Thesis for Degree of Master of fisheries science

# Synergistic effects of dietary vitamin E and arachidonic acid with and without vitamin C supplementation on non-specific immune responses in female broodstock Japanese eel, *Anguilla japonica*

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친어용 암컷 뱀장어에서 사료 내 비타민 C 의 첨가유무에 따른 비타민 E 및 아라키돈산 복합첨가가 비특이적 면역반응에 미치는 시너지 효과

Advisors : Sungchul C. Bai

by

Doyoung Kim

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Synergistic effects of dietary vitamin E and arachidonic acid with and without vitamin C supplementation on non-specific immune responses in female broodstock Japanese eel, *Anguilla japonica*

A dissertation

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# 요 약 문

본 논문은 친어용 암컷 뱀장어사료 내 비타민 C의 첨가유무에 따른 비타민 E 및 아라키돈산의 복합첨가가 비특이적 면역반응에 미치는 시너지 효과를 평가하기 위하여 실시되었다. 실험사료는 상업용 사료에 비타민 C, E 및 아라키돈산을 각각 2가지 수준으로 비타민 C(0과 800mg ascorbic acid/kg 사료, $C_0$ 와 $C_{800}$), 비타민 E(0과 200mg α-tocopherol/kg 사료, $E_0$ 와 $E_{200}$), 아라키돈산(0과 5g Arachidonic acid/kg 사료, $ARA_0$ 와 $ARA_5$)을 첨가하여 8가지 사료를 제작 및 표기 하였다. 실험1은, 비타민C를 추가 첨가한 $C_{800}E_0ARA_0$, $C_{800}E_0ARA_5$, $C_{800}E_{200}ARA_0$ 과 $C_{800}E_{200}ARA_5$를 4가지 사료를 2x2 factorial design으로 설계되었으며, 평균무게 277±7.0g(mean±SD) 인 뱀장어 암컷 친어를 사용하여 12주간 사육이 실시되었다. 실험2는, 비타민C를 추가 첨가하지 않은 $C_0E_0ARA_0$, $C_0E_0ARA_5$, $C_0E_{200}ARA_0$ 과 $C_0E_{200}ARA_5$를 4가지 사료를 2x2 factorial design으로 설계되었으며, 평균무게 279±9.0g(mean±SD) 인 뱀장어 암컷 친어를 사용하여 12주간 사육이 실시되었다. 각각 12주간의 두 개의 사육실험 종료 후, 종합적인 실험결과는 뱀장어 암컷 친어에서 사료 내 비타민 C, E 및 아라키돈산의 복합첨가가 각각 첨가 또는 두 가지씩의 혼합첨가 보다 성장 및 면역증진에 시너지 효과를 보이는 것으로 확인되었다. 실험별 요약은 다음과 같다.

## 실험 1 : 친어용 암컷 뱀장어에서 비타민 C 를 추가 첨가한 사료 내 비타민 E 와 아라키돈산 복합첨가에 따른 시너지효과

실험 1은 친어용 암컷 뱀장어에서 비타민C를 추가 첨가한 사료 내 비타민 E와 아라키돈산 복합첨가에 따른 시너지효과가 성장, 비특이적 면역과 혈액학적 반응, 그리고 조직 내 첨가된 미량영양소의 축적량 등에 미치는 영향을 평가하기 위하여 실시되었다. 4가지 실험사료는 비타민C가 추가첨가(800mg ascorbic acid/kg 사료) 된 상업용 사료에 비타민 E 및 아라키돈산을 각각 2가지 수준으로 비타민 E(0과 200mg α-tocopherol/kg 사료, $E_0$ 와 $E_{200}$), 아라키돈산(0과 5g Arachidonic acid/kg 사료, $ARA_0$ 와 $ARA_5$)을 첨가하여 $C_{800}E_0ARA_0$, $C_{800}E_0ARA_5$, $C_{800}E_{200}ARA_0$, $C_{800}E_{200}ARA_5$의 4가지 사료를 제작 및 표기하고 2x2 factorial design으로 실험설계를 하였다. 실험어는 평균무게 277±7.0g(mean±SD) 인 뱀장어 암컷 친어를 사용하였으며, 12주간 사육이 실시되었다. 12주간의 사육실험 종료 후, 증체율(WG), 일간성장률(SGR)에 있어서, $C_{800}E_{200}ARA_5$실험구가 나머지 실험구보다 유의적으로 높은 것으로 나타났으며, 사료효율(FE)에 있어서는, $C_{800}E_{200}ARA_5$실험구가 $C_{800}E_0ARA_0$와 $C_{800}E_0ARA_5$실험구보다 유의하게 높게 나타났다. Superoxide dismutase (SOD)활성에 있어서도, $C_{800}E_{200}ARA_5$실험구가 나머지 실험구에 비해 유의하게 높게 나타났으며, 비타민E와 아라키돈산 사이에 유의한 상호작용이 있는 것으로 나타났다. 또한, 간에서의 비타민 C 축적량은 실험구간 유의한 차이는 없

었으나, $C_{800}E_{200}ARA_5$실험구가 다른 실험구에 비해 가장 높은 값을 나타내었다. 따라서, 뱀장어 암컷 친어에서 사료 내 비타민 C, E 및 아라키돈산의 복합첨가가 각각 첨가 또는 두 가지씩의 혼합첨가 보다 성장 및 면역증진에 시너지 효과를 보이는 것으로 판단된다.

## 실험 2 : 친어용 암컷 뱀장어에서 비타민 C 를 추가 첨가하지 않은 사료 내 비타민 E 와 아라키돈산 복합첨가에 따른 시너지효과

본 실험은 친어용 암컷 뱀장어에서 비타민C를 추가 첨가하지 않은 사료 내 비타민 E와 아라키돈산 복합첨가에 따른 시너지효과가 성장, 비특이적 면역과 혈액학적 반응, 그리고 조직 내 첨가된 미량영양소의 축적량 등에 미치는 영향을 평가하기 위하여 실시되었다. 4가지 실험사료는 상업용 사료에 비타민 E 및 아라키돈산을 각각 2가지 수준으로 비타민 E(0과 200mg α -tocopherol/kg 사료, $E_0$ 와 $E_{200}$), 아라키돈산(0과 5g arachidonic acid/kg 사료, $ARA_0$ 와 $ARA_5$)을 첨가하여 $C_0E_0ARA_0$, $C_0E_0ARA_5$, $C_0E_{200}ARA_0$, $C_0E_{200}ARA_5$의 4가지 사료를 제작 및 표기하였으며 2x2 factorial design으로 실험을 설계하였다. 실험어는 평균무게 279±9.0g(mean±SD) 인 뱀장어 암컷 친어를 사용하였으며, 12주간 사육이 실시되었다. 사육실험 종료 후, 성장에 있어서는 실험구간 유의한 차이가 나타나지 않았다. Superoxide dismutase (SOD) 활성에 있어서,

$C_0E_{200}ARA_5$실험구가 $C_0E_0ARA_0$와 $C_0E_{200}ARA_0$실험구보다 유의적으로 높게 나타났지만, $C_0E_0ARA_5$실험구와는 유의한 차이가 나타나지 않았으며, 비타민E와 아라키돈산 사이에 유의한 상호작용이 있는 것으로 나타났다. 또한, 간에서의 비타민 C 축적량은 실험구간 유의한 차이는 없었으나, 비타민E와 아라키돈산을 첨가함에 따라 증가하는 경향을 보였다. 따라서, 뱀장어 암컷 친어에서 사료 내 비타민 E 및 아라키돈산의 복합첨가가 면역증진에 시너지 효과를 보이는 것으로 판단된다.

# Synergistic effects of dietary vitamin E and arachidonic acid with and without vitamin C supplementation on non-specific immune responses in female broodstock Japanese eel, *Anguilla japonica*

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## Abstract

Two feeding trials were conducted to evaluate the synergistic effects of dietary vitamin E and arachidonic acid with and without vitamin C supplementation on non-specific immune responses in female broodstock Japanese eel, *Anguilla japonica*. Eight experimental diets were formulated to contain two different vitamin C levels as L-Ascorbic acid (0 and 800mg AA/kg diet, $C_0$ and $C_{800}$), two different vitamin E levels as α-tocopherol (0 and 200mg α-Toc/kg diet, $E_0$ and $E_{200}$) and two different arachidonic acid levels (0, 5g ARA/kg diet, $ARA_0$ and $ARA_5$). In experiment 1, four experimental diets, in a 2x2 factorial design ($C_{800}E_0ARA_0$, $C_{800}E_0ARA_5$, $C_{800}E_{200}ARA_0$ and $C_{800}E_{200}ARA_5$) were formulated. And experimental diets were fed to triplicate groups of fish averaging 277±7.0g(mean±SD) for 12 weeks. In experiment 2, four experimental diets, in a 2x2 factorial design ($C_0E_0ARA_0$, $C_0E_0ARA_5$,

$C_0E_{200}ARA_0$ and $C_0E_{200}ARA_5$) were formulated. And experimental diets were fed to triplicate groups of fish averaging 279±9.0g(mean±SD) for 12 weeks. After 12 weeks of the feeding trial, combined supplementation of vitamin C, E and/or arachidonic acid could improve growth performance, nonspecific immune responses and liver vitamin concentrations in female broodstock Japanese eel.

## Experiment 1 : Synergistic effects of dietary vitamin E and arachidonic acid with vitamin C supplementation in Japanese eel, *Anguilla japonica*

A 12-week of feeding trial was conducted to evaluate the synergistic effects of dietary vitamin E and arachidonic acid (ARA) with vitamin C (Ascorbic Acid, AA) supplementation in Japanese eel, *Anguilla japonica*. Four experimental diets were formulated to contain two different levels of vitamin E (0 and 200mg $\alpha$-Toc/kg diet) and ARA (0, 5g ARA/kg diet) with AA supplementation (800mg ascorbic acid/kg diet). Four experimental diets, in a 2x2 factorial design ($C_{800}E_0ARA_0$, $C_{800}E_0ARA_5$, $C_{800}E_{200}ARA_0$ and $C_{800}E_{200}ARA_5$) were fed to triplicate groups of fish averaging 277±7.0g(mean±SD) for 12 weeks. At the end of the feeding trial, weight

gain and specific growth rate of fish fed $C_{800}E_{200}ARA_5$ diet were significantly higher than those of fish fed the other diets. Feed efficiency values of fish fed $C_{800}E_{200}ARA_5$ diet were significantly higher than those of fish fed $C_{800}E_0ARA_0$ and $C_{800}E_0ARA_5$ diets. Superoxide dismutase activity (SOD) values of fish fed $C_{800}E_{200}ARA_5$ diet were significantly higher than those of fish fed the other diets. And significant interactions between vitamin E and ARA also were observed in SOD values. There were no significant differences in liver AA concentrations among fish fed all the diets. However, liver AA concentrations of fish fed $C_{800}E_{200}ARA_5$ diet were higher than those of fish fed the other diets. Therefore, combined supplementation of vitamin E and arachidonic acid with vitamin C supplementation could improve growth performances and nonspecific immune responses in female broodstock Japanese eel.

