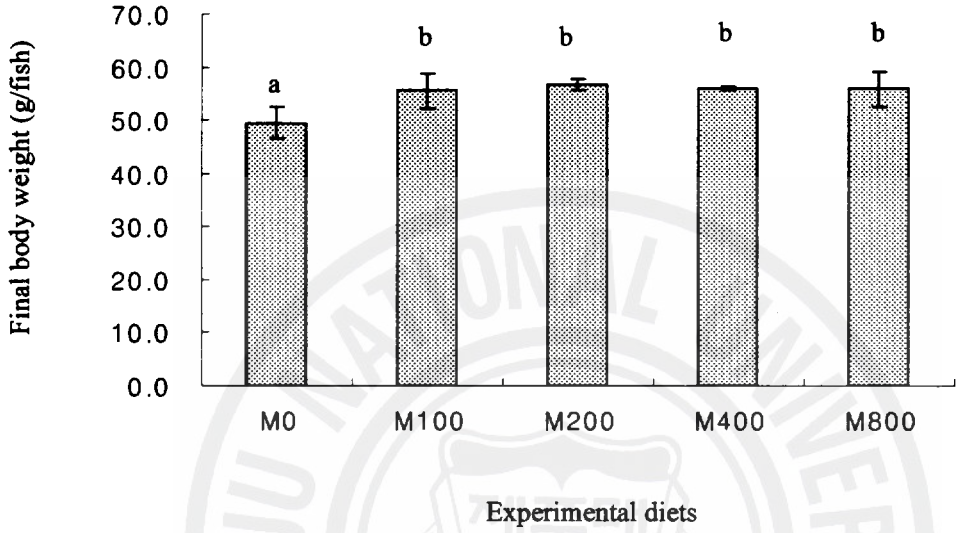

Figure 2.1. Final body weight of juvenile parrot fish fed the experimental diets for 18 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

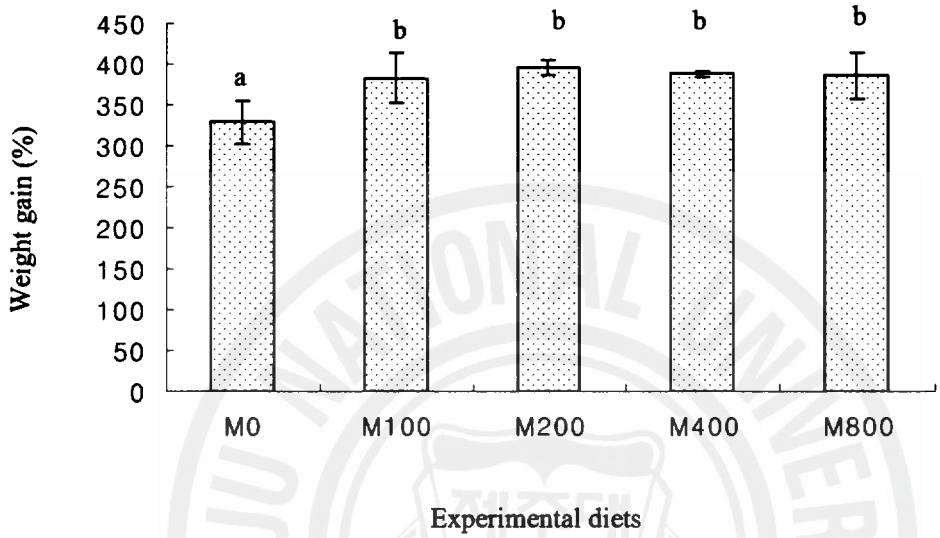

Figure 2.2. Weight gain (%) of juvenile parrot fish fed the experimental diets for 18 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