## Experiment 2 : Synergistic effects of dietary vitamin E and arachidonic acid without vitamin C supplementation in Japanese eel, *Anguilla japonica*

A 12-week of feeding trial was conducted to evaluate the synergistic effects of dietary vitamin E and arachidonic acid (ARA) without vitamin C (Ascorbic Acid, AA) supplementation in Japanese eel, *Anguilla japonica*. Four experimental diets were formulated to contain two different levels of

vitamin E (0 and 200mg α-Toc/kg diet) and ARA (0, 5g ARA/kg diet) without AA supplementation. Four experimental diets, in a 2x2 factorial design ($C_0E_0ARA_0$, $C_0E_0ARA_5$, $C_0E_{200}ARA_0$ and $C_0E_{200}ARA_5$) were fed to triplicate groups of fish averaging 279±9.0g(mean±SD) for 12 weeks. At the end of the feeding trial, Superoxide dismutase activity (SOD) values of fish fed $C_0E_{200}ARA_5$ diet were significantly higher than those of fish fed $C_0E_0ARA_0$ and $C_0E_{200}ARA_0$ diets. But, there were no significant differences among SOD values of fish fed $C_0E_{200}ARA_5$ and $C_0E_0ARA_5$ diets. And significant interactions between vitamin E and ARA also were observed in SOD values. There were no significant differences in liver AA concentrations among fish fed all the diets. However, AA concentrations in liver of fish fed $C_0E_0ARA_0$ diet were lower than those of fish fed the other diets. As vitamin E and ARA were supplemented, liver vitamin C concentrations of fish increased. Therefore, combined supplementation of vitamin E and arachidonic acid without vitamin C supplementation could improve nonspecific immune responses in female broodstock Japanese eel.

# Ⅰ. Introduction

The Japanese eel, *Anguilla japonica*, is a very popular freshwater fish, being one of the important foods in East asia. Specially, Domestic production of the Japanese eel was 5,716 tonnes, ranked it first among Korean freshwater fish species in 2014 (KOSTAT, 2015). Nevertheless, the eel farming industry relies exclusively on wild-caught juveniles (Butts et al. 2014). Broodstock nutrition, particularly in the case of Japanese eel, is one of the most poorly understood and researched areas of finfish nutrition (Izquierdo et al. 2001). To a large extent, this has been due to the necessity of suitable indoor or outdoor culture facilities for maintaining large groups of broodstock fish and the consequent higher cost of running and conducting extended broodstock feeding trials. However, as in human and livestock nutrition, it is clear that the dietary nutrient requirements of broodstock will be different from those of rapidly growing juvenile animals (Leboulanger 1977). Moreover, it is also clear that many of the deficiencies and problems encountered during the early rearing phases of newly hatched finfish larvae are directly related to the feeding regime (including nutrient level and duration) of the broodstock.

Vitamins are organic compounds that can be synthesized from other essential nutrients to spare a portion of the dietary requirements; however, they are required in trace amounts from an exogenous source for normal growth,

reproduction and health (Erfan et al. 2015). Vitamin C (L-Ascorbic acid, AA) is an essential vitamin for normal growth and physiological function of fish (Zhou et al. 2002) and a strong antioxidant that is capable of scavenging reactive oxygen species (Bae et al. 2012). It has also been proposed to be an important nutrient correlating with fish immunuity (Ren et al. 2007; Ai et al. 2004; Roberts et al. 1995; Lin and Shiau 2005). It plays an important role in the growth, immunity, collagen formation, internal haemorrhaging, mortality and reproduction (Ai et al. 2006; Al-Amoudi et al. 1992; Dabrowski 1992; Gouillou-Coustans et al. 1998; Soliman et al. 1986; Zehra and Khan 2012). One frequently overlooked problem is that AA uptake is highly dose dependent. Inadequate supply of dietary AA usually results in a number of deficiency symptoms such as abnormal swimming, ascites, dark skin coloration, deformities, hemorrhage and reduced bone collagen (NRC 2011). This and several other issues should be taken into account while drawing conclusions from randomized controlled trials with the purpose of studying the effects of AA.

Vitamin E (α-tocopherol, α-Toc) is also nutrient related with immune system function which acts as an antioxidant in biological membranes (Montero et al. 1999) and stop the production of reactive oxygen species (Bae et al. 2013). It has also been proposed to be potentially beneficial in reducing oxidative damage to tissues, increase immune response and enhance the resistance to stress (Adham et al. 2000, Puangkaew et al. 2004 and Kiron et al. 2004). One frequently

overlooked problem is that α-Toc uptake is highly dose dependent. Inadequate supply of dietary α-Toc usually results in a number of deficiency symptoms such as muscular dystrophy, fatty liver degeneration, depigmentation, anemia, exudative diathesis, erythrocyte hemolysis and hemorrhages (NRC 2011). However, subjects saturated with α-Toc through their daily diet will increase antibody titers and improve the quality of fish fillets (Gatlin et al. 1992; Chaiyeapechara et al. 2003; Ruff et al. 2003).

Lipids play a significant role in fish nutrition as sources of metabolic energy, components of membrane phospholipids and as precursors of bioactive molecules (Tocher 2003). Highly unsaturated fatty acids (HUFAs) have unique roles in controlling and regulating growth performance, lipid metabolism, cell membrane fluidity and immune function in fish (Higgs and Dong 2000; Kiron et al. 2011). Among n-6 HUFAs, arachidonic acid (ARA, 20:4n-6) is the main fatty acid precursor of eicosanoids in fish (Henderson and Sargent 1985; Henderson et al., 1985; Bell et al., 1994). Several studies have reported that appropriate levels of dietary ARA were needed to improve growth performance, survival, stress resistance (Carrier et al. 2011; Rezek et al. 2010), tissue fatty acid composition (Bessonart et al. 1999; Castell et al. 1994; Estévez et al. 1997) and physiological characteristics (Van Anholt et al. 2004). Furthermore, ARA is involved in the modulation of the immune system via modulation of eicosanoids synthesis and release of cytokines (Furne et al. 2013; Pirante et al. 2002).

To our knowledge, there is no available study on regarding to combinated supplementation of vitamin C, E and/or arachidonic acid in female broodstock Japanese eel. Therefore, the present study was undertaken to evaluate the synergistic effects of dietary vitamin C, E and/or arachidonic acid in female broodstock Japanese eel, *Anguilla japonica*.

## Ⅱ. Synergistic effects of dietary vitamin E and arachidonic acid with vitamin C supplementation in Japanese eel, *Anguilla japonica*


### Abstract

A 12-week of feeding trial was conducted to evaluate the synergistic effects of dietary vitamin E and arachidonic acid (ARA) with vitamin C (Ascorbic Acid, AA) supplementation in Japanese eel, *Anguilla japonica*. Four experimental diets were formulated to contain two different levels of vitamin E (0 and 200mg $\alpha$-Toc/kg diet) and ARA (0, 5g ARA/kg diet) with vitamin C supplementation (800mg ascorbic acid/kg diet). Four experimental diets, in a 2x2 factorial design ($C_{800}E_0ARA_0$, $C_{800}E_0ARA_5$, $C_{800}E_{200}ARA_0$ and $C_{800}E_{200}ARA_5$) were fed to triplicate groups of fish averaging 277±7.0g(mean±SD) for 12 weeks. At the end of the feeding trial, weight gain and specific growth rate of fish fed $C_{800}E_{200}ARA_5$ diet were significantly higher than those of fish fed the other diets. Feed efficiency values of fish fed $C_{800}E_{200}ARA_5$ diet were significantly higher than those of fish fed $C_{800}E_0ARA_0$ and $C_{800}E_0ARA_5$ diets. Superoxide dismutase activity (SOD) values of fish fed $C_{800}E_{200}ARA_5$ diet were significantly higher than those of fish fed the other diets. And significant interactions between vitamin E

and ARA also were observed in SOD values. There were no significant differences in liver AA concentrations among fish fed all the diets. However, liver AA concentrations of fish fed $C_{800}E_{200}ARA_5$ diet were higher than those of fish fed the other diets. Therefore, combined supplementation of vitamin E and arachidonic acid with vitamin C supplementation could improve growth performances and nonspecific immune responses in female broodstock Japanese eel.

## Materials and Methods

1. Experimental diets

A basal commercial diet (Table 1) with Ascorbic Acid (AA) supplementation (800 mg AA $kg^{-1}$) was used as a control diet. Three other diets were prepared by supplementing two different vitamin E (0 and 200 mg α-Toc $kg^{-1}$) and arachidonic acid (0 and 5% ARA) levels to the basal diet. Four experimental diets, in a 2x2 factorial design ($C_{800}E_0ARA_0$, $C_{800}E_0ARA_5$, $C_{800}E_{200}ARA_0$, $C_{800}E_{200}ARA_5$) were formulated. In this study L-ascorbyl-2-monophosphate (AMP) (Sigma-Aldrich, Germany) was used as AA sources due to its high heat resistance in comparison to unprotected AA and its excellent availability to fish (Dabrowski et al. 1994). DL-α-tochpheryl acetate (TA) (Sigma-Aldrich, Switzerland) was used as α-Toc sources. ARA was supplied by Australia company, Bulk Nutrients. The actual AMP and TA levels of the experimental diets were determined by High-Pressure Liquid Chromatography (HPLC; Dionex, Softron, USA) and the levels of dietary ARA analyzed by Gas Chromatography. The analyzed AA, TA and ARA concentrations of the diets were $C_{800}E_0ARA_0$ = 782 and 66.3 mg $kg^{-1}$ and 0.00%, $C_{800}E_0ARA_5$ = 796 and 69.3 mg $kg^{-1}$ and 0.53%, $C_{800}E_{200}ARA_0$ = 788 and 181 mg $kg^{-1}$ and 0.00% and $C_{800}E_{200}ARA_5$ = 783 and 184 mg $kg^{-1}$ and 0.47% diet, respectively. The targeting levels of AMP, TA and ARA were mixed with the powdered commercial diet at the expense of cellulose, and then stored at −20 °C

until diet preparation. Tap water was added to the feeds (1.3% of diet weight) and a ball-shaped dough was made before feeding.

2. Experimental fish and feeding trial

Two-years-old female broodstock of Japanese eel were obtained from an Eel Research Center (Yeonggwang, South Korea). Prior to the start of the feeding trial, the health status of the fish was checked and they were starved for 24 h. All the fish were fed the basal commercial diet for four weeks to become acclimatized to the experimental conditions and facilities. 240 fish averaging at 277 ± 7g (mean ± SD) were weighed and randomly distributed into 24 indoor fiberglass tanks (10 fish/tank) with 150-L volume receiving a constant flow (4 L $min^{-1}$) of filtered freshwater. Each tank was then randomly assigned to one of three replicates of 4 dietary treatments. During the experiment, supplemental aeration was provided in each tank to maintain enough dissolved oxygen, and also water heated by electric heaters in a concrete reservoir. Water temperature and pH during the experiment were maintained at 28.3 ± 0.05 ℃ and 7.68 ± 0.04, respectively. Fish were fed twice daily (06:00 and 18:00 h) for 12 weeks at a fixed rate of 1% body weight per day. Dead fish were removed immediately and weighed, and the amounts of feed for the tanks adjusted to the proper percentage of the remaining fish weight in the tanks. Uneaten feed was siphoned out after 1 hour of feeding and tank inside was scrubbed once per week to minimize algal

and fungal growth.

3. Sample collection and analysis

At the end of the feeding trial, fish were starved for 24 h, and the total number and weight of fish in each tank were determined for calculation of final weight, specific growth rate, feed efficiency and survival. Three fish per tank were randomly selected, individually weighed, then dissected to obtain liver and ovary for determination of hepatosomatic index and gonadosomatic index. Thereafter, the same liver and ovary and dissected muscle were used for determining AA and TA concentrations and fatty acid composition. Three additional fish per tank were randomly captured, anesthetized with ethylene glycol phenyl ether (200 mg $l^{-1}$ for 5–10 min), and blood samples were collected from the caudal vein with heparinized syringes. Plasma was separated by centrifugation at 5000 × *g* for 10 min and stored at −70°C for determination of blood biochemical parameters including plasma alanine aminotransferase, aspartate aminotransferase and glucose. Another set of blood samples of the same fish were taken without heparin and allowed to clot at room temperature for 30 min. Then, the serum was separated by centrifugation at 5000 × *g* for 10 min and stored at −70°C for the analysis of non-specific immune responses including lysozyme, myeloperoxidase and superoxide dismutase activities.

Analysis of moisture, crude protein, lipid, ash and fiber in the feed were

performed using standard methods (AOAC 1995), and carbohydrate content was measured by Bomb Calorimeter (PARR 1351, Co., Illinois, USA). The energy value was determined on the basis of physiological fuel value, i.e., 3.99 kcal $g^{-1}$ proteins or carbohydrates and 9.01 kcal $g^{-1}$ lipids (Lee and Putnam, 1973). Samples of diets, fish and liver were dried to constant weights at 105°C to determine their moisture contents. Ash was determined by incineration at 550°C, crude lipid was determined by soxhlet extraction using the Soxtec system 1046 (Tecator AB, Hoganas, Sweden), and crude protein content was determined by the Kjeldahl method (N×6.25) after acid digestion.

Tissue Ascorbic Acid concentrations were determined by High-Performance Liquid Chromatography (HPLC; Dionex, Softron, USA) with an ultraviolet detector at 254 nm. The mobile phase was 0.05 M $KH_2PO_4$ at pH 2.8, and the flow rate was 1.0 mL $min^{-1}$. Weighed samples were homogenized in 10% cold metaphosphoric acid. Homogenates were centrifuged at 3000 × *g* for 20 min, and supernatants were analyzed on HPLC after being filtered through a 0.45 μm pore size syringe filter.

Tissue α-Toc concentrations were determined by High-Performance Liquid Chromatography (HPLC; Dionex, Softron, USA) with an ultraviolet detector at 290 nm. The mobile phase was Hexane: Isopropanol (98:2, v/v) and the flow rate was 1.0 ml $min^{-1}$. Weighed samples were homogenized in 5 (ml) ethanol. Homogenates were centrifuged at 3000 ×g for 5 min, and supernatants were

analyzed on HPLC after being filtered through a 0.45 μm pore size syringe filter.

The composition of fatty acid methyl esters was determined by Gas Chromatography (Trace GC, TheromoFinnigan, USA) with flame ionization detector, equipped with a Carbowax 007 capillary column (30 m×0.25 mm i.d., film thickness 0.25μm, QUADREX, USA). Injector and detector temperatures were 250°C. The column temperature was programmed from 100°C to 220°C at a rate of 5°C $min^{-1}$ and 220°C to 240°C at a rate of 3°C $min^{-1}$. Helium was used as the carrier gas. Fatty acids were identified by comparison with known standards.

The plasma levels of glucose and activities of ALT and AST were measured by a chemical analyzer (Fuji DRI-CHEM 3500i, Fuji Photo Film, Ltd., Tokyo, Japan).

A turbidometric assay was used for determination of serum lysozyme level by the method described by Hultmark (1980) with slight modifications. Briefly, *Micrococcus lysodeikticus* (0.75 mg $ml^{-1}$) was suspended in sodium phosphate buffer (0.1 M, pH 6.4), 200 μl of suspension was placed in each well of 96-well plates, and 20 μl serum was added subsequently. The reduction in absorbance of the samples was recorded at 570 nm after incubation at room temperature for 0 and 30 min in a microplate reader (UVM 340, Biochrom, Cambridge, UK). A reduction in absorbance of 0.001 $min^{-1}$ was regarded as one unit of lysozyme activity.

MPO activity was measured according to Quade and Roth (1997). Briefly, 20 μl of serum was diluted with HBSS (Hanks Balanced Salt Solution) without $Ca^{2+}$ or $Mg^{2+}$ (Sigma-Aldrich, USA) in 96-well plates. Then, 35 μl of 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB, 20 mM) (Sigma-Aldrich, USA) and $H_2O_2$ (5 mM) were added. The color change reaction was stopped after 2 min by adding 35 μl of 4 M sulfuric acid. Finally, the optical density was read at 450 nm in a microplate reader.

SOD activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma-Aldrich, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 °C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

4. Statistical analysis

All data were analyzed by two-way ANOVA to test for the effects of the dietary t reatments. When significant differences were found, a least significant difference (LSD) test used to identify differences among experimental groups. Treatment eff ects were considered with the significance level at $P < 0.05$. All statistical analyse s were carried out by SAS version 9.1 (SAS Institute, Cary, NC, USA).

## Results

At the end of the feeding trial, weight gain and specific growth rate of fish fed $C_{800}E_{200}ARA_5$ diet were significantly higher than those of fish fed the other diets. Feed efficiency (FE) values of fish fed $C_{800}E_{200}ARA_5$ diet were significantly higher than those of fish fed $C_{800}E_0ARA_0$ and $C_{800}E_0ARA_5$ diets. However, there were no significant differences among the FE of fish fed $C_{800}E_{200}ARA_0$ and $C_{800}E_{200}ARA_5$ diets. Fish survival rate varied from 96.7 to 100% and no significant differences were observed among dietary treatments. There were significant differences in hepatosomatic index and gonadosomatic index among fish fed all the diets.

There were no significant differences in the proximate compositions of the whole-body of fish between dietary treatments.

The α-Toc concentrations in liver of fish fed 200 mg vitamin E/kg diets ($C_{800}E_{200}ARA_0$ and $C_{800}E_{200}ARA_5$) were significantly higher than those of fish fed without vitamin E/kg diets ($C_{800}E_0ARA_0$ and $C_{800}E_0ARA_5$). So, liver α-Toc concentrations indicated accordingly to the dietary supplementation level of vitamin E. There were no significant differences in liver ascorbic acid (AA) concentrations among fish fed all the diets. However, AA concentrations in liver of fish fed $C_{800}E_0ARA_0$ and $C_{800}E_0ARA_5$ diet showed the lower values than those of fish fed $C_{800}E_{200}ARA_0$ and $C_{800}E_{200}ARA_5$ diets. As vitamin E was supplemented, liver vitamin C concentration was increased. The arachidonic acid

(ARA) concentrations in liver indicated accordingly to the dietary supplementation level of ARA.

Non-specific immune responses are shown in table 7. Superoxide dismutase activity (SOD) values of fish fed $C_{800}E_{200}ARA_5$ diet were significantly higher than those of fish fed the other diets. And significant interactions between vitamin E and ARA also were observed in SOD values. Myeloperoxidase activity (MPO) values of fish fed $C_{800}E_{200}ARA_5$ diet were significantly higher than those of fish fed $C_{800}E_0ARA_0$ diet. But, there were no significant differences among the MPO values of fish fed $C_{800}E_0ARA_5$, $C_{800}E_{200}ARA_5$ and $C_{800}E_{200}ARA_5$ diets. There were no significant differences in lysozyme activity between dietary treatments.

Table 8 shows hematological parameters of female broodstock eel fed different experimental diets. There were no significant differences in aspartate aminotransferase, alanine aminotransferase and glucose level of fish fed all the diets.

## Discussion

In the present experiment, weight gain, specific growth rate and feed efficiency were significantly increased by increment dietary vitamin E and arachidonic acid (ARA) supplementation. Similarly, Several experiments showed that dietary vitamin E improved growth performance in hybrid striped bass (Kocabas & Gatlin 1999) and appropriate dietary ARA level could be improved growth performance in Japanese eel (Bae et al. 2010).

Non-specific immune parameters play a critical role in controlling the balance of release and clear of reactive oxygen species in immune cells (Xu et al. 2010). Superoxide dismutase (SOD) is one of the critical antioxidant enzymes in the body, plays an important role in the self-defense system, and it also possesses a vital function in the immune system (Calvin and Muscatine 1997; Lin et al. 2011). it is considered to be an indicator of the antioxidant status of organisms and a biomarker of oxidative stress (Kohen and Nyska 2002; Shen et al. 2010). Myeloperoxidase (MPO) is also an important enzyme utilizing one of oxidative radicals to produce hypochlorous acid with ability to kill pathogens. During oxidative respiratory burst, the MPO was mostly released by the azurophilic granules of neutrophils (Wang et al. 2008). Lysozyme has anti-inflammatory and antiviral properties, besides its high potential for bactericidal or bacteriolytic

activity against Gram-positive and Gram-negative bacteria (Saurabh & Sahoo, 2008). In this study, SOD and MPO activities significantly increased by increment dietary α-Toc and ARA supplementation. Similarly, Several experiments showed that dietary α-Toc improved the non-specific immune responses in turbot, *Scophthalmus maximus* (Tocher et al. 2002), black sea bream, *Sparus macrocephalus* (Zhang et al. 2007), grouper, *E. malabaricus* (Lin and Shiau 2005) and cobia, *R. canadum* (Zhou et al. 2013).

Liver α-Toc concentrations indicated accordingly to the dietary supplementation level of vitamin E. This result showed an increase in α-Toc concentration by dietary α-Toc increment due to fish cannot synthesize vitamin E and must rely on a dietary supply (Peng and Gatlin 2009). Similarly, vitamin E concentrations in liver showed strong linear relationships to dietary vitamin E concentrations as observed in other studies with channel catfish (Bai & Gatlin 1993) and hybrid striped bass (Sealey & Gatlin 2002). The ARA concentrations in liver indicated accordingly to the dietary supplementation level of arachidonic acid. Similarly, Bae et al. (2010) reported an increase in liver ARA concentrations by dietary ARA concentrations in juvenile eel. The vitamin C concentrations in liver of fish fed $C_{800}E_0ARA_0$ and $C_{800}E_0ARA_5$ diet showed the lower values than those of fish fed $C_{800}E_{200}ARA_0$ and $C_{800}E_{200}ARA_5$ diets. As vitamin E was supplemented, liver vitamin C concentration was increased. Lee et al.(2003) reported the hypothesis that vitamin C spares and/or regenerates vitamin

E in yellow perch. However there were no synergistic effects of both vitamins. Sealey & Gatlin (2002) also reported that no significant dietary vitamin E effect on liver ascorbate was observed in hybrid striped bass. Therefore, combinated supplementation of vitamin E and arachidonic acid with vitamin C supplementation could improve growth performances and nonspecific immune responses in female broodstock Japanese eel.