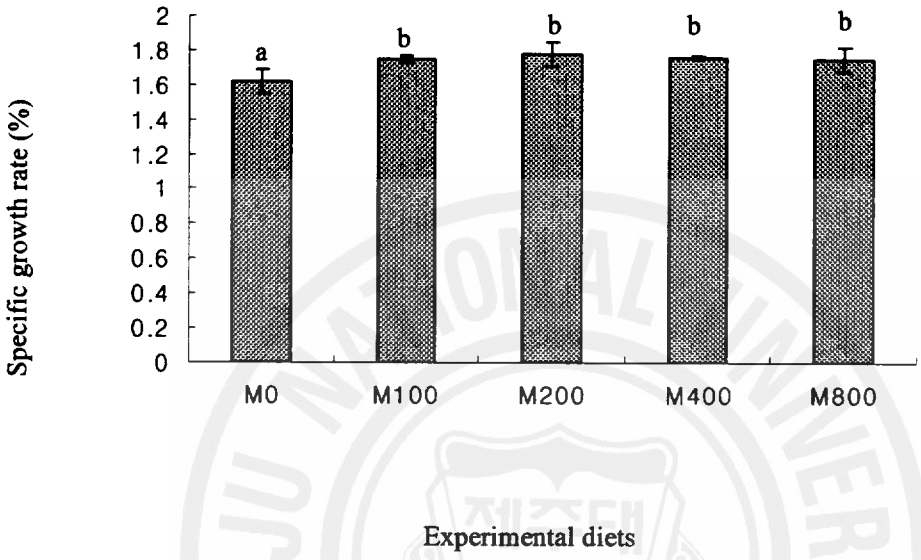

Figure 2.3. Specific growth rate (%) of juvenile parrot fish fed the experimental diets for 18 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

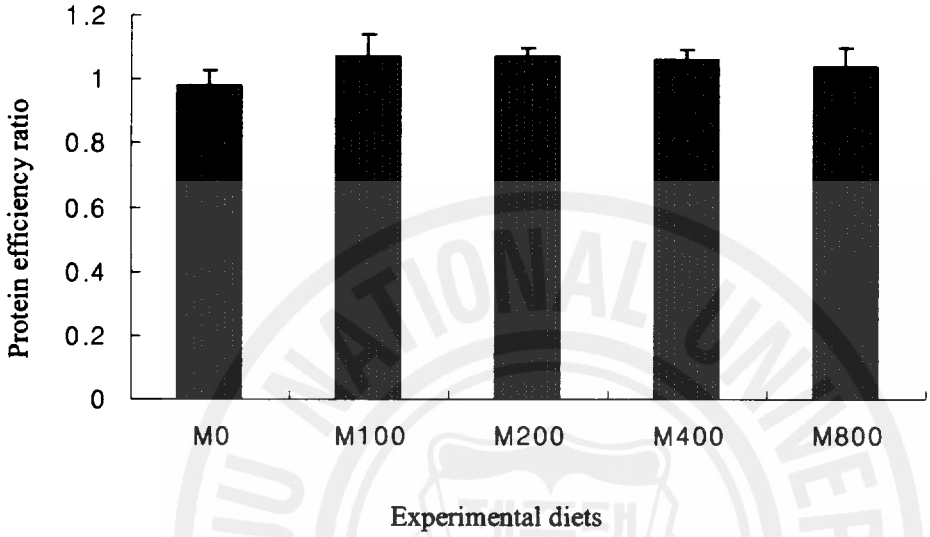

Figure 2.4. Protein efficiency ratio of juvenile parrot fish fed the experimental diets for 18 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

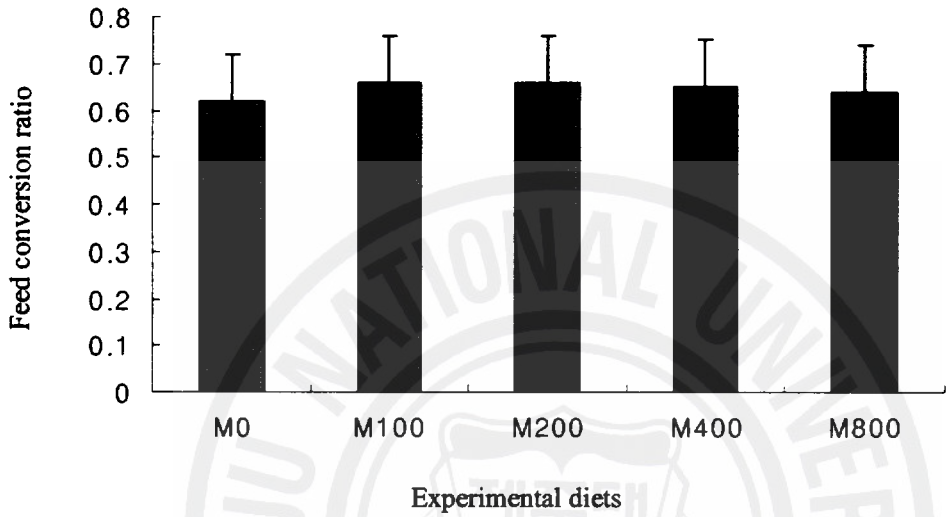

Figure 2.5. Feed conversion ratio of juvenile parrot fish fed the experimental diets for 18 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$). Values are presented as mean ± SD.