## Tables and Figures

**Table 1.** Proximate analyses (dry matter basis) of the basal commercial diet.

| Calculated composition | Amount |
|---|---|
| Moisture (%) | 4.25 |
| Crude Protein (%) | 51.8 |
| Crude lipid (%) | 6.37 |
| Crude ash (%) | 11.4 |
| Crude fiber (%) | 2.13 |
| Carbohydrate (%) | 24.1 |
| Gross energy (Kcal $g^{-1}$) | 3.61 |

[1] Values are mean of duplicate samples.

**Table 2.** Actual concentrations of vitamin C, E and arachidonic acid (ARA) for the experimental diets.

| Diets | Vit.C[1] (ppm) | Vit.E[2] (ppm) | ARA[3] (%) |
|---|---|---|---|
| $C_{800}E_0ARA_0$ | 782 | 66.3 | 0.00 |
| $C_{800}E_0ARA_5$ | 796 | 69.3 | 0.53 |
| $C_{800}E_{200}ARA_0$ | 788 | 181 | 0.00 |
| $C_{800}E_{200}ARA_5$ | 783 | 184 | 0.47 |

[1]Vit. C source : L-ascorbyl-2-monophosphate.

[2]Vit. E source : dl- α-tocopheryl acetate.

[3]Supplied by Bulk Nutrients. Australia.

**Table 3.** Growth performance, feed efficiency, organosomatic indices and survival of broodstock Japanese eel fed the experimental diets for 12 weeks[1].

| | Diets | | | | Pooled SEM[9] |
|---|---|---|---|---|---|
| | $C_{800}E_0ARA_0$ | $C_{800}E_0ARA_5$ | $C_{800}E_{200}ARA_0$ | $C_{800}E_{200}ARA_5$ | |
| IBW[2] | 277 | 277 | 280 | 276 | 1.15 |
| FBW[3] | 307 | 311 | 320 | 332 | 3.89 |
| WG[4] | 11.3[b] | 11.0[b] | 14.3[b] | 21.3[a] | 1.55 |
| SGR[5] | 0.13[b] | 0.12[b] | 0.16[b] | 0.23[a] | 0.02 |
| FE[6] | 14.9[b] | 14.6[b] | 20.2[ab] | 29.3[a] | 2.31 |
| GSI[7] | 0.40 | 0.47 | 0.38 | 0.56 | 0.03 |
| HSI[8] | 1.16 | 1.09 | 1.36 | 1.24 | 0.06 |
| Survival (%) | 100 | 96.7 | 100 | 96.7 | 1.12 |

[1]Values are mean ± SD of three replicates. Values with different letters within the same row are significantly different.

[2]Initial body weight (g).

[3]Final body weight (g).

[4]Weight gain(%) = (final wt. – initial wt.) × 100 / initial wt.

[5]Specific growth rate (%) = (ln final weight − ln initial weight) × 100 / d.

[6]Feed efficiency (%) = wet weight gain × 100 / dry feed intake.

[7]Gonadosomatic index (%) = gonad weight × 100/body weight.

[8]Hepatosomatic index (%) = liver weight × 100 / body weight.

[9]Pooled SEM= SD/$\sqrt{n}$.

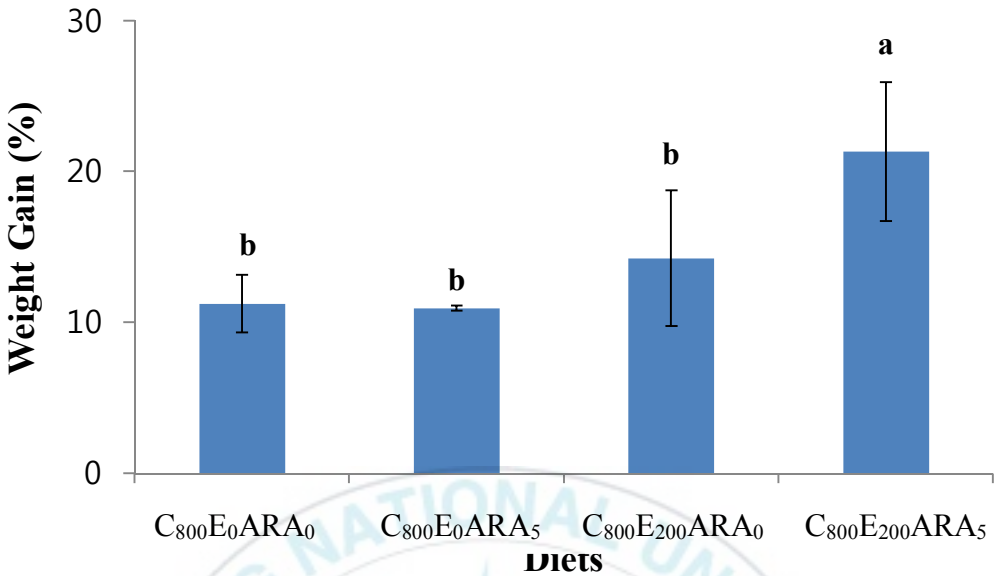


**Fig 1.** Weight gain of broodstock Japanese eel fed the experimental diets for 12 weeks.

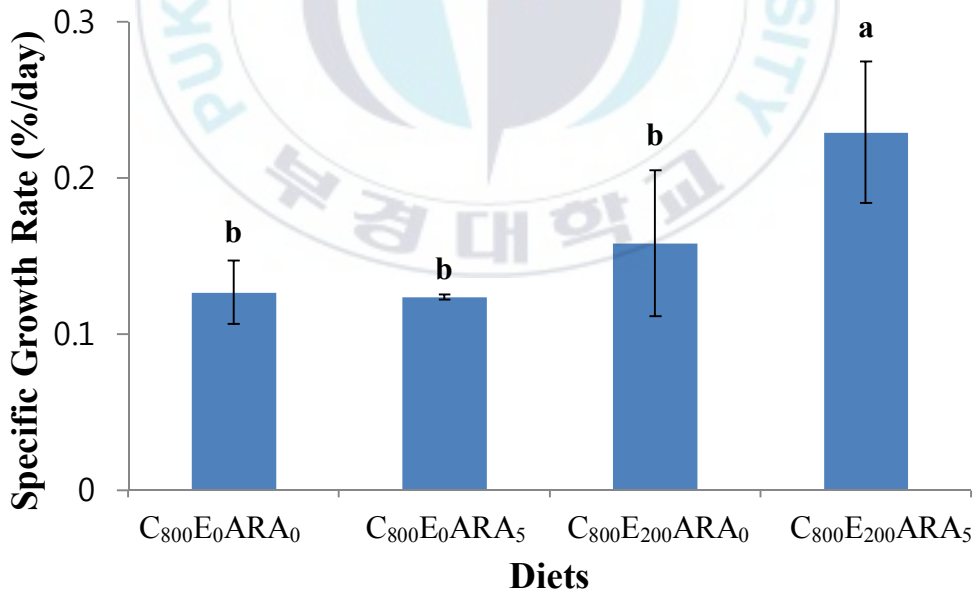


**Fig 2.** Specific growth rate of broodstock Japanese eel fed the experimental diets for 12 weeks.

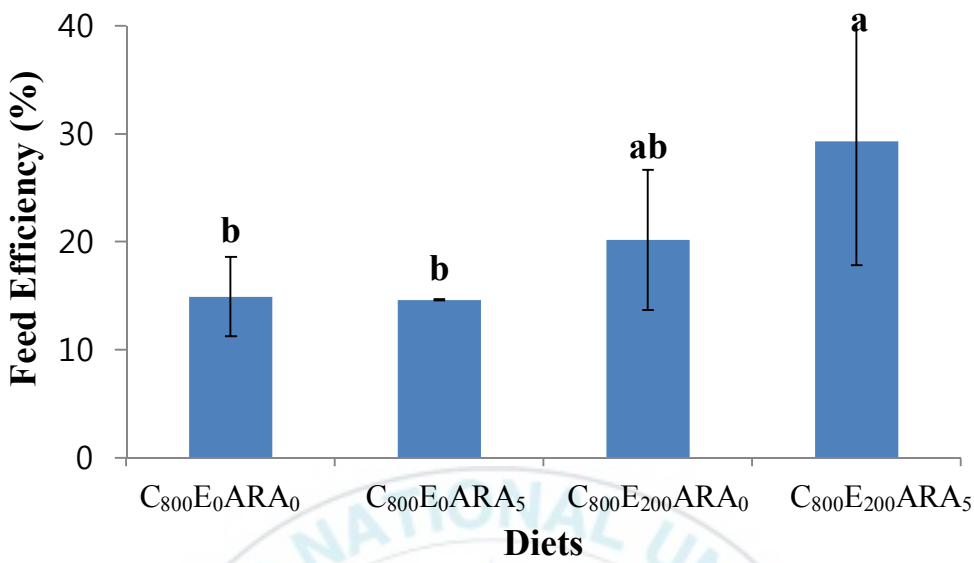


**Fig 3.** Feed efficiency of broodstock Japanese eel fed the experimental diets for 12 weeks.

**Table 4.** Whole-body proximate compositions (%, DM) of broodstock Japanese eel fed the experimental diets for 12 weeks[1].

| | Moisture | Protein | Lipid | Ash |
|---|---|---|---|---|
| $C_{800}E_0ARA_0$ | 62.4 | 46.5 | 47.0 | 5.88 |
| $C_{800}E_0ARA_5$ | 61.4 | 47.0 | 45.2 | 5.45 |
| $C_{800}E_{200}ARA_0$ | 60.7 | 47.6 | 44.9 | 5.83 |
| $C_{800}E_{200}ARA_5$ | 63.0 | 47.6 | 44.8 | 5.34 |
| Pooled SEM[2] | 0.51 | 0.27 | 0.51 | 0.13 |

[1]Values are mean ± SD of three replicates. Values with different letters within the same row are significantly different.

[2]Pooled SEM= SD/$\sqrt{n}$.

**Table 5.** Vitamin C and E concentrations (mg/kg ; DM) in liver of broodstock Japanese eel fed the experimental diets for 12 weeks[1]

| | Vitamin C (AA) | Vitamin E (α-Toc ) |
|---|---|---|
| $C_{800}E_0ARA_0$ | 64.6 | 18.4[b] |
| $C_{800}E_0ARA_5$ | 64.3 | 21.4[b] |
| $C_{800}E_{200}ARA_0$ | 68.7 | 39.2[a] |
| $C_{800}E_{200}ARA_5$ | 74.3 | 39.8[a] |
| Pooled SEM[2] | 2.32 | 3.57 |

[1]Values are mean ± SD of three replicates. Values with different letters within the same row are significantly different.

[2]Pooled SEM= SD/√*n*.

**Table 6.** Liver fatty acid concentrations (%) in liver of broodstock Japanese eel fed the experimental diets for 12 weeks[1].

| | Diets | | | | Pooled SEM[2] |
|---|---|---|---|---|---|
| | $C_{800}E_0ARA_0$ | $C_{800}E_0ARA_5$ | $C_{800}E_{200}ARA_0$ | $C_{800}E_{200}ARA_5$ | |
| 14:0 | 1.32 | 1.02 | 1.03 | 1.14 | 0.07 |
| 16:0 | 19.29 | 19.35 | 20.35 | 19.76 | 0.24 |
| 18:0 | 11.96 | 12.37 | 11.69 | 12.41 | 0.17 |
| Σ saturated | 33.8 | 27.5 | 29.4 | 27.8 | 0.17 |
| 16:1n-7 | 3.57 | 4.24 | 3.52 | 4.57 | 0.26 |
| 18:1n-9 | 35.22 | 34.70 | 35.26 | 35.33 | 0.15 |
| Σ monoenes | 39.0 | 39.9 | 39.8 | 38.8 | 0.27 |
| 18:2n-6 | 5.18 | 4.04 | 4.92 | 4.27 | 0.27 |
| 18:3n-6 | 0.35 | 0.58 | 0.27 | 0.11 | 0.10 |
| 20:3n-6 | 0.48 | 0.50 | 0.26 | 0.14 | 0.09 |
| 20:4n-6 | $4.89^b$ | $7.21^a$ | $5.12^b$ | $7.39^a$ | 0.67 |
| Σ n-6 PUFA | 10.4 | 11.4 | 10.0 | 12.8 | 0.41 |
| 18:3n-3 | 0.21 | 0.33 | 0.29 | 0.72 | 0.11 |
| 20:5n-3 | 5.86 | 4.04 | 5.18 | 3.98 | 0.46 |
| 22:6n-3 | 11.65 | 11.62 | 12.11 | 10.18 | 0.42 |
| Σ n-3 PUFA | 16.9 | 14.5 | 17.2 | 15.4 | 0.68 |
| ∑n-3/∑n-6 | 1.62 | 1.27 | 1.72 | 1.20 | 0.11 |
| ∑Total | 100 | 100 | 100 | 100 | |

[1]Values are mean ± SD of three replicates. Values with different letters within the same row are significantly different.

[2]Pooled SEM= SD/√*n*.

**Table 7.** Non-specific immune responses of broodstock Japanese eel fed the experimental diets for 12 weeks[1].

| | SOD[2] (% inhibition) | MPO[3] (absorbance) | Lysozyme (U $ml^{-1}$) |
|---|---|---|---|
| $C_{800}E_0ARA_0$ | 62.5[b] | 1.97[b] | 0.59 |
| $C_{800}E_0ARA_5$ | 64.1[b] | 2.21[ab] | 0.67 |
| $C_{800}E_{200}ARA_0$ | 63.3[b] | 2.58[ab] | 0.68 |
| $C_{800}E_{200}ARA_5$ | 81.2[a] | 2.61[a] | 0.67 |
| Pooled SEM[4] | 2.69 | 0.11 | 0.03 |
| Two way ANOVA | | | |
| Vit E | 0.0190 | 0.0314 | 0.5212 |
| ARA | 0.0130 | 0.5208 | 0.5941 |
| Vit E*ARA | 0.0289 | 0.5995 | 0.5112 |

[1]Values are mean ± SD of three replicates. Values with the different letters within the same row are significantly different.

[2] Superoxide dismutase (% inhibition).

[3] Myeloperoxidase (absorbance).

[4]Pooled SEM= SD/$\sqrt{n}$.

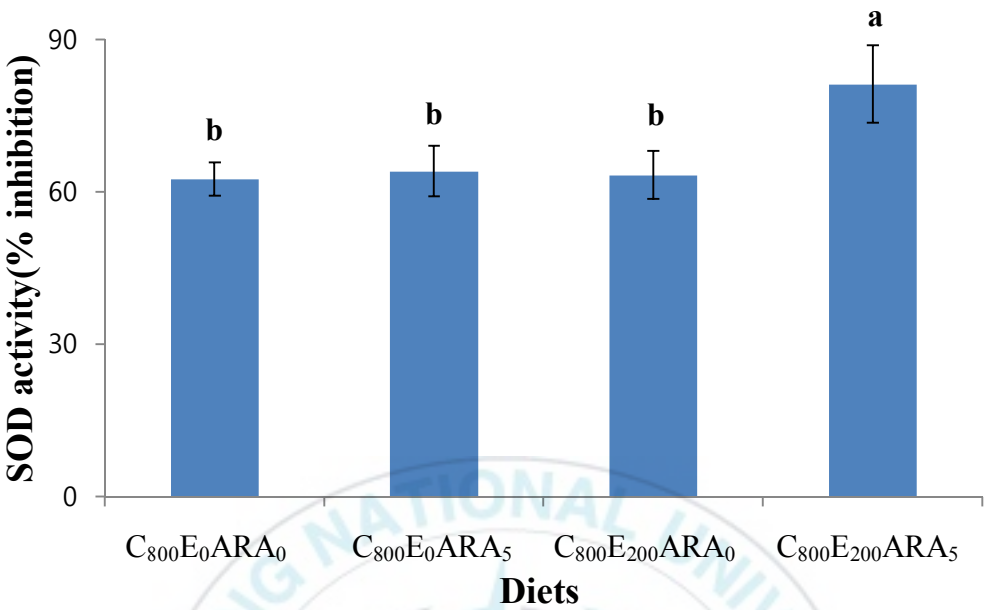


**Fig 4.** Superoxide dismutase activity of broodstock Japanese eel fed the experimental diets for 12 weeks.

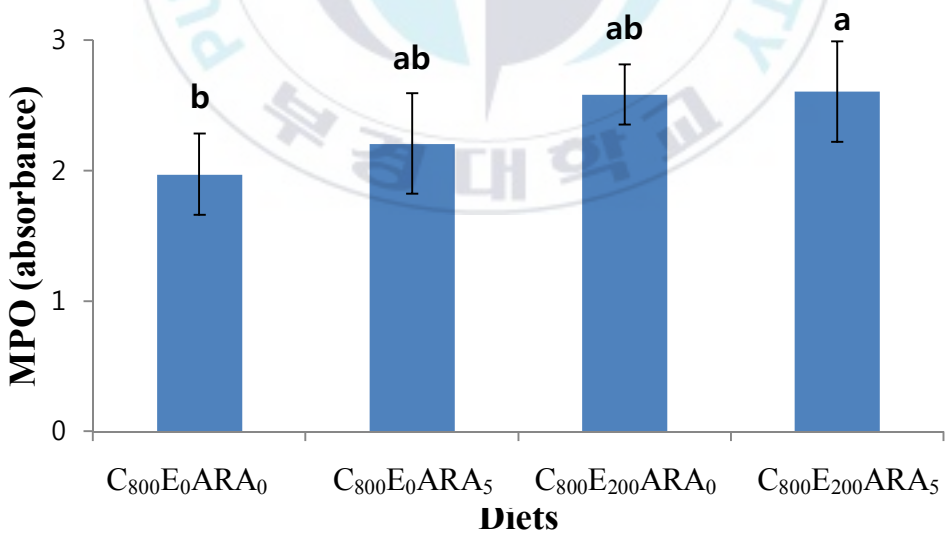


**Fig 5.** Myeloperoxidase activity of broodstock Japanese eel fed the experimental diets for 12 weeks.

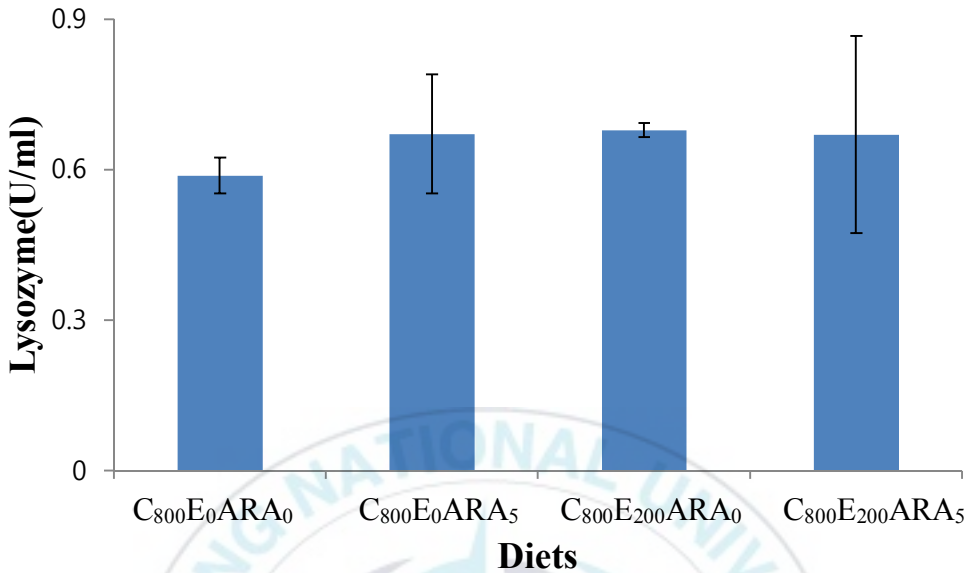


**Fig 6.** Lysozyme activity of broodstock Japanese eel fed the experimental diets for 12 weeks.

**Table 8.** Biochemical characteristics of broodstock Japanese eel fed the experimental diets for 12 weeks[1].

| | AST[2] | ALT[3] | Glucose(mg $dl^{-1}$) |
|---|---|---|---|
| $C_{800}E_0ARA_0$ | 41.7 | 14.3 | 45.0 |
| $C_{800}E_0ARA_5$ | 55.0 | 17.0 | 67.0 |
| $C_{800}E_{200}ARA_0$ | 58.7 | 16.3 | 74.0 |
| $C_{800}E_{200}ARA_5$ | 57.0 | 16.0 | 59.7 |
| Pooled SEM[4] | 3.73 | 0.47 | 6.22 |

[1]Values are mean ± SD of three replicates. Values with the different letters within the same row are significantly different.

[2]AST (U $l^{-1}$): Aspartate aminotransferase.

[3]ALT (U $l^{-1}$): Alanine aminotransferase.

[4]Pooled SEM= SD/$\sqrt{n}$.