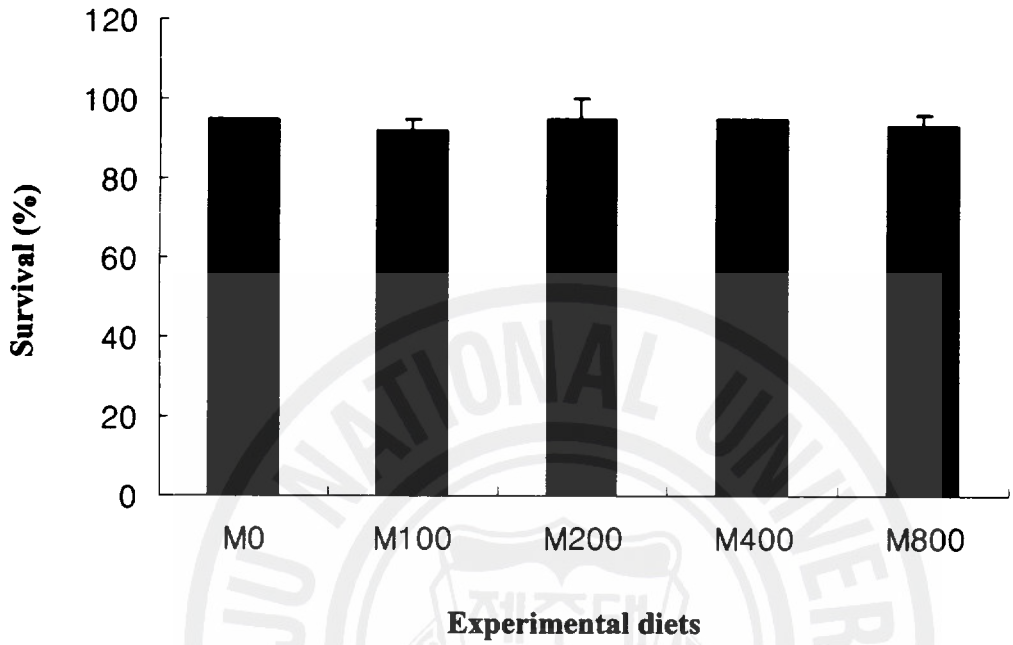

Figure 2.6. Survival (%) of juvenile parrot fish fed the experimental diets for 18 weeks. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

Table 2.2. Hematological parameters of juvenile parrot fish (IBW, 11.5 ± 0.01g) after 3 and 24 h feeding experimental diets with different levels of *myo*-inositol for 18 weeks

Diets	M0(0)	M100(83)	M200(212)	M400(410)	M800(943)
After 3 h feeding experimental diets					
Hematocrits (%)	39.6±2.80	41.8±1.20	41.7±0.80	42.3±2.10	40.0±2.10
Hemoglobin (g/dl)	7.5±0.60	7.9±1.20	8.2±0.50	8.7±0.20	8.0±0.10
AST	15.8±3.30^{a}	10.9±0.10^{b}	13.9±3.76^{ab}	15.7±1.90^{a}	14.2±0.90^{a}
ALT	10.0±0.65^{a}	7.7±0.30^{b}	7.7±0.70^{b}	7.7±0.80^{b}	7.6±0.70^{b}
Triglyceride (mg/dl)	3.5±0.20	3.8±0.60	3.6±0.50	4.0±0.60	3.6±0.10
Cholesterol (mg/dl)	67.3±9.10^{a}	90.9±5.60^{cd}	79.4±4.20^{b}	100.4±5.20^{d}	89.1±2.60^{bc}
MPO	2.92±0.02	2.89±0.08	2.91±0.02	2.91±0.11	3.00±0.06
After 24 h feeding experimental diets					
Hematocrits (%)	40.3±2.20^{a}	41.4±2.00^{ab}	43.2±0.90^{b}	44.2±0.90^{b}	44.1±0.30^{b}
Hemoglobin (g/dl)	7.6±0.50	7.8±0.60	8.4±0.30	7.9±0.20	7.9±0.20
AST	20.0±4.74	18.5±3.10	20.6±1.90	20.9±3.10	18.5±3.00
ALT	10.0±4.00	11.2±4.70	12.4±3.30	12.0±4.00	13.0±9.50
Triglyceride (mg/dl)	3.5±0.80	3.4±0.70	3.6±0.40	3.5±0.60	4.0±0.30
Cholesterol (mg/dl)	82.1±10.7	67.0±14.9	66.5±5.30	69.8±13.3	72.8±8.20
MPO	2.56±0.09	2.47±0.11	2.52±0.11	2.70±0.18	2.31±0.36

Mean values of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P<0.05$).

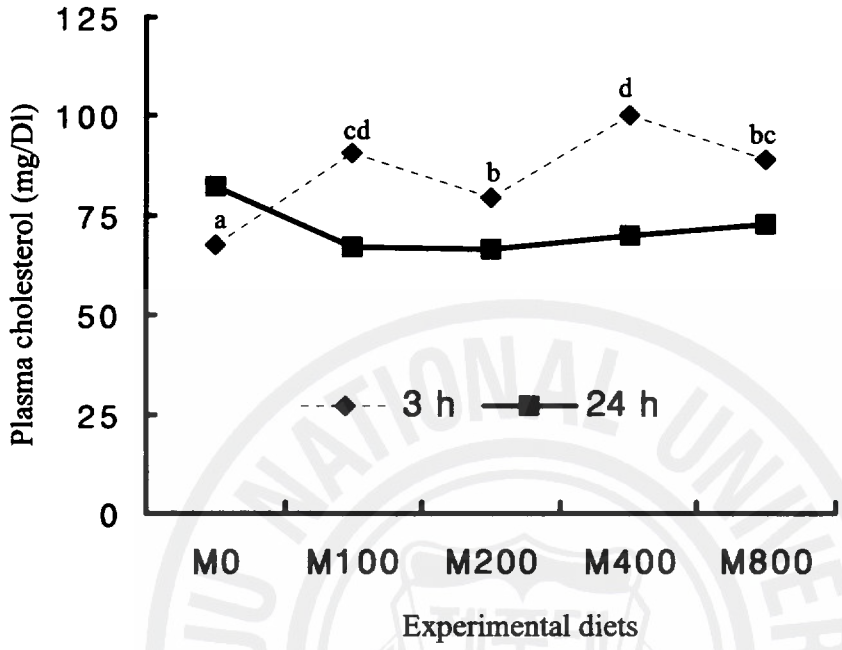

Figure 2.7. Plasma cholesterol of juvenile parrot fish fed the experimental diets for 18 weeks, after 3 and 24 hours feeding. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

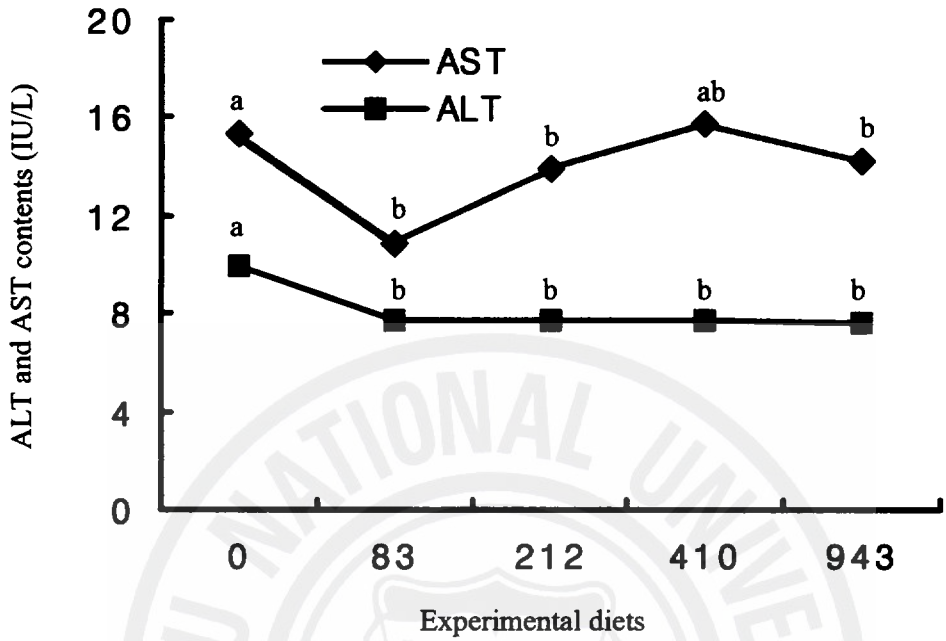

Figure 2.8. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity (IU/L) of juvenile parrot fish fed the experimental diets for 18 weeks, after 3 hours feeding. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