# Ⅲ. Synergistic effects of dietary vitamin E and arachidonic acid without vitamin C supplementation in Japanese eel, *Anguilla japonica*

## Abstract

A 12-week of feeding trial was conducted to evaluate the synergistic effects of dietary vitamin E and arachidonic acid (ARA) without vitamin C (Ascorbic Acid, AA) supplementation in Japanese eel, *Anguilla japonica*. Four experimental diets were formulated to contain two different levels of vitamin E (0 and 200mg α-Toc/kg diet) and ARA (0, 5g ARA/kg diet) without AA supplementation. Four experimental diets, in a 2x2 factorial design ($C_0E_0ARA_0$, $C_0E_0ARA_5$, $C_0E_{200}ARA_0$ and $C_0E_{200}ARA_5$) were fed to triplicate groups of fish averaging 279±9.0g(mean±SD) for 12 weeks. At the end of the feeding trial, Superoxide dismutase activity (SOD) values of fish fed $C_0E_{200}ARA_5$ diet were significantly higher than those of fish fed $C_0E_0ARA_0$ and $C_0E_{200}ARA_0$ diets. But, there were no significant differences among SOD values of fish fed $C_0E_{200}ARA_5$ and $C_0E_0ARA_5$ diets. And significant interactions between vitamin E and ARA also were observed in SOD values. There were no significant differences in liver AA concentrations among fish fed all the diets. However, AA concentrations in liver of fish fed $C_0E_0ARA_0$ diet were lower than those of fish fed the other diets. As vitamin E and ARA were supplemented, liver vitamin C concentrations of fish increased. Therefore,

combined supplementation of vitamin E and arachidonic acid without vitamin C supplementation could improve nonspecific immune responses in female broodstock Japanese eel.

## Materials and Methods

1. Experimental diets

A basal commercial diet (Table 1) without ascorbic acid (AA), α-Toc, arachidonic acid (ARA) supplementation was used as a control diet. Three other diets were prepared by supplementing two different vitamin E (0 and 200 mg α-Toc $kg^{-1}$) and arachidonic acid (0 and 5% ARA) levels to the basal diet. Four experimental diets, in a 2x2 factorial design ($C_0E_0ARA_0$, $C_0E_0ARA_5$, $C_0E_{200}ARA_0$, $C_0E_{200}ARA_5$) were formulated. DL-α-tocohpheryl acetate (TA) (Sigma-Aldrich, Switzerland) was used as α-Toc sources. ARA was supplied by Australia company, Bulk Nutrients. The actual L-ascorbyl-2-monophosphate (AMP) and TA levels of the experimental diets were determined by High-Pressure Liquid Chromatography (HPLC; Dionex, Softron, USA) and the levels of dietary ARA analyzed by Gas Chromatography. The analyzed AA, TA and ARA concentrations of the diets were $C_0E_0ARA_0$ = 32.5 and 77.6 mg $kg^{-1}$ and 0.00%, $C_0E_0ARA_5$ = 32.3 and 73.2 mg $kg^{-1}$ and 0.51%, $C_0E_{200}ARA_0$ = 31.1 and 182 mg $kg^{-1}$ and 0.00% and $C_0E_{200}ARA_5$ = 33.2 and 192 mg $kg^{-1}$ and 0.43% diet, respectively (table. 2). The targeting levels of AMP, TA and ARA were mixed with the powdered commercial diet at the expense of cellulose, and then stored at −20 °C until diet preparation. Tap water was added to the feeds (1.3% of diet weight) and a ball-shaped dough was made before feeding.

2. Experimental fish and feeding trial

Two-years-old female broodstock of Japanese eel were obtained from an Eel Research Center (Yeonggwang, South Korea). Prior to the start of the feeding trial, the health status of the fish was checked and they were starved for 24 h. All the fish were fed the basal commercial diet for four weeks to become acclimatized to the experimental conditions and facilities. 240 fish averaging at 279 ± 9g (mean ± SD) were weighed and randomly distributed into 24 indoor fiberglass tanks (10 fish/tank) with 150-L volume receiving a constant flow (4 L $min^{-1}$) of filtered freshwater. Each tank was then randomly assigned to one of three replicates of 4 dietary treatments. During the experiment, supplemental aeration was provided in each tank to maintain enough dissolved oxygen, and also water heated by electric heaters in a concrete reservoir. Water temperature and pH during the experiment were maintained at 28.3 ± 0.05 ℃ and 7.68 ± 0.04, respectively. Fish were fed twice daily (06:00 and 18:00 h) for 12 weeks at a fixed rate of 1% body weight per day. Dead fish were removed immediately and weighed, and the amounts of feed for the tanks adjusted to the proper percentage of the remaining fish weight in the tanks. Uneaten feed was siphoned out after 1 hour of feeding and tank inside was scrubbed once per week to minimize algal and fungal growth.

3. Sample collection and analysis

At the end of the feeding trial, fish were starved for 24 h, and the total number and weight of fish in each tank were determined for calculation of final weight, specific growth rate, feed efficiency and survival. Three fish per tank were randomly selected, individually weighed, then dissected to obtain liver and ovary for determination of hepatosomatic index and gonadosomatic index. Thereafter, the same liver and ovary and dissected muscle were used for determining AA and TA concentrations and fatty acid composition. Three additional fish per tank were randomly captured, anesthetized with ethylene glycol phenyl ether (200 mg $l^{-1}$ for 5–10 min), and blood samples were collected from the caudal vein with heparinized syringes. Plasma was separated by centrifugation at 5000 × *g* for 10 min and stored at −70°C for determination of blood biochemical parameters including plasma alanine aminotransferase, aspartate aminotransferase and glucose. Another set of blood samples of the same fish were taken without heparin and allowed to clot at room temperature for 30 min. Then, the serum was separated by centrifugation at 5000 × *g* for 10 min and stored at −70°C for the analysis of non-specific immune responses including lysozyme, myeloperoxidase and superoxide dismutase activities.

Analysis of moisture, crude protein, lipid, ash and fiber in the feed were performed using standard methods (AOAC 1995), and carbohydrate content was measured by Bomb Calorimeter (PARR 1351, Co., Illinois, USA). The energy

value was determined on the basis of physiological fuel value, i.e., 3.99 kcal $g^{-1}$ proteins or carbohydrates and 9.01 kcal $g^{-1}$ lipids (Lee and Putnam, 1973). Samples of diets, fish and liver were dried to constant weights at 105°C to determine their moisture contents. Ash was determined by incineration at 550°C, crude lipid was determined by soxhlet extraction using the Soxtec system 1046 (Tecator AB, Hoganas, Sweden), and crude protein content was determined by the Kjeldahl method (N×6.25) after acid digestion.

Tissue ascorbic acid concentrations were determined by High-Performance Liquid Chromatography (HPLC; Dionex, Softron, USA) with an ultraviolet detector at 254 nm. The mobile phase was 0.05 M $KH_2PO_4$ at pH 2.8, and the flow rate was 1.0 mL $min^{-1}$. Weighed samples were homogenized in 10% cold metaphosphoric acid. Homogenates were centrifuged at 3000 × *g* for 20 min, and supernatants were analyzed on HPLC after being filtered through a 0.45 μm pore size syringe filter.

Tissue α-Toc concentrations were determined by High-Performance Liquid Chromatography (HPLC; Dionex, Softron, USA) with an ultraviolet detector at 290 nm. The mobile phase was Hexane: Isopropanol (98:2, v/v) and the flow rate was 1.0 ml $min^{-1}$. Weighed samples were homogenized in 5 (ml) ethanol. Homogenates were centrifuged at 3000 ×g for 5 min, and supernatants were analyzed on HPLC after being filtered through a 0.45 μm pore size syringe filter.

The composition of fatty acid methyl esters was determined by Gas

Chromatography (Trace GC, TheromoFinnigan, USA) with flame ionization detector, equipped with a Carbowax 007 capillary column (30 m×0.25 mm i.d., film thickness 0.25μm, QUADREX, USA). Injector and detector temperatures were 250°C. The column temperature was programmed from 100°C to 220°C at a rate of 5°C $min^{-1}$ and 220°C to 240°C at a rate of 3°C $min^{-1}$. Helium was used as the carrier gas. Fatty acids were identified by comparison with known standards.

The plasma levels of glucose and activities of ALT and AST were measured by a chemical analyzer (Fuji DRI-CHEM 3500i, Fuji Photo Film, Ltd., Tokyo, Japan).

A turbidometric assay was used for determination of serum lysozyme level by the method described by Hultmark (1980) with slight modifications. Briefly, *Micrococcus lysodeikticus* (0.75 mg $ml^{-1}$) was suspended in sodium phosphate buffer (0.1 M, pH 6.4), 200 μl of suspension was placed in each well of 96-well plates, and 20 μl serum was added subsequently. The reduction in absorbance of the samples was recorded at 570 nm after incubation at room temperature for 0 and 30 min in a microplate reader (UVM 340, Biochrom, Cambridge, UK). A reduction in absorbance of 0.001 $min^{-1}$ was regarded as one unit of lysozyme activity.

MPO activity was measured according to Quade and Roth (1997). Briefly, 20 μl of serum was diluted with HBSS (Hanks Balanced Salt Solution) without $Ca^{2+}$ or $Mg^{2+}$ (Sigma-Aldrich, USA) in 96-well plates. Then, 35 μl of 3,3′,5,5′-

tetramethylbenzidine hydrochloride (TMB, 20 mM) (Sigma-Aldrich, USA) and $H_2O_2$ (5 mM) were added. The color change reaction was stopped after 2 min by adding 35 μl of 4 M sulfuric acid. Finally, the optical density was read at 450 nm in a microplate reader.

SOD activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma-Aldrich, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 ℃. The percent inhibition was normalized by mg protein and presented as SOD activity units.

4. Statistical analysis

All data were analyzed by two-way ANOVA to test for the effects of the dietary treatments. When significant differences were found, a least significant difference (LSD) test used to identify differences among experimental groups. Treatment effects were considered with the significance level at $P < 0.05$. All statistical analyses were carried out by SAS version 9.1 (SAS Institute, Cary, NC, USA).

## Results

At the end of the feeding trial, there were no significant differences in Final weight, weight gain (WG), specific growth rate (SGR) and feed efficiency (FE) among fish fed all the diets. Fish survival rate varied from 90 to 100% and no significant differences were observed among dietary treatments. There were significant differences in hepatosomatic index (HSI) and gonadosomatic index (GSI) among fish fed all the diets. However, fish fed $C_0E_{200}ARA_0$ diet showed the higher values of WG, SGR, FE, GSI and HSI than those of fish fed the other diets.

There were no significant differences in the proximate compositions of the whole-body of fish between dietary treatments.

The α-Toc concentrations in liver of fish fed 200 mg vitamin E/kg diets ($C_0E_{200}ARA_0$ and $C_0E_{200}ARA_5$) were significantly higher than those of fish fed without vitamin E/kg diets ($C_0E_0ARA_0$ and $C_0E_0ARA_5$). So, liver α-Toc concentrations indicated accordingly to the dietary supplementation level of vitamin E. There were no significant differences in liver ascorbic acid (AA) concentrations among fish fed all the diets. However, the AA concentrations in liver of fish fed $C_0E_0ARA_0$ diet showed the lower values than those of fish fed the other diets. As vitamin E and arachidonic acid (ARA) were supplemented, liver vitamin C concentration was increased. The ARA concentrations in liver indicated accordingly to the dietary supplementation level of arachidonic acid.

Non-specific immune responses are shown in table 7. Superoxide dismutase activity (SOD) values of fish fed $C_0E_{200}ARA_5$ diet were significantly higher than those of fish fed $C_0E_0ARA_0$ and $C_0E_{200}ARA_0$ diets. But, there were no significant differences among SOD values of fish fed $C_0E_{200}ARA_5$ and $C_0E_0ARA_5$ diets. And significant interactions between vitamin E and ARA also were observed in SOD values. There were no significant differences in myeloperoxidase (MPO) and lysozyme activities between dietary treatments. However, MPO and lysozyme activities of fish fed $C_0E_0ARA_0$ diet showed the lower values than those of fish fed the other diets.