Table 2.3. Whole body composition of juvenile parrot fish fed five experimental diets with different levels of *myo*-inositol for 18 weeks

Diets	M0(0)	M100(83)	M200(212)	M400(410)	M800(943)
Moisture, %	69.2±1.4	68.7±0.4	69.2±0.5	68.7±0.5	68.0±1.6
Protein, %	62.4±0.5[a]	56.9±0.8[b]	60.1±0.7[b]	59.7±3.2[b]	58.2±2.7[b]
Lipid, %	18.4±2.7	18.6±1.6	19.6±3.4	19.5±2.9	17.9±2.5
Ash, %	5.8±0.6	6.2±0.2	5.5±0.3	5.9±0.5	6.3±1.0

Mean values of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

Table 2.4. Inositol and lipid concentration in the liver of juvenile parrot fish fed five experimental diets with different levels of *myo*-inositol for 18 weeks

Diet	Inositol (ug/g wet tissue)	Lipid (%, wet tissue)
M0(0)	225 ± 31.6^{a}	15.9 ± 1.3^{a}
M100(83)	399 ± 61.1^{b}	13.7 ± 2.3^{ab}
M200(212)	467 ± 22.0^{bc}	12.9 ± 2.0^{ab}
M400(410)	463 ± 18.1^{bc}	12.3 ± 1.2^{bc}
M800(943)	507 ± 47.8^{c}	9.8 ± 0.1^{c}

Mean values of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

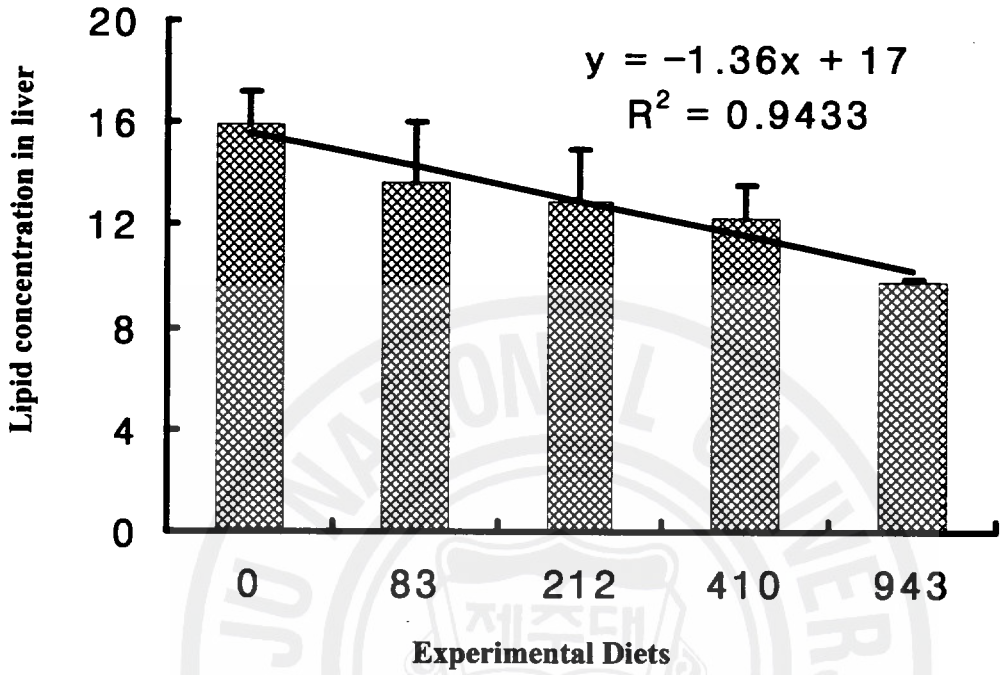

Figure 2.9. Whole lipid content (%) of juvenile parrot fish fed the experimental diets for 18 weeks, after 3 hours feeding. Values are mean of three replicates per treatment. Bars with different letters are significantly different ($P<0.05$).

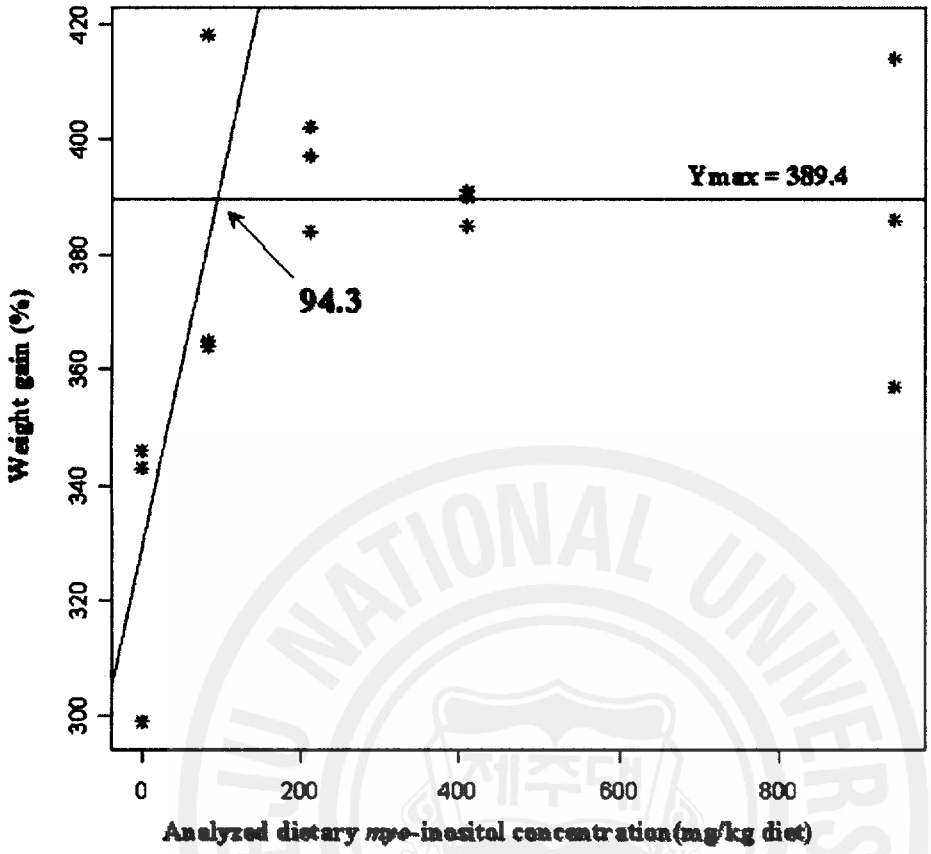

Figure 2.10. Broken-line regression analysis of weight gain (%) to the analyzed dietary myo-inositol levels. Dietary requirement of myo-inositol for weight gain are 94.3 mg/kg diet.