Table 8 shows hematological parameters of female broodstock eel fed different experimental diets. There were no significant differences in aspartate aminotransferase, alanine aminotransferase and glucose level of fish fed all the diets.

## Discussion

In the present experiment, no abnormally behaved fish and structural deformity were observed. Weight gain, specific growth rate, feed efficiency and hepatosomatic index were not significantly affected by dietary vitamin E and arachidonic acid (ARA) supplementation. Fish survival rate varied from 90 to 100% and no significant differences were observed among dietary treatments.

Non-specific immune parameters play a critical role in controlling the balance of release and clear of ROS in immune cells (Xu et al. 2010). In this study, Superoxide dismutase (SOD) activity was significantly increased by increment dietary α-Toc and ARA supplementation. Specially, SOD activity of fish fed 5g ARA/kg diets ($C_0E_0ARA_5$ and $C_0E_{200}ARA_5$) were significantly higher than those of fish fed without ARA/kg diets ($C_0E_0ARA_0$ and $C_0E_{200}ARA_0$). This showed that dietary ARA improved SOD activity. Similarly, Xu et al.(2010) reported significant enhancement of SOD activity by supplementation of ARA in diet for juvenile Japanese seabass, *Lateolabrax japonicas*.

Liver micro-nutrients concentrations showed similar trend to experiment 1. Liver α-Toc concentrations indicated accordingly to the dietary supplementation level of vitamin E. This result showed an increase in α-Toc concentration by dietary α-Toc increment due to fish cannot synthesize vitamin E and must rely on a dietary supply (Peng and Gatlin 2009). Similarly, Lin & Shiau (2005) reported an increase in liver vitamin E concentrations by dietary vitamin E concentrations

in grouper, *Epinephelus malabaricus*. The ARA concentrations in liver indicated accordingly to the dietary supplementation level of arachidonic acid. This is in agreement with previous studies (Bransden et al. 2004, 2005; Lee et al. 2003; Villalta et al. 2005; Xu et al. 2010) indicating that the tissue fatty acids composition of fish generally reflect dietary fatty acids contents (Bransden et al. 2003; Francis et al. 2006; Rezek et al. 2009). Ascorbic acid concentrations in liver of fish fed $C_0E_0ARA_0$ and $C_0E_0ARA_5$ diet showed the lower values than those of fish fed $C_0E_{200}ARA_0$ and $C_0E_{200}ARA_5$ diets. As vitamin E was supplemented, liver vitamin C concentration was increased. Lee et al. (2003) reported the hypothesis that vitamin C spares and/or regenerates vitamin E in yellow perch. However there were no synergistic effects of both vitamins. Sealey & Gatlin (2002) also reported that no significant dietary vitamin E effect on liver ascorbate was observed in hybrid striped bass.

Therefore, combined supplementation of vitamin E and arachidonic acid without vitamin C supplementation could improve nonspecific immune responses in female broodstock Japanese eel.

## Tables and Figures

**Table 1.** Proximate analyses (dry matter basis) of the basal commercial diet[1].

| Calculated composition | Amount |
|---|---|
| Moisture (%) | 4.25 |
| Crude Protein (%) | 51.8 |
| Crude lipid (%) | 6.37 |
| Crude ash (%) | 11.4 |
| Crude fiber (%) | 2.13 |
| Carbohydrate (%) | 24.1 |
| Gross energy (Kcal $g^{-1}$) | 3.61 |

[1] Values are mean of duplicate samples.

**Table 2.** Actual concentration of vitamin C, E and arachidonic acid (ARA) for the experimental diets.

| Diets | Vit.C[1] (ppm) | Vit.E[2] (ppm) | ARA[3] (%) |
|---|---|---|---|
| $C_0E_0ARA_0$ | 32.5 | 77.6 | 0.00 |
| $C_0E_0ARA_5$ | 32.3 | 73.2 | 0.51 |
| $C_0E_{200}ARA_0$ | 31.1 | 182 | 0.00 |
| $C_0E_{200}ARA_5$ | 33.2 | 192 | 0.43 |

[1]Vit. C source : L-ascorbyl-2-monophosphate

[2]Vit. E source : dl- α-tocopheryl acetate

[3]Supplied by Bulk Nutrients. Australia

**Table 3.** Growth performance, feed efficiency, organosomatic indices and survival of broodstock Japanese eel fed the experimental diets for 12 weeks[1].

| | Diets | | | | Pooled SEM[9] |
|---|---|---|---|---|---|
| | $C_0E_0ARA_0$ | $C_0E_0ARA_5$ | $C_0E_{200}ARA_0$ | $C_0E_{200}ARA_5$ | |
| IBW[2] | 275 | 281 | 277 | 275 | 1.49 |
| FBW[3] | 307 | 314 | 311 | 305 | 2.82 |
| WG[4] | 11.7 | 11.6 | 12.5 | 11.2 | 0.69 |
| SGR[5] | 0.13 | 0.13 | 0.14 | 0.13 | 0.01 |
| FE[6] | 15.9 | 16.4 | 16.5 | 15.3 | 0.88 |
| GSI[7] | 0.52 | 0.46 | 0.58 | 0.56 | 0.05 |
| HSI[8] | 1.14 | 1.28 | 1.33 | 1.10 | 0.05 |
| Survival (%) | 100 | 96.7 | 93.3 | 90.0 | 1.49 |

[1]Values are mean ± SD of three replicates. Values with different letters within the same row are significantly different.

[2]Initial body weight (g).

[3]Final body weight (g).

[4]Weight gain(%) = (final wt. – initial wt.) × 100 / initial wt.

[5]Specific growth rate (%) = (ln final weight − ln initial weight) × 100 / d.

[6]Feed efficiency (%) = wet weight gain × 100 / dry feed intake.

[7]Gonadosomatic index (%) = gonad weight × 100/body weight.

[8]Hepatosomatic index (%) = liver weight × 100 / body weight.

[9]Pooled SEM= SD/$\sqrt{n}$.

**Table 4** Whole-body proximate compositions (%, DM) of broodstock Japanese eel fed the experimental diets for 12 weeks[1].

| | Moisture | Protein | Lipid | Ash |
|---|---|---|---|---|
| $C_0E_0ARA_0$ | 64.2 | 46.2 | 50.4 | 5.69 |
| $C_0E_0ARA_5$ | 60.6 | 46.7 | 46.2 | 5.66 |
| $C_0E_{200}ARA_0$ | 62.8 | 45.4 | 47.9 | 6.18 |
| $C_0E_{200}ARA_5$ | 67.5 | 44.0 | 48.8 | 5.43 |
| Pooled SEM[2] | 1.46 | 0.59 | 0.86 | 0.16 |

[1]Values are mean ± SD of three replicates. Values with different letters within the same row are significantly different.

[2]Pooled SEM= SD/$\sqrt{n}$.

**Table 5.** Vitamin C and E concentrations (mg/kg ; DM) in liver of broodstock Japanese eel fed the experimental diets for 12 weeks[1]

| | Vitamin C (AA) | Vitamin E (α-Toc ) |
|---|---|---|
| $C_0E_0ARA_0$ | 31.5 | 16.2[b] |
| $C_0E_0ARA_5$ | 32.9 | 15.6[b] |
| $C_0E_{200}ARA_0$ | 34.0 | 36.1[a] |
| $C_0E_{200}ARA_5$ | 38.2 | 37.5[a] |
| Pooled SEM[2] | 2.03 | 3.29 |

[1]Values are mean ± SD of three replicates. Values with different letters within the same row are significantly different.

[2]Pooled SEM= SD/√*n*.

**Table 6.** Liver fatty acid concentrations (%) in liver of broodstock Japanese eel fed the experimental diets for 12 weeks[1].

| | Diets | | | | Pooled SEM[2] |
|---|---|---|---|---|---|
| | $C_0E_0ARA_0$ | $C_0E_0ARA_5$ | $C_0E_{200}ARA_0$ | $C_0E_{200}ARA_5$ | |
| 14:0 | 1.12 | 1.14 | 1.03 | 1.06 | 0.03 |
| 16:0 | 19.7 | 19.8 | 20.4 | 19.2 | 0.23 |
| 18:0 | 13.0 | 13.3 | 11.7 | 12.8 | 0.35 |
| Σ saturated | 33.8 | 27.5 | 29.4 | 27.8 | 0.28 |
| 16:1n-7 | 3.69 | 4.54 | 3.52 | 3.48 | 0.25 |
| 18:1n-9 | 35.3 | 35.3 | 36.3 | 35.3 | 0.23 |
| Σ monoenes | 39.0 | 39.9 | 39.8 | 38.8 | 0.27 |
| 18:2n-6 | 4.85 | 4.27 | 4.82 | 4.97 | 0.15 |
| 18:3n-6 | 0.13 | 0.11 | 0.20 | 0.26 | 0.04 |
| 20:3n-6 | 0.78 | 0.14 | 0.26 | 0.35 | 0.14 |
| 20:4n-6 | $4.61^b$ | $6.89^a$ | $4.72^b$ | $7.19^a$ | 0.69 |
| Σ n-6 PUFA | 10.4 | 11.4 | 10.0 | 12.8 | 0.62 |
| 18:3n-3 | 0.13 | 0.72 | 0.29 | 0.20 | 0.13 |
| 20:5n-3 | 4.81 | 3.61 | 5.18 | 3.77 | 0.39 |
| 22:6n-3 | 11.9 | 10.2 | 11.7 | 11.4 | 0.38 |
| Σ n-3 PUFA | 16.9 | 14.5 | 17.2 | 15.4 | 0.62 |
| ∑n-3/∑n-6 | 1.62 | 1.27 | 1.72 | 1.20 | 0.13 |
| ∑Total | 100 | 100 | 100 | 100 | |

[1]Values are mean ± SD of three replicates. Values with different letters within the same row are significantly different.

[2]Pooled SEM= SD/√*n*.

**Table 7.** Non-specific immune responses of broodstock Japanese eel fed the experimental diets for 12 weeks[1].

| | SOD[2] (% inhibition) | MPO[3] (absorbance) | Lysozyme (U $ml^{-1}$) |
|---|---|---|---|
| $C_0E_0ARA_0$ | 40.9[c] | 1.88 | 0.52 |
| $C_0E_0ARA_5$ | 65.6[ab] | 2.12 | 0.70 |
| $C_0E_{200}ARA_0$ | 62.3[b] | 2.25 | 0.57 |
| $C_0E_{200}ARA_5$ | 71.4[a] | 2.39 | 0.64 |
| Pooled SEM[4] | 3.61 | 0.10 | 0.04 |
| Two way ANOVA | | | |
| Vit E | 0.0003 | 0.1309 | 0.9621 |
| ARA | >.0001 | 0.3438 | 0.1507 |
| Vit E*ARA | 0.0085 | 0.7971 | 0.5332 |

[1]Values are mean ± SD of three replicates. Values with the different letters within the same row are significantly different.