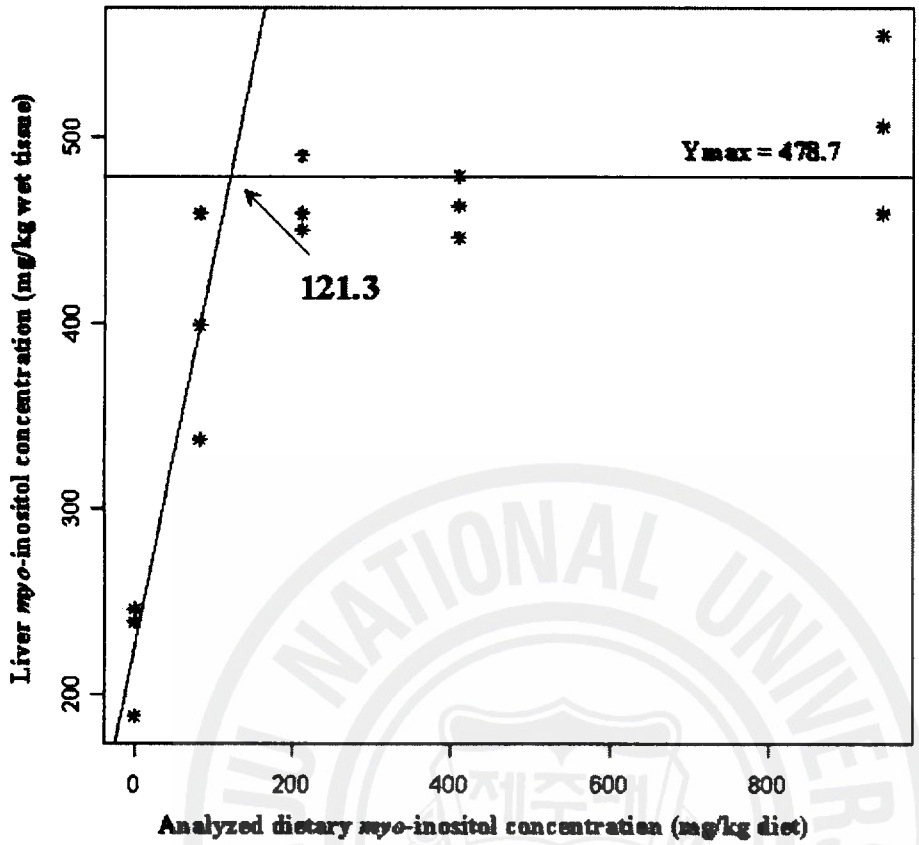

Figure 2.11. Broken-line regression analysis of liver myo-inositol concentration to the analyzed dietary myo-inositol levels. Dietary requirements of myo-inositol for liver myo-inositol concentration are 121.3 mg/kg diet.

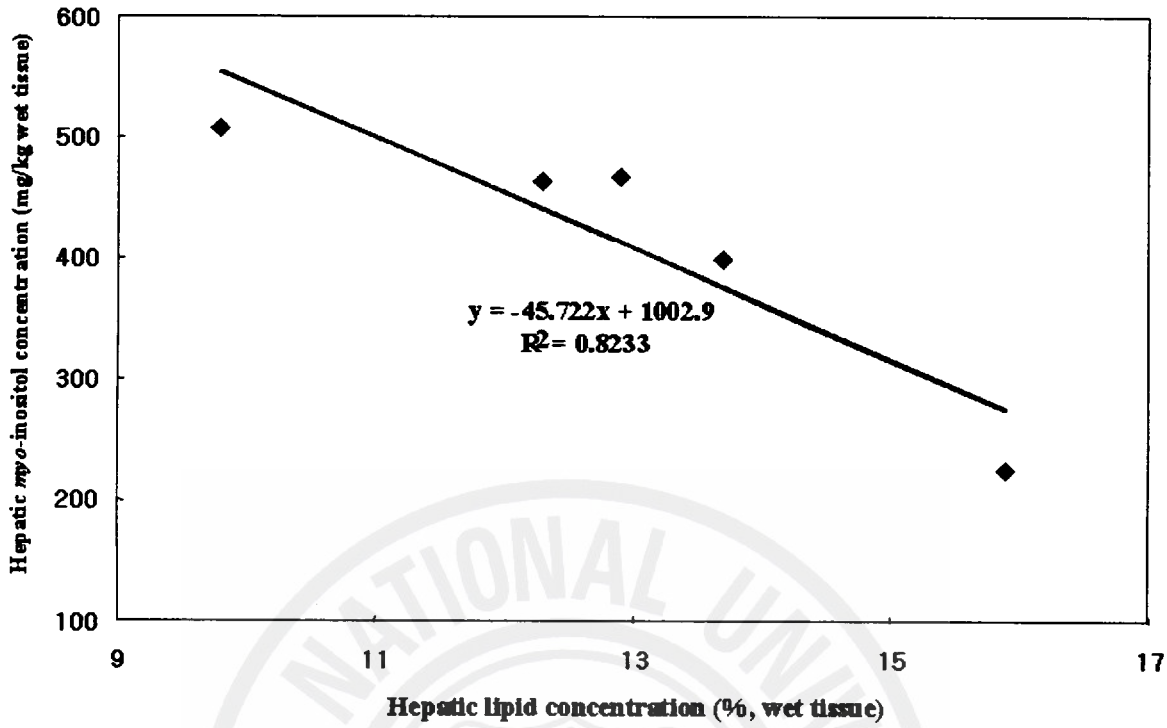

Figure 2.12. Correlation between *myo*-inositol and lipid concentration in the liver of juvenile parrot fish fed five experimental diets with different levels of *myo*-inositol for 18 weeks. Each dot represents the average of three groups of fish.

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LIST OF PUBLICATIONS

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