[2] Superoxide dismutase (% inhibition).

[3] Myeloperoxidase (absorbance).

[4]Pooled SEM= SD/$\sqrt{n}$.

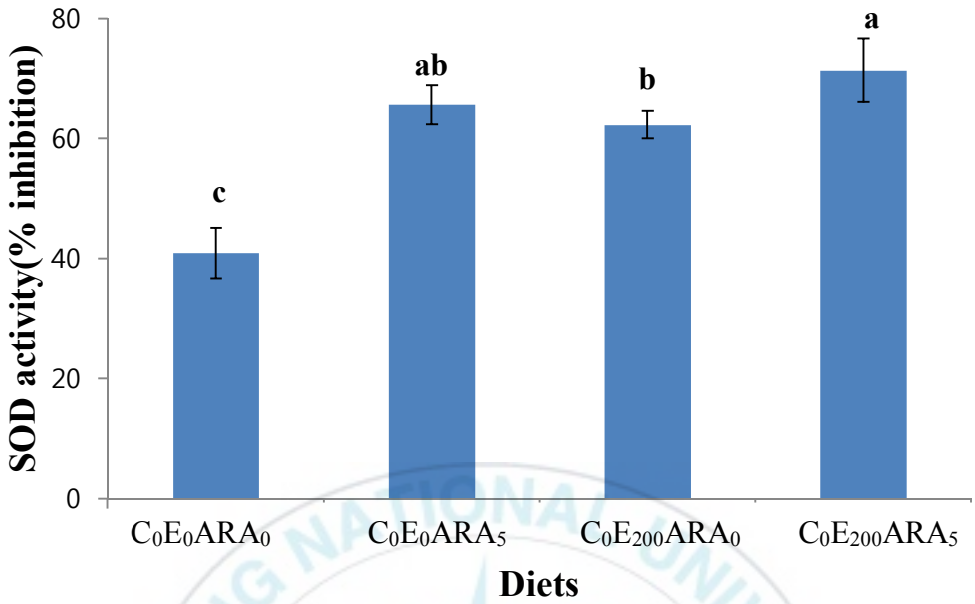


**Fig 1.** Superoxide dismutase activity of broodstock eel fed the experimental diets for 12 weeks

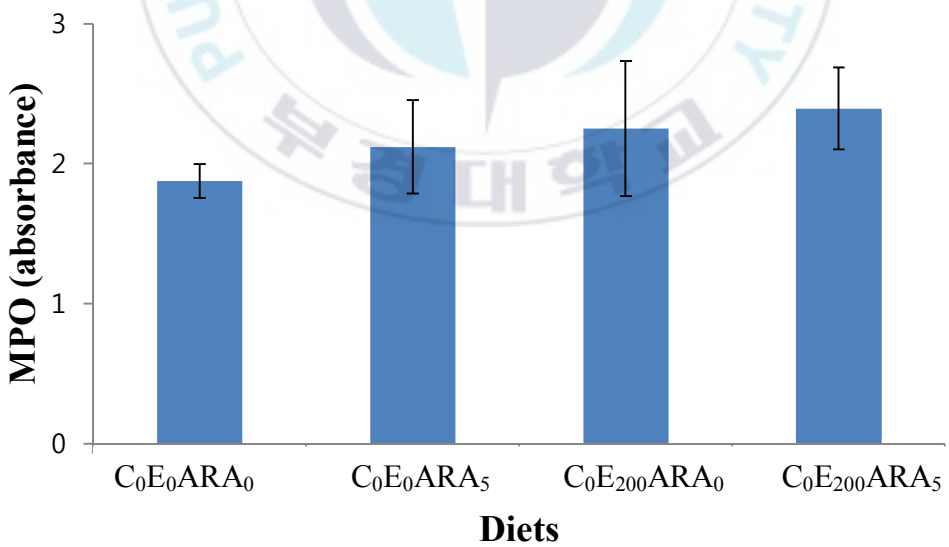


**Fig 2.** Myeloperoxidase activity of broodstock eel fed the experimental diets for 12 weeks

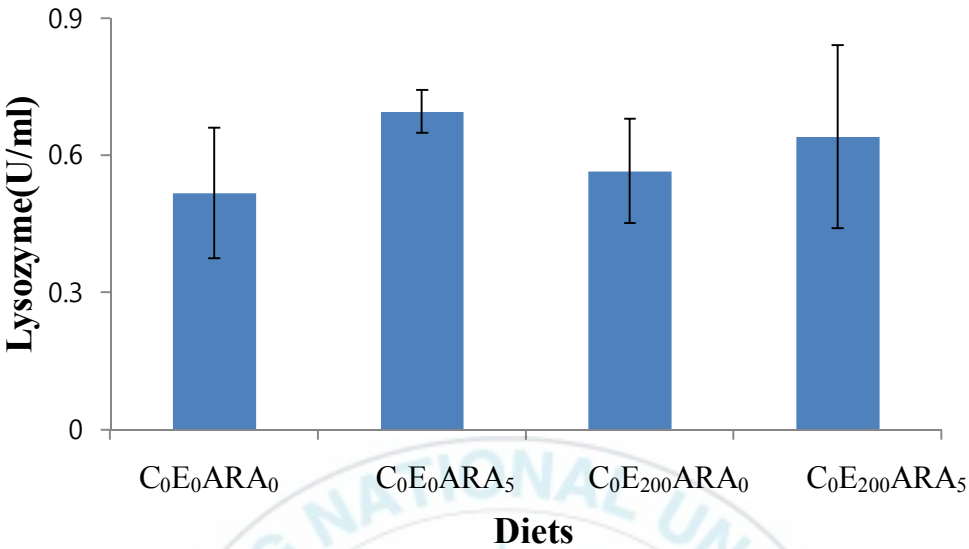


**Fig 3.** Lysozyme activity of broodstock eel fed the experimental diets for 12 weeks

**Table 8.** Biochemical characteristics of broodstock Japanese eel fed the experimental diets for 12 weeks[1].

| | AST[2] | ALT[3] | Glucose(mg $dl^{-1}$) |
|---|---|---|---|
| $C_0E_0ARA_0$ | 35.7 | 14.0 | 65.7 |
| $C_0E_0ARA_5$ | 38.7 | 14.7 | 64.0 |
| $C_0E_{200}ARA_0$ | 47.3 | 14.7 | 57.3 |
| $C_0E_{200}ARA_5$ | 49.3 | 17.3 | 59.7 |
| Pooled SEM[4] | 3.22 | 0.46 | 3.54 |

[1]Values are mean ± SD of three replicates. Values with the different letters within the same row are significantly different.

[2]AST (U $l^{-1}$): Aspartate aminotransferase.

[3]ALT (U $l^{-1}$): Alanine aminotransferase.

[4]Pooled SEM= SD/$\sqrt{n}$.

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**Appendix**

Exp.1 Initial data

| | Tank No | Number | Individual weight | Total feeding | Individual feeding |
|---|---|---|---|---|---|
| $C_0E_0ARA_0$ | 12 | 10 | 280 | 112 | 11.2 |
| | 17 | 10 | 272 | 109 | 10.9 |
| | 10 | 10 | 272 | 109 | 10.9 |
| $C_0E_0ARA_5$ | 13 | 10 | 276 | 110 | 11.0 |
| | 8 | 10 | 280 | 112 | 11.2 |
| | 1 | 10 | 288 | 115 | 11.5 |
| $C_0E_{200}ARA_0$ | 24 | 10 | 280 | 112 | 11.2 |
| | 6 | 10 | 270 | 108 | 10.8 |
| | 11 | 10 | 280 | 112 | 11.2 |
| $C_0E_{200}ARA_5$ | 23 | 10 | 272 | 109 | 10.9 |
| | 9 | 10 | 278 | 111 | 11.1 |
| | 4 | 10 | 274 | 110 | 11.0 |

$12^{th}$ weeks data

| | Tank No | Number | Individual weight | Total feeding | Individual feeding |
|---|---|---|---|---|---|
| $C_0E_0ARA_0$ | 12 | 10 | 312 | 2090 | 208.6 |
| | 17 | 10 | 316 | 2026 | 202.6 |
| | 10 | 10 | 292 | 1863 | 186.3 |
| $C_0E_0ARA_5$ | 13 | 10 | 304 | 1932 | 193.2 |
| | 8 | 10 | 314 | 1939 | 193.9 |
| | 1 | 9 | 324 | 1915 | 212.8 |
| $C_0E_{200}ARA_0$ | 24 | 10 | 314 | 1988 | 198.8 |
| | 6 | 9 | 302 | 1863 | 207.0 |
| | 11 | 9 | 318 | 2030 | 225.5 |
| $C_0E_{200}ARA_5$ | 23 | 9 | 293 | 1796 | 199.5 |
| | 9 | 10 | 314 | 1988 | 198.8 |
| | 4 | 9 | 309 | 1835 | 203.9 |

Exp.2 Initial data

| | Tank No | Number | Individual weight | Total feeding | Individual feeding |
|---|---|---|---|---|---|
| $C_{800}E_0ARA_0$ | 14 | 10 | 274 | 110 | 11.0 |
| | 22 | 10 | 280 | 112 | 11.2 |
| | 20 | 10 | 278 | 111 | 11.1 |
| $C_{800}E_0ARA_5$ | 3 | 10 | 278 | 111 | 11.1 |
| | 21 | 10 | 272 | 109 | 10.9 |
| | 15 | 10 | 282 | 113 | 11.3 |
| $C_{800}E_{200}ARA_0$ | 18 | 10 | 284 | 113 | 11.3 |
| | 19 | 10 | 278 | 111 | 11.1 |
| | 16 | 10 | 278 | 111 | 11.1 |
| $C_{800}E_{200}ARA_5$ | 5 | 10 | 280 | 112 | 11.2 |
| | 7 | 10 | 270 | 108 | 10.8 |
| | 2 | 10 | 278 | 111 | 11.1 |

$12^{th}$ weeks data

| | Tank No | Number | Individual weight | Total feeding | Individual feeding |
|---|---|---|---|---|---|
| $C_{800}E_0ARA_0$ | 14 | 10 | 310 | 2055 | 205.5 |
| | 22 | 10 | 307 | 1811 | 181.1 |
| | 20 | 10 | 304 | 2213 | 221.3 |
| $C_{800}E_0ARA_5$ | 3 | 10 | 308 | 2043 | 204.3 |
| | 21 | 10 | 311 | 2243 | 224.3 |
| | 15 | 9 | 313 | 1931 | 214.6 |
| $C_{800}E_{200}ARA_0$ | 18 | 10 | 338 | 2045 | 204.5 |
| | 19 | 10 | 306 | 2085 | 208.5 |
| | 16 | 10 | 316 | 1835 | 183.5 |
| $C_{800}E_{200}ARA_5$ | 5 | 10 | 332 | 1802 | 180.2 |
| | 7 | 10 | 340 | 1869 | 186.9 |
| | 2 | 9 | 324 | 1974 | 219.3 |